

LIPID OXIDATION IN A MODEL SYSTEM AND IN  
MEAT

Andrew Richard Arnold

A Thesis Submitted for the Degree of PhD  
at the  
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LIPID OXIDATION IN  
A MODEL SYSTEM  
AND IN MEAT

A Thesis presented for the degree of  
DOCTOR OF PHILOSOPHY  
in the faculty of Science of the  
University of St Andrews

by

ANDREW RICHARD ARNOLD, BSc (Hons)

89.

United College of St Salvator  
and St Leonard, St Andrews

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# CERTIFICATE

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I certify that the following thesis is based on the results of research carried out by me, that it is my own composition, and has not previously been presented for a higher degree.

.....



## ACKNOWLEDGEMENTS

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Finally, thanks go to Lorraine Berry for typing this thesis and Cathy for her constant help and encouragement.

## ABSTRACT

Lipid oxidation is the main factor which limits the shelf-life of meat when held under frozen storage. Research undertaken used pork phospholipid liposomes as a model for studying lipid oxidation in meat. Oxidation was followed by monitoring the decrease in the phospholipid unsaturated fatty acyl chains. It was found that the greater the level of unsaturation of the phospholipid fatty acyl chain the greater was their susceptibility to peroxidation. However, the results were not consistent and several reasons for the variation in rate are provided. At ambient temperatures copper (II) was found to be pro-oxidant in the peroxidation of liposomes. At temperatures below 0°C the pro-oxidant activity of copper (II) was significantly reduced. However copper again became highly pro-oxidant if sodium chloride was present. It is suggested that salt controls the copper ion concentration at sub-zero temperatures as the pro-oxidant activity of copper (II) is reduced on increasing the copper (II) concentration from 0.9 to 90 ppm. Other experiments found sodium nitrite and polyphosphate to act as antioxidant and that liposome structure was an important factor in the rate of peroxidation.

Four storage trials on pork burgers were undertaken to determine whether salt was also pro-oxidant in the stability of pork when held under frozen storage. The oxidative deterioration of the meat was followed by the following methods of analysis:-

1. The decrease in the unsaturated acyl chains of both total lipid and phospholipid.
2. The change in the colour parameters of the meat using reflectance spectroscopy.
3. The analysis of neutral lipid oxidation products by HPLC.
4. The organoleptic qualities of the pork using a trained panel of food assessors.

The results from these storage trials showed that the deterioration of pork was minimised by storing the burgers at lower temperatures within the range 0 to -30°C. Salt was found to accelerate the oxidative deterioration of both uncooked and cooked pork when stored at -20°C. Nitrite was found to exhibit some antioxidant behaviour and reduce the pro-oxidant effect of salt.

## ABBREVIATIONS

ECL	Equivalent Chain Length
EDTA	Ethylenediamine-acetic acid
FAME	Fatty Acid Methyl Ester
FID	Flame ionisation Detector
GLC	Gas Liquid Chromatography
GMP	Guanosine - 5 - monophosphate
HPLC	High Performance Liquid Chromatography
IMP	Inosine - 5 - Monophosphate
LOX	Lipoxygenase
NMR	Nuclear Magnetic Resonance
OI	Oxidation Index
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipid
PI	Phosphatidylinositol
PS	Phosphatidylserine
PUFA	Polyunsaturated Fatty Acids
PV	Peroxide Value
Substrate	1-16:0-2-20:4-PC
TBA	Thiobarbituric Acid
TLC	Thin Layer Chromatography
UV	Ultra Violet

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# CHAPTER 1

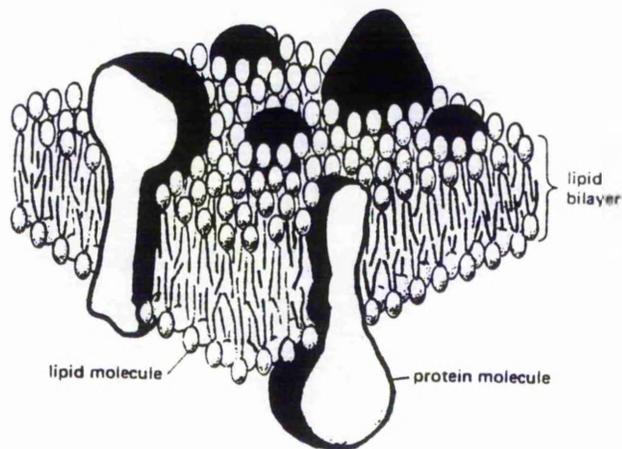
## INTRODUCTION

### 1.1 GENERAL INTRODUCTION

Lipid oxidation is believed to be responsible for the deterioration of meat and meat products when held under frozen storage. This leads to the development of rancidity which lowers the quality, safety and therefore shelf life of a product.

Lipid or fat can be found as constituent parts in either adipose tissue (the white fat we normally associate with meat), or as an integrated part of cell membranes, Figure (1.1), which occur both in adipose tissue and in the lean of the meat.

FIGURE 1.1



Lipids are defined as 'fatty acids and their derivatives'. They may be divided into two classes :-

- 1 Neutral Lipids and
- 2 Polar Lipids

(Tables 1.1 and 1.2 respectively)

TABLE 1.1

Neutral Lipids

Triacylglycerols

Diacylglycerols

Monoacylglycerols

Free fatty acids

Sterols

Sterol esters

TABLE 1.2

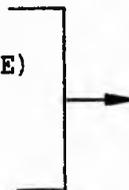
Polar Lipids

Phosphatidylcholine (PC)

Phosphatidylethanolamine (PE)

Phosphatidylserine (PS)

Phosphatidylinositol (PI)



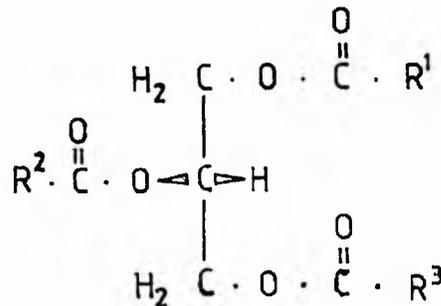
Phosphoglycerides

Sphingolipids

Neutral Lipids

Triacylglycerols are the major neutral lipids and are the predominate constituents of adipose tissue. Biologically their primary function is as a source of chemical energy.<sup>(1)</sup> They are converted to carbon dioxide and water when metabolised and yield twice as much energy as do carbohydrates or proteins. Triacylglycerols are esters of glycerol in which all three glycerol hydroxyl groups are esterified to fatty acids, Figure (1.2).

FIGURE 1.2



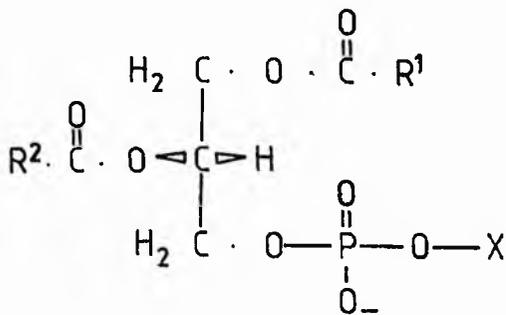
1,2,3-triacyl-sn-glycerol

$\text{R}^1 \text{ R}^2 \text{ R}^3$  — fatty alkyl units

Polar Lipids

The phosphoglycerides are the most abundant polar lipids and can be found in every living cell. They contain a hydrophobic tail and a hydrophilic polar head group which allows them to exist as a bilayer within cell membranes. Figure (1.3) shows their structures. Sphingolipids are less abundant but serve an important function. Together with proteins and polysaccharides they make up myelin, the protective coating that encloses nerve fibres.<sup>(2)</sup>

FIGURE 1.3



$\text{R}^1 \text{R}^2$ -fatty alkyl units

X = — H

(PA) Phosphatidic acid

—  $\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$

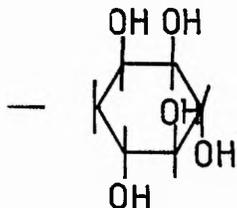
(PC) Phosphatidylcholine

—  $\text{CH}_2\text{CH}_2\text{NH}_2$

(PE) Phosphatidylethanolamine

—  $\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$

(PS) Phosphatidylserine



(PI) Phosphatidylinositol

It is the fatty acid moieties of lipids which makes them vulnerable to oxidation. Phosphoglycerides which contain a high concentration of polyunsaturated fatty acids would be expected to be most at risk from oxidative damage.

## 1.2 FATTY ACID AUTOXIDATION

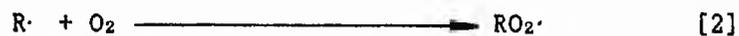
This is believed to be a free radical process consisting of initiation, propagation and termination steps,<sup>(3-11)</sup> Figure (1.4).

FIGURE 1.4

### Initiation



### Propagation

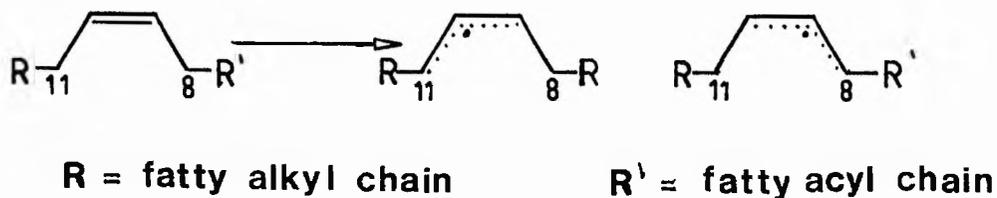


### Termination



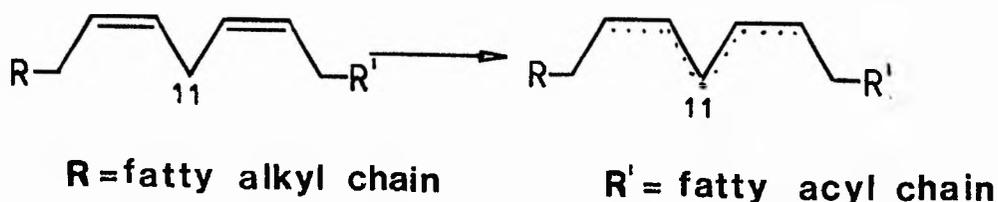
The susceptibility of unsaturated fatty acids to initiation varies according to the lability of their allylic hydrogens. This accounts for the significant difference in rates between oleate and linoleate. Oleate autoxidation involves hydrogen abstraction from carbon - 8 or carbon - 11 with the formation of the two allylic radicals, Figure (1.5).

FIGURE 1.5



Initiation of oleate autoxidation

FIGURE 1.6



Initiation of linoleate autoxidation

Linoleate autoxidation occurs at the reactive doubly allylic carbon - 11 of the fatty acid yielding a pentadienyl radical, Figure (1.6). For both oleate and linoleate the radicals react with oxygen and gain a hydrogen atom producing hydroperoxides.

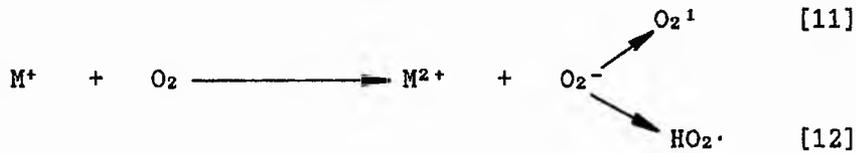
The process of initiation is not well understood but metal ions, heat, light and hydrogen atom abstractors are thought to be responsible. In biological systems, hydroxyl radicals have been shown to be efficient initiators and may be produced by the Fenton Reaction.<sup>(12,13)</sup> In this reaction trace amounts of ferrous ions ( $\text{Fe}^{2+}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) form hydroxyl radicals ( $\text{OH}\cdot$ ). The hydrogen peroxide may have been formed by dismutation of superoxide anions ( $\text{O}_2^-$ ). The production of the latter compound is thought to be formed by the action of ferrous ion on oxygen.<sup>(14)</sup> A sequence of reactions leading to hydrogen abstraction of unsaturated fatty acids in the presence of ferrous ions and oxygen may be represented as follows, Figure (1.7).

FIGURE 1.7



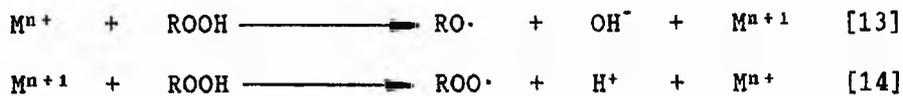
Other metals may also react with oxygen producing superoxide radical anions which can either lose an electron giving singlet oxygen or react with a proton to form a hydroperoxy radical, a good chain initiator <sup>(15)</sup>, Figure (1.8).

FIGURE 1.8



It is believed that in food systems minute quantities of hydroperoxide can decompose in the presence of transition metal ions to produce peroxy or alkoxy radicals which can act as initiators for further oxidation, Figure (1.10)

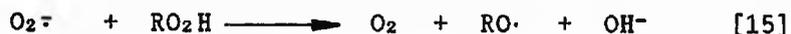
FIGURE 1.10



Pryor <sup>(16)</sup> has recently shown that lipid diene hydroperoxide reacts with superoxide anion to produce alkoxy radicals that subsequently initiate further lipid peroxidation. If the production of superoxide anions by ferrous and oxygen

reaction [7] takes place in the presence of lipid hydroperoxides the following reaction occurs, Figure (1.11).

FIGURE 1.11



Pryor showed that reaction [15] is sufficiently fast to compete with reaction [8] - dismutation of superoxide radical.

Recently researchers have questioned the role of OH in autoxidation. Chan et al.<sup>(17)</sup> found that OH scavengers had no effect in the  $\text{Cu}^{\text{II}}/\text{H}_2\text{O}_2$  induced peroxidation of erythrocyte membranes while dimethylfuran, a singlet oxygen scavenger was slightly inhibitory. A more recent paper by the same research group showed that incubation in  $\text{D}_2\text{O}$  which significantly increases the half life of singlet oxygen resulted in a three fold increase in peroxidation.<sup>(18)</sup>

The most important termination reaction for secondary peroxy radicals at room temperature is that proposed by Russell.<sup>(12)</sup> The mechanism is believed to involve a tetroxide intermediate which decomposes forming a ketone, an alcohol and oxygen.

### 1.3 HYDROPEROXIDE FORMATION

The initial products of autoxidation are lipid hydroperoxides.

#### Oleate 18:1 (n-9) Figure (1.11)

The mechanism involves hydrogen abstraction on carbon-8 or carbon-11 producing two resonance stabilised allylic radicals. They react with oxygen, resulting finally in a mixture of 8-, 9-, 10- and 11- allylic hydroperoxides.

#### Linoleate 18:2 (n-6) Figure (1.12)

Carbon atom 11 is doubly allylic and consequently considerably more reactive. A hydrogen atom is removed at carbon-11 to produce a pentadienyl radical. This reacts at either end to produce a mixture of 9- and 13- diene hydroperoxides.

#### Arachidonate 20:4 (n-6) Figure (1.13)

Hydrogen abstraction can occur at the doubly allylic carbon -7, -10 and -13 positions of arachidonate producing three pentadienyl radicals reacting with oxygen at their end positions C-5 and C-9, C-8 and C-12, and C-11 and C-13. Experimentally it is found that the external 5- and 15- hydroperoxides are present in higher concentrations than the internal 8-, 9-, 11- and 12-

hydroperoxides.<sup>(19-22)</sup> The structure of the internal hydroperoxide allows cyclisation and explains their relatively low concentrations compared to the external 5- and 15- hydroperoxide isomers.

FIGURE 1.11

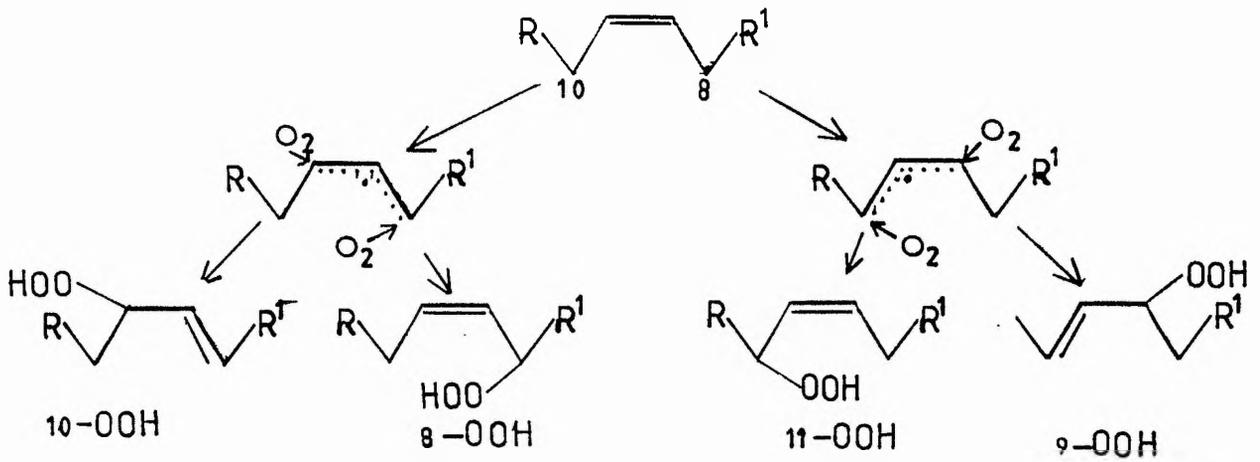
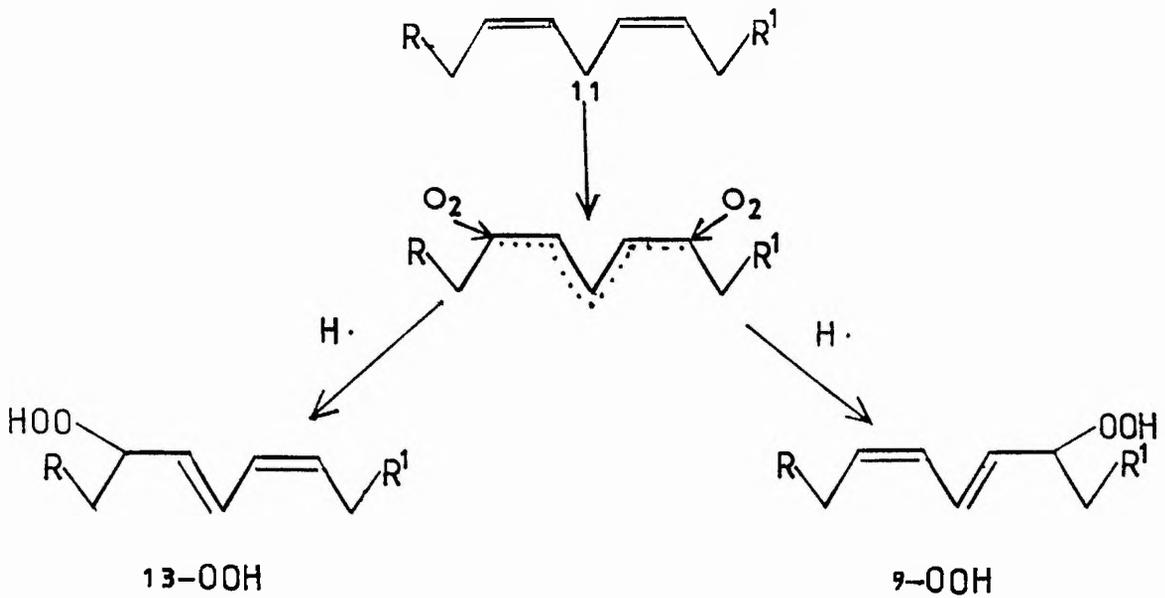
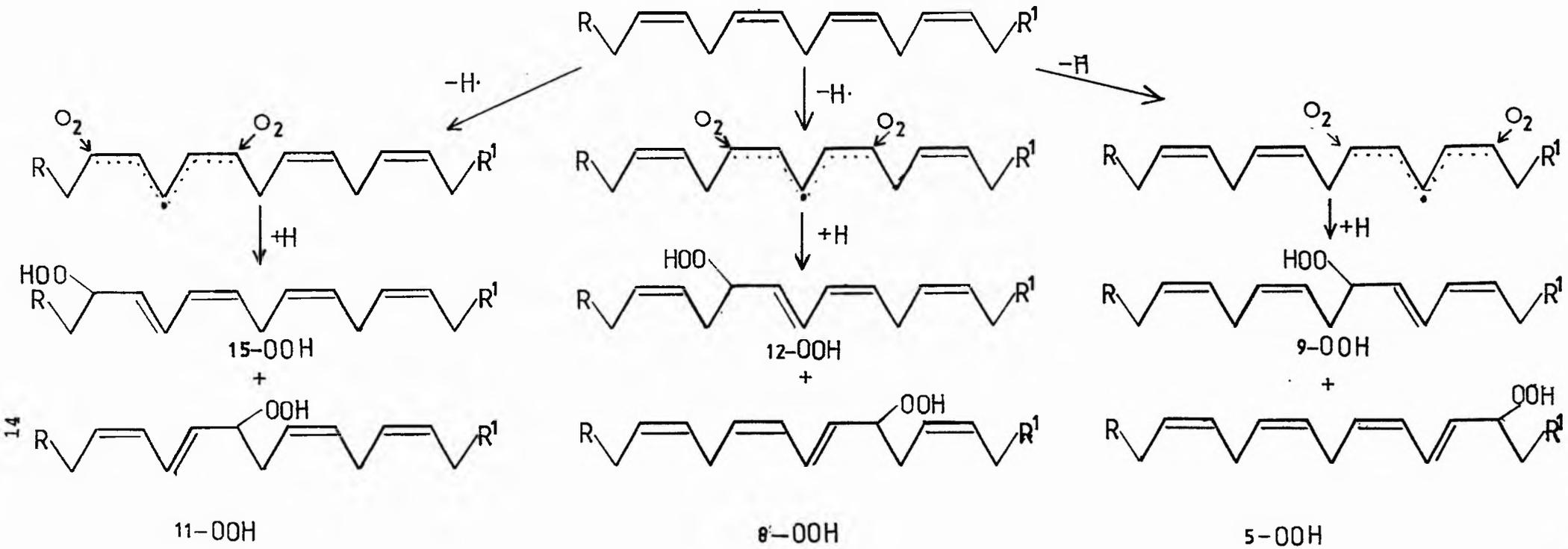


FIGURE 1.12



$R, R^1 = \text{fatty alkyl units}$

FIGURE 1.13



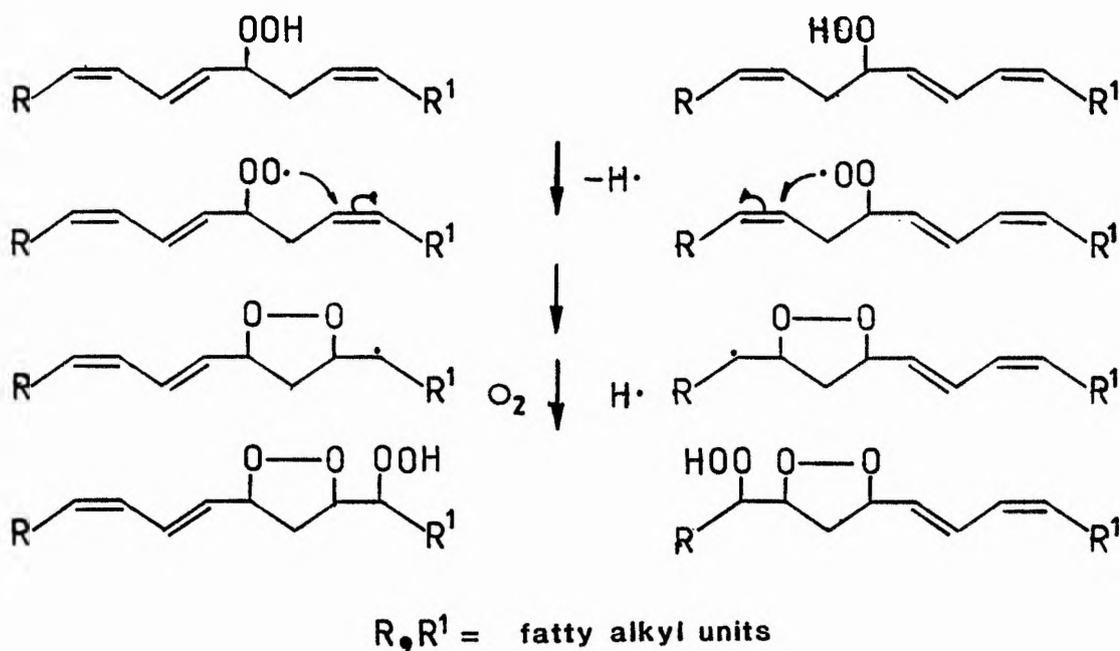
$\text{R}, \text{R}' = \text{fatty alkyl units}$

## 1.4 SECONDARY REACTION PRODUCTS

### 1.4.1 Hydroperoxide Cyclisation

It has been known for some time that in the autoxidation of linolenate the external 9- and 16- hydroperoxides are found in higher concentrations than the internal 12- and 13- isomers. It is thought that the internal 12- and 13- hydroperoxides cyclise into hydroperoxy cyclic peroxides, Figure (1.14).

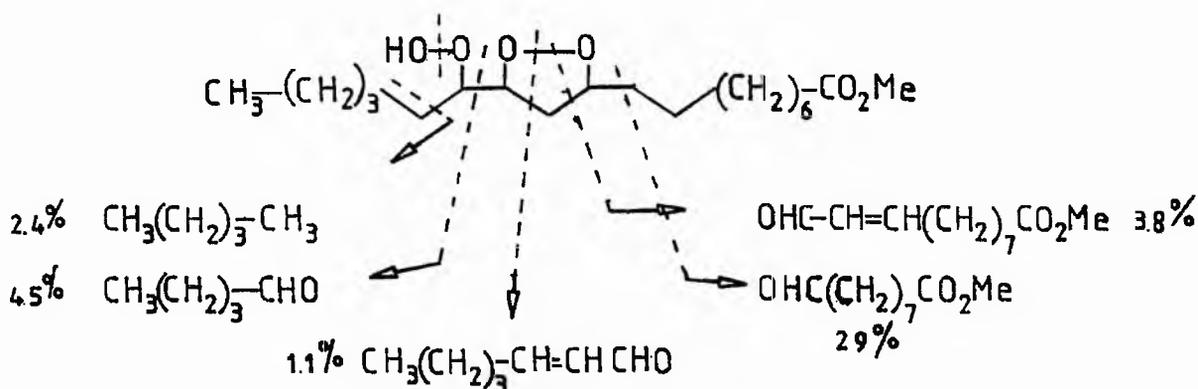
FIGURE 1.14



1,3- Cyclisation of 12- and 13- linolenate hydroperoxides

This process has also been observed in linoleate and arachidonate hydroperoxides. These cyclic peroxides may also breakdown to produce a mixture of products as illustrated in Figure (1.15) for linoleate.<sup>(23)</sup>

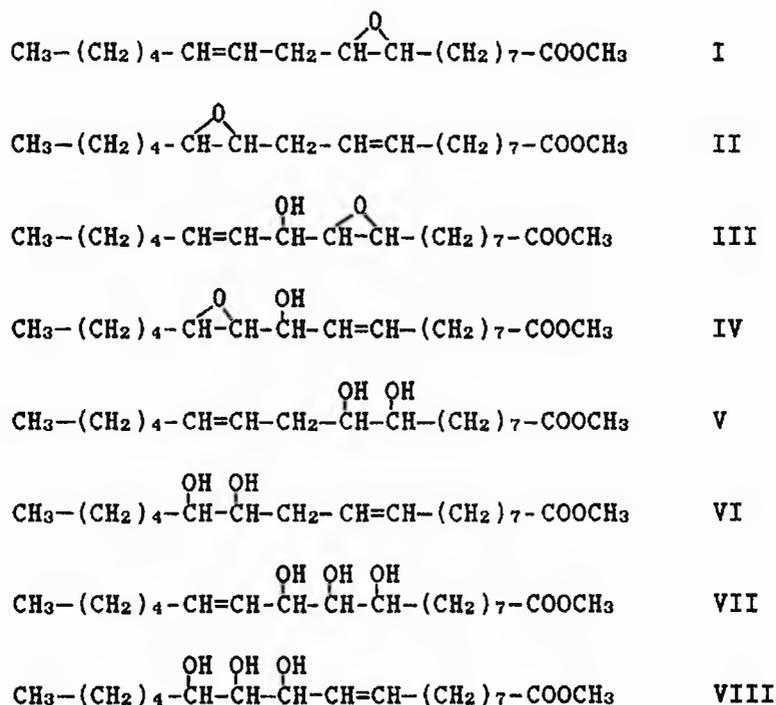
FIGURE 1.15



Mead et al<sup>(24)</sup> used a monolayer of linoleic acid on silica gel as a model for membrane autoxidation. He found that epoxides and not hydroperoxides were the major products of oxidation. Hydroperoxides appeared to be intermediate products but did not accumulate. Mead found that by changing the phospholipid composition of liposomes he could change the kinetics and products from autoxidation. By incorporating dipalmitoylphosphatidylcholine into soyabean phosphatidylcholine liposomes at a molar ratio 4:1, he found a large variety of products, Figure (1.16). They included epoxy (I

and II), hydroxyepoxy (III and IV), dihydroxy (V and VI), and trihydroxy (VII and VIII) fatty esters in the amounts 5, 13, 10 and 45% respectively. Hydroperoxides accounted for 26%. In pure soyabean phosphatidylcholine liposomes these products were found in only small concentrations. These findings may play an important part in elucidating the chemistry occurring in cell membranes.

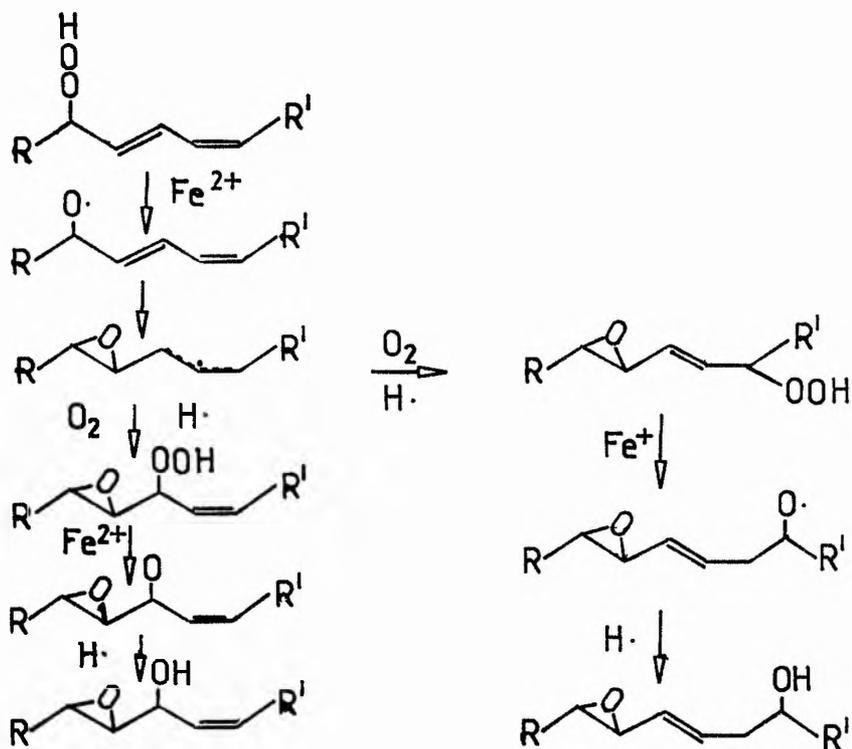
FIGURE 1.16



### 1.4.2 Ferrous-catalyzed Hydroperoxide Decomposition

It has been shown that linoleic acid hydroperoxides can be decomposed to epoxy alcohols by ferrous ions.<sup>(25)</sup> Figure (1.17) shows the ferrous - catalyzed decomposition of a hydroperoxide to an alkoxy radical. This is followed by addition of oxygen then hydrogen abstraction to yield the isomeric epoxy hydroperoxides. Formation of the epoxy alcohol is thought to occur by iron catalyzed decomposition of the hydroperoxide via an alkoxy radical.

FIGURE 1.17



$\text{R}, \text{R}'$  — fatty alkyl units

#### 1.4.3 Volatile Decomposition Products

Due to the volume of literature available on secondary oxidation products only an outline of the more salient features shall be given in this introduction.

Hydroperoxides, the primary oxidation products, may take part in a complex array of secondary reactions which are deteriorative in food systems. Numerous volatile and nonvolatile products have been identified from the further reaction of hydroperoxides at elevated temperatures. This is best illustrated by looking at the volatile decomposition products obtained from oxidised methyl linoleate hydroperoxides, Table (1.3)

TABLE 1.3

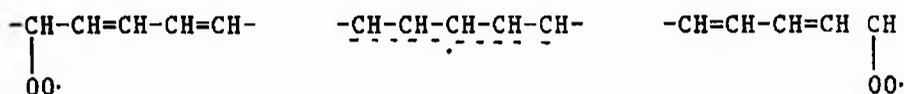
<u>Hydroperoxides</u>	<u>Volatiles</u>	<u>Autoxidation%</u>
9-OOH	Me octanoate	15
	2, 3 - Nonenal	1.4
	2, 4 - Decadienal	14
	Me 9 - oxononanoate	19
10-OOH	1 - Octen - 3 -ol	Tr
	Me 10-oxo-8-decanoate	4.9
12-OOH	2 - Heptenal	Tr
13-OOH	Pentane	9.9
	Pentanal	0.8
	1 - Pentanol	1.3
	Hexanal	15
	Other volatiles	18.7

The fragmentation route for the decomposition of monohydroperoxides involves homolytic cleavage of the hydroperoxy group to yield alkoxy (RO·) radicals and hydroxy (·OH) radicals, Figure (1.18). The next step is the carbon-carbon cleavage,  $\beta$  - scission, on either side



However, more recent research by Chan et al<sup>(26)</sup> found that the same major volatile cleavage products were obtained from either the 9- or 13- hydroperoxide isomer of linoleate. They suggest that this was due to positional isomerisation of the hydroperoxides via carbon oxygen scission, Figure (1.19).

FIGURE 1.19



#### Positional rearrangement of linoleate hydroperoxide

Porter et al<sup>(27)</sup> recently showed using <sup>18</sup>O labelled hydroperoxides that oleic acid hydroperoxides rearrange by a concerted rearrangement via a five-membered ring transition state. The reason for the differences is that for linoleate hydroperoxide rearrangement C-O scission would produce a stabilised pentadienyl radical, whereas oleate hydroperoxide would produce a simple allylic radical with significantly lower stabilization energy.

Research by Frankel et al<sup>(28)</sup> compared homolytic versus heterolytic cleavage of primary and secondary oxidation

products. The volatile thermal oxidation products were those expected from B-scission on both sides of the hydroperoxide group but no dialdehydes were found. In contrast, dialdehydes including malonaldehyde and 2, 4-hexadienediol were obtained from the acid catalyzed decomposition of cyclic peroxides and dihydroperoxides.

It is apparent from these studies that oxidation pathways are complex and there is the need for further research to determine which mechanistic pathways are relevant to the particular problem under investigation.

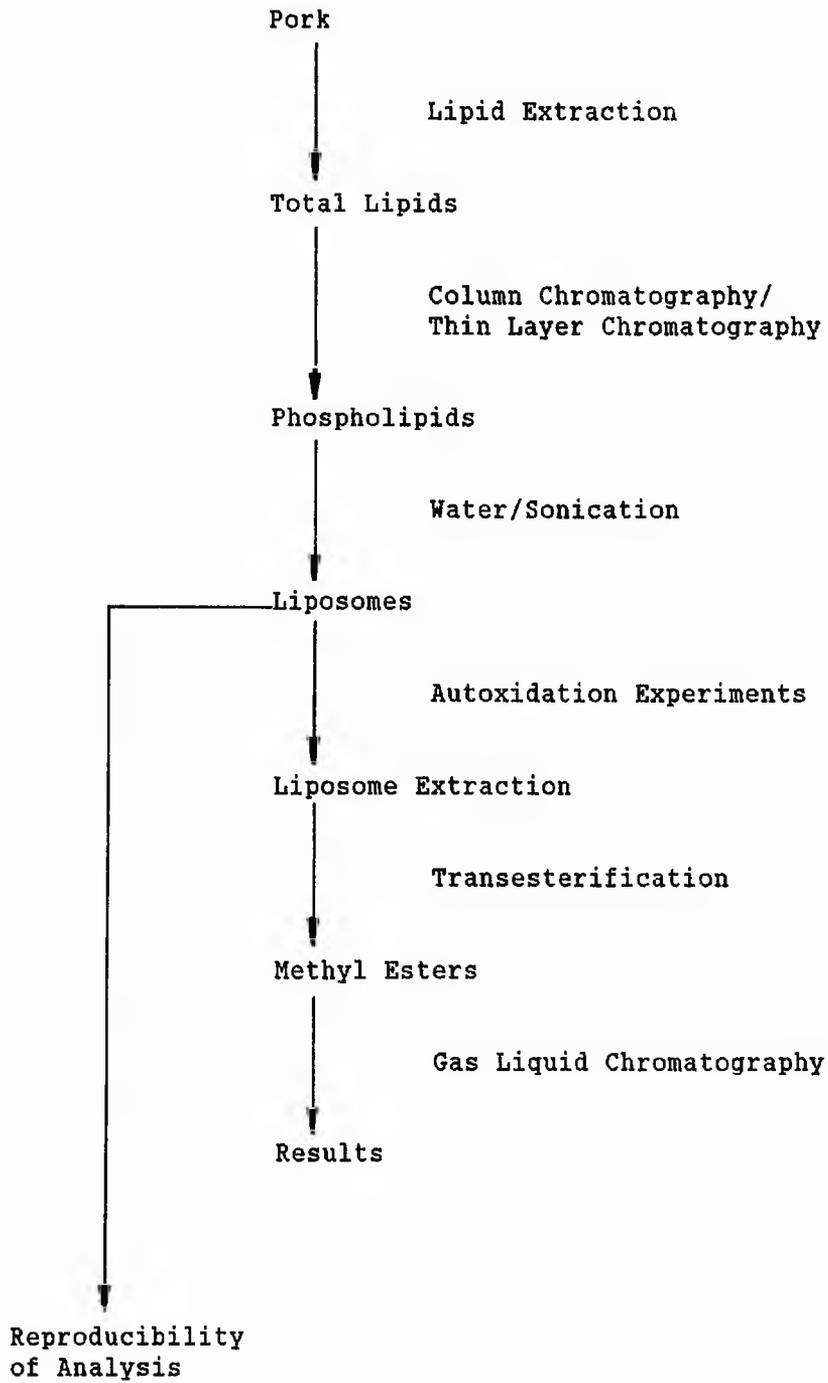
**CHAPTER 2**

## 2.1 INTRODUCTION

In carrying out autoxidation experiments it was of vital importance to establish reliable methodology. Presented in this chapter are methods to extract, isolate and optimise the analysis of phospholipids and their fatty acyl chains.

Initially, total lipid was extracted from pork by the method of Bligh and Dyer.<sup>(29)</sup> The phospholipids were separated from neutral lipids by column chromatography and their purity checked by thin-layer chromatography. The purified phospholipids were suspended in water and sonicated to produce liposomes. Autoxidation studies were then carried out on the freshly prepared liposomes. Oxidation was followed by monitoring the decrease in the phospholipid fatty acid methyl esters by gas-liquid chromatography. A summary of the techniques and procedures is provided in Figure (2.1).

FIGURE 2.1



## 2.2 EXPERIMENTAL

### 2.2.1 Lipid Extraction

100 g of meat (pork steaks) were homogenised for 4 minutes with 300 mls of chloroform-methanol (1:2). If the mixture had two liquid phases more chloroform-methanol was added until a single phase was obtained. The mixture was filtered through a sintered glass funnel and the tissue residue rehomogenised with 100 ml of chloroform and filtered once more. The two filtrates were combined, transferred to a 1 litre separating funnel containing 100 ml of 0.88 per cent potassium chloride in distilled water and the mixture shaken thoroughly before being allowed to settle. The solution was then biphasic (if not, further aqueous solution was added to ensure this). The lower phase was separated and the solvent removed using a rotary film evaporator. Acetone was sometimes necessary to remove water. The lipid was stored under nitrogen in a small volume of chloroform at  $-20^{\circ}\text{C}$  and used within two weeks of purification.

Total lipid extracts carried out by this method were in good agreement with literature values.<sup>(30)</sup>

2.2 - 3.6%

Own figures

1.85-3.27%

Yamouchi et al<sup>(30)</sup>

Variation in the lipid content did occur which was probably due to differences in the animals' diet and in the cut of meat.

### 2.2.2 Column Chromatography

Phospholipids were separated from total lipids by column chromatography. Silica was used as adsorbent (30 mg lipid per g silica)<sup>(31)</sup> and the chromatographic conditions which provided the best separation are provided in Table (2.1).

TABLE 2.1

<u>Class of Lipid</u>	<u>Solvent</u>	<u>Column Vols</u>
Neutral Lipid	Chloroform	10
	↓ increasing proportions of methanol	
Phospholipids	Methanol	10

### 2.2.3 Thin Layer Chromatography (TLC)

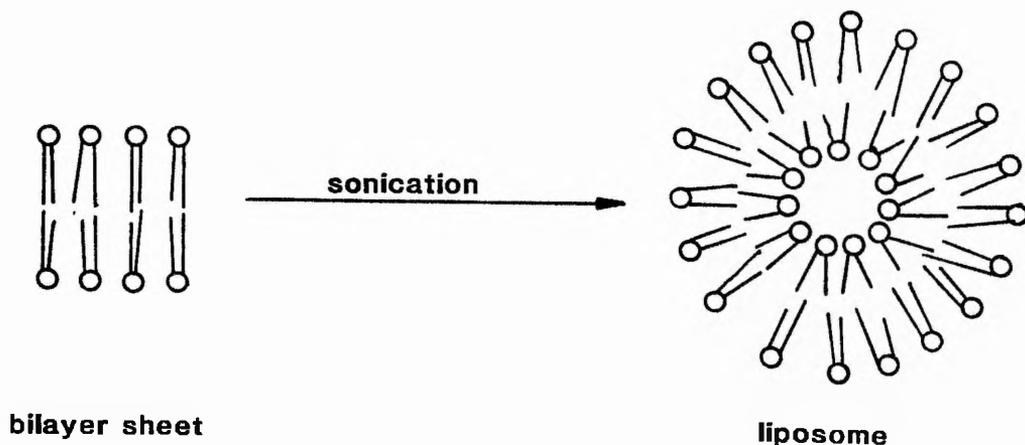
The purity of the isolated phospholipids was checked by TLC using silica gel G impregnated plates. A 0.2% 2,7-dichlorofluorescein solution in ethanol was used as a non destructive UV active spray reagent and 10% phosphomolybdic acid in ethanol was used as a destructive spray reagent which

charred all lipid components after heating at 120°C. Identification of individual lipid classes was obtained by comparison with standards. (32, 33)

#### 2.2.4 Liposome Preparation

Liposomes were prepared by adding an appropriate amount of deionised and distilled water to the dry phospholipid in a glass buchi flask. Plastic film was placed over the surface of the sonicating probe to prevent metal fragments entering the solution. The phospholipid/water mixture was then sonicated under nitrogen for approximately 20 minutes or until the originally milky suspension changed to a translucent one. Figure (2.4) illustrates the structural changes associated with this transformation. The liposome suspension was then centrifuged for 10 minutes in a MSE desk top centrifuge at speed-6 to remove any lipid not incorporated into the liposomes. The liposomes were then used immediately in oxidation experiments.

FIGURE 2.4



### 2.2.5 Base-Catalyzed Transesterification

The lipid sample, normally 10-50 mg, was dissolved in tetrahydrofuran (1 ml) or dichloromethane (1 ml) in a 10 ml test tube. To this was added 0.5 M sodium methoxide in anhydrous methanol (2 ml). The sample was heated for 10 minutes at 50°C then 0.1 ml glacial acetic acid was added followed by water (5 ml) and the esters were extracted with petrol 40-60 (2x5 ml).<sup>(34)</sup> The petrol layer was removed and stored over anhydrous sodium sulphate containing 10 per cent potassium bicarbonate. If the esters were not analysed immediately they were stored under nitrogen at -20°C. When small sample sizes (5-10 mg) were used the solvent was removed from the esters by blowing nitrogen over the surface then 0.1 ml of petrol added before analysis by gas-liquid chromatography.

### 2.2.6 Gas-Liquid Chromatography (GLC)

All GLC data presented in this thesis have been obtained using the equipment and conditions described below.

### Packed System

Analysis was performed on one of two instruments:-

- 1 A Pye Unicam PU 4500 gas chromatograph connected to a JJ Instruments CR-650 A chart recorder and a LBC data control computing integrator model 308. A 5 ft glass column (id - 4 mm) was packed with 10% SP 2340, as stationary phase, on chromosorb WAW 100-120 mesh. Detector -FID carrier gas - N<sub>2</sub> (40 mls/min). Injector and detector temperatures were both 240°C. Oven temperature programme - 160°C to 205°C, at 2°/min, held at 205°C.
  
- 2 A Hewlett Packard HP 5890 A gas chromatograph coupled to a HP 3393 integrator and recorder. All other conditions were as above.

### Capillary System

The analysis were performed on a Carlo Erba Series 2150 gas chromatograph connected to recorder and integrator as for the packed system instrument 1 above. Column, -25 metre fused silica with carbowax 20M as stationary phase. Carrier gas -H<sub>2</sub>, column flow -1.8 ml/min, septum purge 2.3 ml/min, split

ratio -40. The analysis was performed splitless for 30 seconds. Injector and detector were both set at 240°C.

In deciding which of the two gas chromatographic systems to use in future work a mixture of four fatty acid methyl esters (each 25% by weight, Sigma Chemical Company) were analysed. The mixture contained 18:2, 18:3, 20:4 and 22:6. The esters were run five times on both packed and capillary systems.

The means and standard derivations are presented below, Table (2.2).

TABLE 2.2

<u>PACKED</u>	<u>Ester</u>	<u>Mean</u>	<u>x6n-1</u>
	18:2	24.34	0.12
	18:3	24.23	0.14
	20:4	25.40	0.12
	22:6	26.03	0.30
<u>CAPILLARY</u>	<u>Ester</u>	<u>Mean</u>	<u>x6n-1</u>
	18:2	28.11	0.55
	18:3	27.04	0.44
	20:4	25.47	0.16
	22:6	19.37	0.87

The above results indicate the following:-

- 1 With samples run on the capillary column the greater the boiling point of the ester the lower was the recorded percentage. This may have been due to different volatiles of the esters in the injection port resulting in different amounts entering the column.
- 2 Samples run on the packed system gave results which were more reproducible.
- 3 The packed system had a shorter analysis time of 30 minutes as compared with 50 minutes for the capillary system.

Taking the above factors into consideration it was decided to use a packed column in the analysis of fatty acid methyl esters (FAME's) in future autoxidation experiments. The conditions of analysis were as described above.

#### 2.2.7 Pork Phospholipid Fatty Acids - Identification

To aid the identification of the pork phospholipid FAME's equivalent chain length (ECL) values were obtained. These values were calculated from the equation below where  $R_x$ ,  $R_n$ -

and  $R_{n+2}$  are retention times of the unknown ester and of saturated esters of chain lengths  $n$  and  $n+2$ .

$$\text{ECL} = 2 \frac{\log R_x - \log R_n}{\log R_{n+2} - \log R_n}$$

A standard mixture of cod liver oil and linseed oil whose FAME profile is known was also analysed and compared to that of the phospholipid FAME's. Table (2.3) presents ECL values and identifies the major pork phospholipid FAME's. The remaining FAME's were identified by comparison with literature ECL values. FAME's were analysed isothermally at 190°C on a packed column with 10% Sp 2340 as stationary phase. All other details are as described previously (2.2.6).

TABLE 2.3

n=16

<u>ECL</u>		<u>Identification</u>
<u>CLO/LO</u>	<u>Pork Phospholipid</u>	
[16.00]	[16.00]	16:0
16.76	16.63	16:1
[18.00]	[18.00]	18:0
18.61	18.61	18:1
19.50	19.52	18:2 (n-6)
	20.58	18:3 (n-6)
	22.80	20:4 (n-6)
23.90	23.93	20:5 (n-3)
26.18	26.23	22:5 (n-3)
26.62	26.67	22:6 (n-3)

#### 2.2.8 Presentation of Results

In presenting results an oxidation index (OI) has been used which is the ratio of the percentage composition of saturated esters 16:0 + 18:0, 18:0, or 17:0, divided by the percentage composition of an unsaturated ester x.

$$OI = 16:0 + 18:0/x, 18:0/x \text{ or } 17:0/x$$

This index has been used to follow autoxidation reactions and relies on the stability of 16:0 and 18:0 to autoxidation.

## 2.3 SYNTHESIS OF Di-17:0 PC

### 2.3.1 Isolation of Total Phospholipid from Egg Yolk

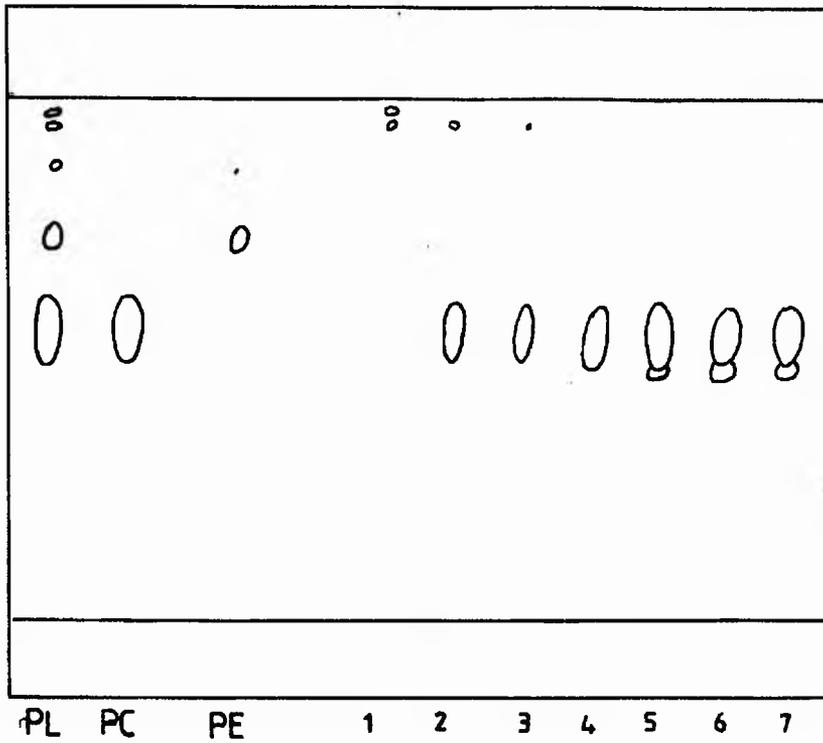
Five egg yolks were blended at room temperature with acetone (200 mls) in a homogenizer for 5 minutes. The homogenate was transferred to a conical flask and kept for 2 hours at 5°C. The mixture was centrifuged for 20 minutes at 3000 rpm (MSE centrifuge) then, the acetone layer was decanted from the residue which was mixed with a further 250 ml of fresh acetone and centrifuged as above. The residue was transferred to a round-bottomed flask and the remaining solvent removed under reduced pressure (water pump). The dry residue was extracted twice with 100 ml portions of chloroform-methanol (1:1 v/v). The solvent from the combined extracts was removed under vacuum then the residue dissolved in 25 ml petrol 40-60 and diluted 10-fold with acetone. The mixture was kept at 0-5°C until the supernatant cleared (approximately 1 hour) and then filtered through a sintered glass filter funnel. The precipitation procedure was repeated and the residue (phospholipid) dried in vacuo at 40°C. The phospholipids were dissolved in 20 ml chloroform-

methanol (19:1 v/v) and stored at  $-20^{\circ}\text{C}$ . The phospholipid was used within 1 week of purification.

### 2.3.2 Isolation of Lecithin from Egg Yolk Total Phospholipids

A slurry of 150 g aluminum oxide in 200 ml chloroform-methanol (19:1 v/v) was poured into a glass column (2.5 x 50 cm) then washed with 400 ml of the same solvent mixture. The chloroform-methanol solution of the total phospholipids (sec:2.3.1) was then applied onto the column which was eluted with chloroform-methanol (1:1 v/v). The eluate was collected in 50 ml fractions which were analysed by TLC. As can be seen from Figure (2.5) phosphatidylcholines were eluted in fractions 3-7. Fraction 4 was the only fraction used in the deacylation reaction as fractions 5-7 were contaminated with other compounds. The fatty acid profile of purified egg yolk phosphatidylcholine fraction 4 was determined, Table (2.4).

FIGURE 2.5



Developing System  $\text{CHCl}_3$  - MeOH -  $\text{H}_2\text{O}$  (65:35:8)

TABLE 2.4

<u>FAME</u>	<u>%COMPOSITION</u>
16:0	33.5
16:1	1.0
18:0	10.8
18:1	32.6
18:2	16.4
20:4	2.7
22:6	1.3

### 2.3.3 Synthesis of Glycerophosphorylcholine

This preparation was based on work carried out elsewhere.<sup>(35)</sup> The purified egg phosphatidylcholine was dissolved in 10 ml of diethyl ether and the solution cleared by centrifugation. 1 ml of 1 M methanolic tetrabutylammonium hydroxide was then added. After 1 hour at room temperature the solvent was decanted from the glassy precipitate of glycerophosphorylcholine then the vessel was rinsed with further portions of diethyl ether. The glycerophosphorylcholine was used immediately.

### 2.3.4 Synthesis of Diheptadecanoylphosphatidylcholine

A mixture of glycerophosphorylcholine (0.256 mmol) and sodium heptadecanoate (0.512 mmol) in 20 ml of methanol were evaporated to dryness in vacuo at 60°C. The powder was dried overnight under vacuum over P<sub>2</sub>O<sub>5</sub> and transferred to a 50 ml round-bottomed flask. Heptadecanoic anhydride (1.024 mmol) was then added and the flask closed under vacuum, and placed in a heating block of 80°C for three days. A thick homogeneous oil was obtained which solidified on cooling. The solid was dissolved in boiling chloroform and cooled in a refrigerator. A precipitate formed which was filtered off and washed with cold chloroform. The chloroform and washings contained almost pure phosphatidylcholine which was further

purified by column chromatography (50 g silica). Elution with 200 ml chloroform followed by 100 ml of chloroform-methanol (9:1). Phosphatidylcholine was eluted with 200 ml of chloroform-methanol (1:9). The purity of the synthesis was checked by TLC (chloroform-methanol-water 65:35:4 as developing solvent) which showed pure product. The purity was also checked by gas chromatography of the methyl esters. The product was over 99% pure. The yield of di (17:0) PC was 24.1 mg which represents 13.1%. The low yield in this reaction may have been because no rotation of the reaction mixture took place under vacuum. This may have been important in ensuring good contact between reactants. The synthesis of diheptadecanoylphosphatidylcholine was based on work by other researchers.<sup>(35)</sup>

#### 2.4 REPRODUCIBILITY OF ANALYSIS

To test the reproducibility and error in the experimental procedure a series of analyses were performed. Liposomes were prepared as described previously and divided into five portions. Each portion was extracted and transesterified to produce methyl esters. The five ester preparations were analysed twice each giving a total of ten sets of results. The mean was determined for each sample and the overall mean, sample standard deviation and error bars for all five samples collectively. The results are provided in Appendix (2.1) and on summary in Table (2.5). Results

were expressed as oxidation indexes and the statistical error bars determined for these indexes which may be used as an indication of the reproducibility of the method.

In analyzing the results it is assumed that experimental data follow a normal distribution.<sup>(36)</sup> From this we may determine a statistical error bar around a sample mean. This is expressed as follows:-

$$\text{upper limit} = u + 1.96 \quad \sigma / n$$

$$\text{lower limit} = u - 1.96 \quad \sigma / n$$

u = mean

$\sigma$  = standard deviation

n = number of data used in determination of 6

1.96 = factor which gives a 95% probability that a result shall lie within the error bar.

TABLE 2.5

FAME	OI		
	mean u	$\times 6n-1$	error bar
18:1	2.14	0.06	0.04
18:2	0.96	0.05	0.03
20:4	1.88	0.23	0.14
20:5	5.91	1.08	0.67
22:5	8.22	1.04	0.64
22:6	7.54	1.36	0.84

In order to see if the error bars were different at high levels of oxidation the experiment was repeated with oxidised liposomes. The results are presented in Appendix (2.2) and summarised in Table (2.6). They suggest that as oxidation progresses so the error increases. Variation would also be expected to occur with column ageing which may increase the error further.

TABLE 2.6

<u>FAME</u>	<u>OI mean u</u>	<u>x6n-1</u>	<u>error bar</u>
18:1	2.00	0.02	0.01
18:2	1.93	0.12	0.07
20:4	23.92	0.92	0.57
20:5	41.18	3.71	2.30
22:5	52.69	4.44	2.75
22:6	94.56	22.56	13.97

2.5 LIPOSOME AUTOXIDATION USING DIHEPTADECANOYL-  
PHOSPHATIDYLCHOLINE di 17:0 PC

This experiment investigated the stability of individual phospholipid fatty acids to autoxidation at 42°C.

Experimental

Liposomes prepared as described previously were suspended in glass tubes at a concentration of 10 mg/ml. The tubes were sealed and covered with foil to eliminate light then placed in a waterbath at 42 ± 0.2°C.

Samples were removed at the required time and di 17:0 PC (0.48 mg) added before subsequent extraction, transesterification and gas chromatography. Each sample was analysed twice. The percentage composition of the major esters are provided in Appendix (2.3).

Results and Discussion

The results are presented using:-

- 1 an oxidation index as described earlier and
- 2 by monitoring the percentage fall of individual esters.

In obtaining the actual decrease of esters the percentage internal standard (di 17:0 PC) was multiplied by a factor which took into account the obvious percentage increase in the ester. For example, from Appendix (2.3) at  $t=0$  the average value of 17:0 was 4.10% and after 71 hours 17:0 was 6.46%, therefore all data at 71 hours was multiplied by  $4.10/6.46$ . Appendices (2.4), (2.5) and (2.6) present results using (16:0 + 18:0), 17:0 and 18:0 as the basis of oxidation indexes respectively. Appendix (2.7) gives results for method 2 above. The complex kinetics of unsaturated hydrocarbon autoxidation was studied in detail by Farmer et al,<sup>(37)</sup> Bolland<sup>(38)</sup> and Bateman.<sup>(39)</sup> They stated that once the first hydroperoxides were produced monomolecular decomposition occurred producing free radicals. This mechanism is believed to hold up to 0.5% oxidation.<sup>(3)</sup> The reaction then becomes bimolecular and involves hydroperoxide dimer formation (hydrogen bonded) and subsequent decomposition. It can be seen from Figure (2.6) that liposome autoxidation followed classical autocatalytic kinetics. As expected the greater the unsaturation of the fatty acyl chain the more prone to peroxidation. Although the kinetics are complex an attempt was made to try and obtain a straight line from the exponential region of the curve using the first order rate equation below.

$$\ln \left\{ \frac{a}{a-x} \right\} = Kt$$

a = oxidation index at the start of the experiment

a-x = oxidation index after time t

k = rate of reaction

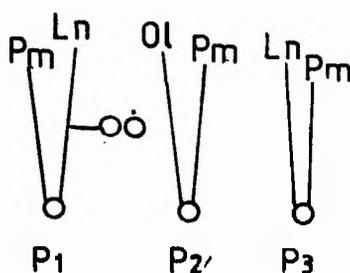
Under no circumstances must this be interpreted as first-order kinetics. The reaction is an autocatalytic chain reaction and the above equation has been used only as a method of comparing relative rates. Figure (2.7) presents straight line plots for several of the FAME's. The data is tabulated in Appendices (2.8-2.11).

Only those points that appeared to lie on a straight line were used in the determination of k.

All the different methods for the presentation of data gave results of similar magnitude. With 17:0 as OI there was an apparent increase in 18:0 and decrease in 16:0. It is doubtful if these changes were real. From Appendix (2.3) changes were seen in the content of 16:0 and 18:0 from two different analysis of the same sample. However, there did seem to be a small overall decrease which was reflected in a slightly lower rate. A possible reason for this observation is that there may have been a minor polyunsaturated ester below palmitate in the chromatogram. This did not explain the apparent increase in stearate but could be the

reason for the overall lower rates. There was a significant increase in rate between oleate (1 double bond) and linoleate (2 double bonds). This was due to the enhanced reactivity of the double allylic methylene group in linoleate, ie, the radical formed is stabilised over a 5-carbon centre whereas in oleate the radical is only stabilised over a 3-carbon centre. However, the difference in rate was considerably less than reported in previous studies (40,41). Zwierzykowski et al (40) found the difference in rate between methyl linoleate and methyl oleate to be temperature dependant, varying from 10 to 30. A more recent study by Wong and Hammond (39) found that linoleate oxidised 10 times faster than oleate. From Appendix (2.11) the difference in rate between linoleate and oleate was only 5.3 (17:0 as internal standard). A decrease in difference of rates of oxidation may be due to the structure within the membrane. Figure (2.6) shows three neighboring phospholipid molecules P<sub>1</sub>, P<sub>2</sub> and P<sub>3</sub> in a bilayer.

FIGURE 2.6



P<sub>1</sub> and P<sub>3</sub> contain fatty acyl chains of linoleate (Ln) and palmitate (Pm). P<sub>2</sub> contains oleate (Ol) and palmitate (Pm). Since these three fatty acyl chains are the most predominant in the FAME profile of the pork phospholipids and that phospholipids normally have a saturated fatty chain adjacent to an unsaturated chain then this may actually represent neighboring phospholipids in the membrane. If a peroxy radical is formed from linoleate (Ln) on phospholipid P<sub>1</sub> hydrogen abstraction by the peroxy radical would preferentially come from the doubly allylic linoleate of P<sub>3</sub>. However, due to its position in the membrane this does not occur and hydrogen abstraction comes from oleate of phospholipid P<sub>2</sub>. If there was no movement of phospholipids in the membrane then the rate of oxidation of linoleate and oleate would probably be of similar magnitude. However, there can be considerable movement of phospholipid within the bilayer which shall be discussed in chapter three.

Recent research by Cosgrove et al<sup>(42)</sup> demonstrated that the rate of autoxidation of polyunsaturated fatty acids (PUFA) was directly related to the number of doubly allylic positions present in the molecule. Therefore docosaheptaenoate (22:6) which has five doubly allylic positions oxidised five times faster than linoleate. Arachidonate (20:4) with three doubly allylic positions oxidised three times faster than linoleate. With reference to Table (2.7) and Figure (2.7) we can see that there were considerable

differences in the rates of autoxidation between the individual polyenes.

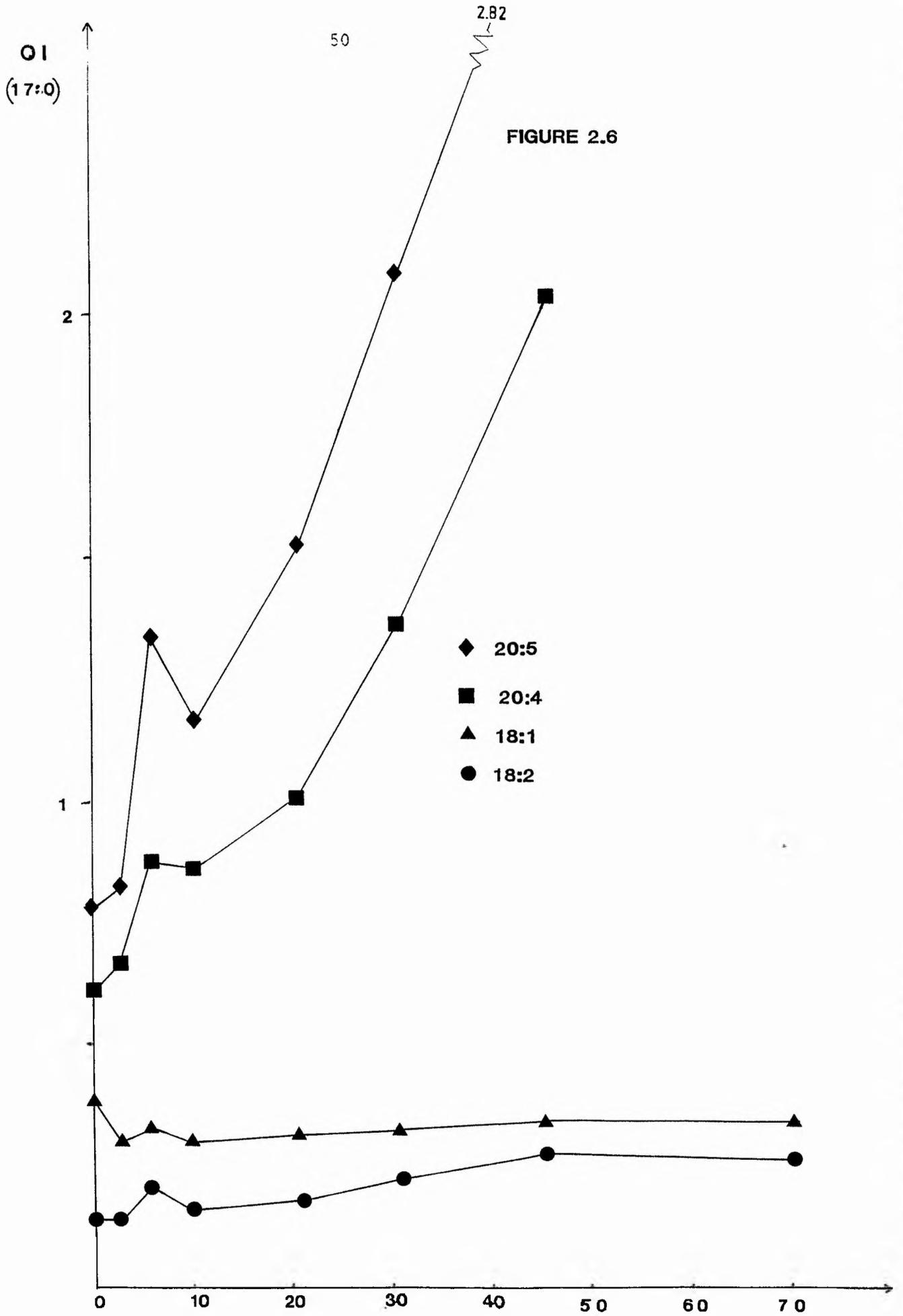
TABLE 2.7

Fatty acyl Chain	No of doubly allylic positions	Relative Rates	
		Cosgrove et al. <sup>(42)</sup>	Liposomes
18:2	1	1	1
20:4	3	3	1.6
20:5	4	4	1.9

As expected the more unsaturated the fatty acyl chain the more rapidly it oxidised. However the differences were significantly lower than reported by Cosgrove. As for oleate/linoleate these differences may again have been due to the restricted movement of the fatty acyl chains of the phospholipids within the bilayer. The relative rates of 22:5 and 22:6 were not determined as the two FAME's were not sufficiently resolved in the chromatogram to give reliable results. However, in other experiments (chapter four) long induction periods were observed which made it difficult to compare relative rates. In addition variation in the relative rates of oxidation of individual fatty acyl chains were observed. For example, in experiment 4.5 the relative rates between 18:2, 20:4 and 20:5 were 1:3.02:4.24 which were in better agreement with Cosgrove et al.<sup>(42)</sup> Factors which may contribute to the variation

in rate of peroxidation are discussed in greater detail in chapter four.

Although there is some doubt over the purity of palmitate in the chromatogram, the results indicate that the course of an autoxidation experiment may be followed by the use of phospholipid saturated fatty acyl chains in an oxidation index.



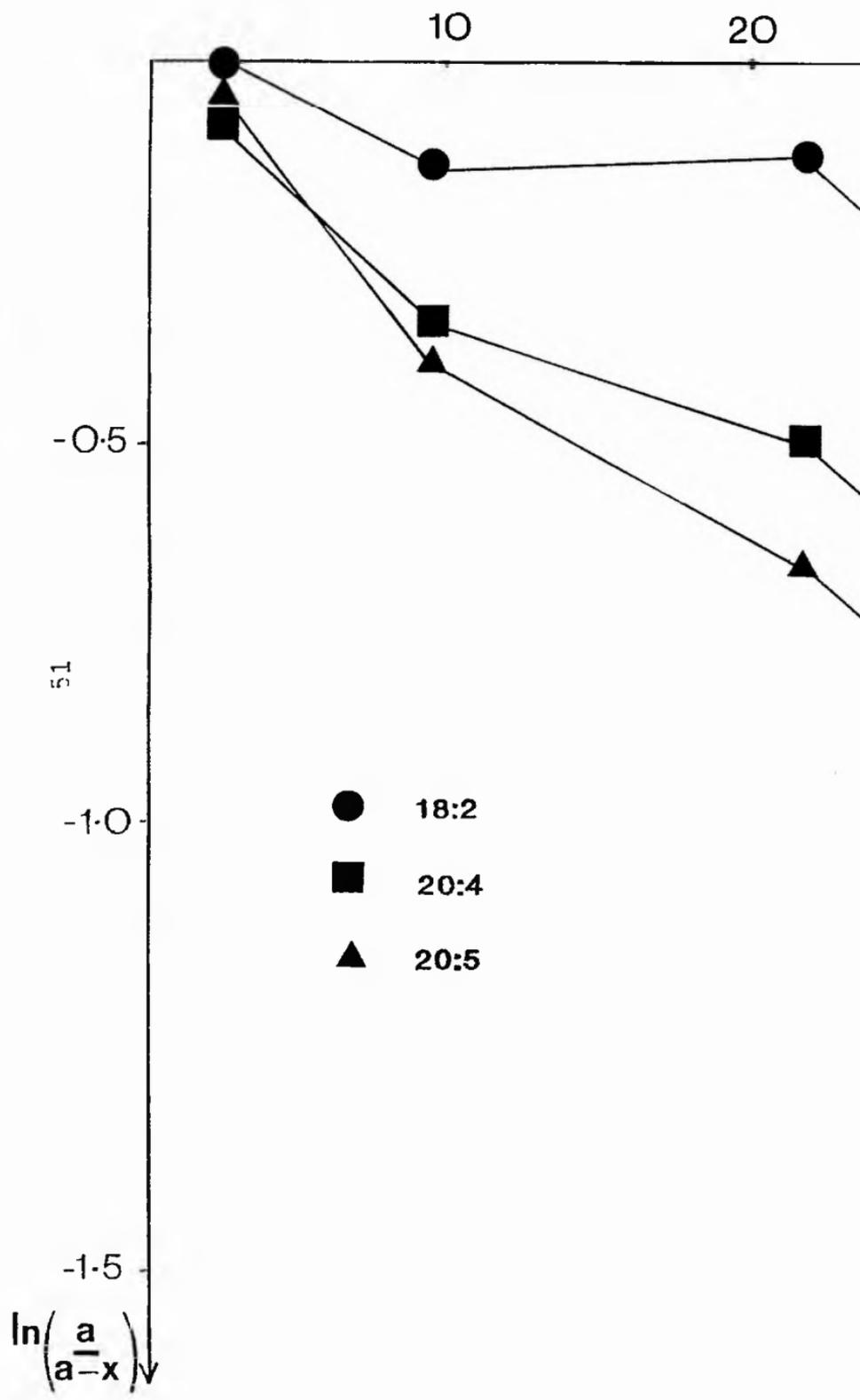


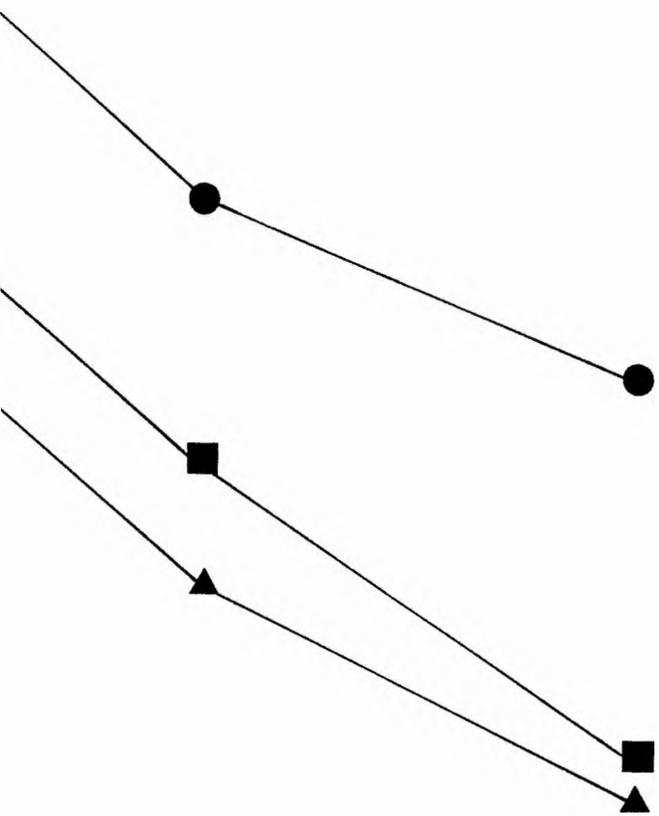
FIGURE 2.7

Time (Hours)

30

40

50

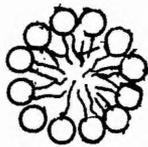


## CHAPTER 3

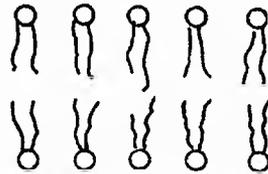
### 3.1 INTRODUCTION

Phospholipids are amphipathic in nature, that is, they are partly hydrophobic and partly hydrophilic. It is this property which allows them to exist as a bilayer and to be described as the basic structural framework of living membranes. In water individual phospholipid classes do not all form liposomal bilayers. The structure they adopt depends largely on their shape and the relative size of the polar head group compared to the hydrocarbon tail.<sup>(43)</sup> For example, lysophospholipids with a wide head group and a narrow hydrocarbon chain form spherical micelles while phosphatidylethanolamines, with saturated fatty chains, a narrow head group and a wide hydrocarbon tail, form planar bilayers, Figure (3.1).

FIGURE 3.1



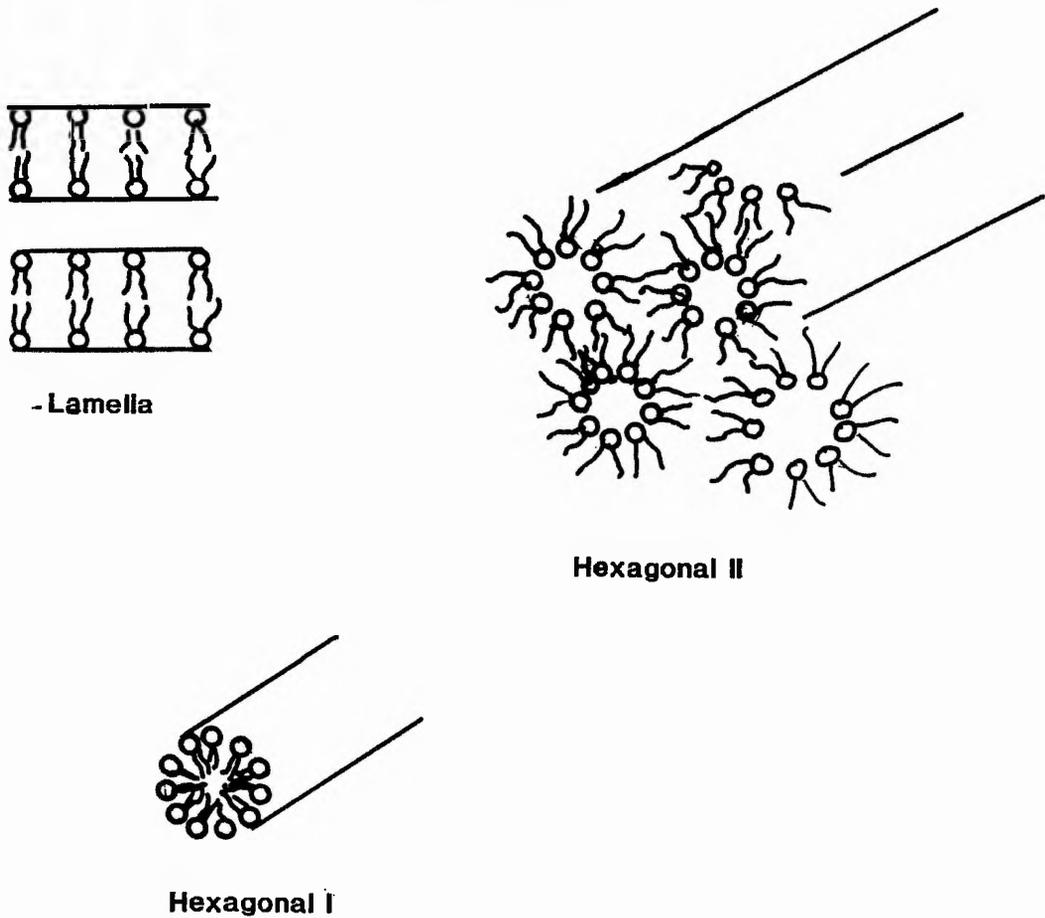
spherical micelle



planar bilayer

In addition to the capillary melting point phospholipids exhibit a number of other phase changes at lower temperatures. These thermotropic changes have been analysed by differential thermal analysis and are similar to those of liquid crystals.<sup>(44)</sup> The transition temperature,  $T_c$ , represents the transformation from gel to liquid-crystal which is an endothermic process. Above  $T_c$  and under the influence of heat, phospholipids in water have been shown to adopt several other different types of phase organization, Figure (3.2).

FIGURE 3.2



There is much evidence to support the existence of different types of phospholipid movement within biological membranes. This has led to bilayers being described as three-dimensional fluids.<sup>(45)</sup> Molecules within a membrane can undergo many different types of movement which under some circumstances can lead to total phase separation. Phospholipids can undergo lateral diffusion (side-ways movement) which may result in like molecules aggregating and crystallising. This can occur from a fall in temperature. Phospholipids can also flip-flop: ie the movement of a molecule on one side of the bilayer to the other side of the same bilayer. Other molecular movements associated with bilayers include rotation within the plane of the membrane and the up and down movement at right angles to the plane, normally referred to as 'bobbing'. Phase separations within liposomes have been observed by numerous researchers.<sup>(46-49)</sup> Ladbroke et al<sup>(46)</sup> showed that liposomes composed of dimyristoyl lecithin and distearoyl lecithin when cooled caused molecular migration within the bilayer to give crystalline regions corresponding to the two compounds. Recently, it has also been shown that  $\text{Ca}^{2+}$  can induce phase separations within mixed PC, PS and PE membranes.<sup>(50)</sup> It is believed that the separation is due to the chelation of  $\text{Ca}^{2+}$  to the negatively charged PS.

Taking all these factors into consideration we realise that the structure of phospholipids in water is dependent on many factors. This study set out to investigate the morphology of liposomes

produced from mixed phospholipids extracted from pork. Further experiments are described looking at structural modifications brought about by autoxidation.

### 3.2 EXPERIMENTAL

Electron microscopy was used to study the morphology of liposomes. This was carried out at Unilever Research, Colworth House. Two techniques were used in the investigation.

#### Negative Staining of liposomes

1. One drop of sample was placed on a Formvar coated 200 mesh copper grid.
2. The sample was washed with 1-2 drops of methylamine tungstate stain.
3. The grid was blotted dry as quickly as possible to minimise interaction between the stain and the liposomes.
4. The sample was then analysed immediately under the electron microscope (JEOL 100 CX).

Freeze-Fracture of Liposomes

1. One drop of sample was placed on a copper stub and plunged into a liquid nitrogen slush ( $-210^{\circ}\text{C}$ ).
2. The stub was transferred into a freeze-fracture machine (Polarow/MRC hybrid), and the temperature was raised to  $-98^{\circ}\text{C}$ .
3. The sample was fractured with a cold knife ( $-180^{\circ}\text{C}$ ) whilst under vacuum  $5 \times 10^{-6}$  TORR or lower.
4. Immediately after the fracture was made the sample was tilted to  $45^{\circ}$  and shadowed with platinum, then tilted to  $90^{\circ}$  and shadowed (backed) with carbon.
5. The replica was floated off the sample by immersing the stub in distilled water.
6. The replica was then picked up on a 400 mesh copper grid, blotted dry and then immersed in chloroform/methanol (2:1) for 10 minutes to remove any residual liposomes.
7. The replica was then examined under the electron microscope (JEOL 100 CX).

In photographing freeze-fracture replicas the samples are illuminated by light from one direction. This has the effect of producing shadows over the surface of the liposomes. When the liposomes suspension is cut, the liposomes may be broken open, in which case we look into them. Or alternatively, they may stay intact as the ice fractures around them, in which case we look over their outer surface.

### 3.3 RESULTS AND DISCUSSION

The first objective in the study was to see if liposomes were produced upon sonication of the mixed phospholipids. Figure (3.3) illustrates a typical preparation visualised by negative staining. Liposomes were seen to be produced but in small multilamellar clusters. It was felt that the negative stain may have interacted with the liposomes to create unrepresentative structures. Therefore samples were visualised by freeze-fracture microscopy to prove or disprove this proposal, Figure (3.4). The liposomes are seen to be small, unilamellar vesicles of varying size. Using the two techniques the liposomes were oxidised for one day at 50°C in darkness. On examination, both methods showed the formation of large vesicles, Figure (3.5). It was not known if this change was due to oxidation or a result of temperature dependent phase changes which induced liposome fusion producing the large vesicles. As mentioned in the introduction phospholipids can exhibit phase organizations in water other than that of a bilayer.

Verkleij et al<sup>(51)</sup> has shown that mixed phospholipid unilamellar vesicles may fuse to form large multilamellar vesicles on increasing the temperature from 0 to 50°C. This process is associated with the formation of lipidic particles which, has been suggested reflect the presence of intra-bilayer inverted micelles representing the transformation from bilayer to hexagonal (H<sub>11</sub>) phases. The group identified lipidic particles as nodules and pits on the liposome surface. Figure (3.6) shows a liposome with small pits which may represent a lipidic particle and help explain the above transformation at 50°C.

Further experiments were performed using the negative staining technique to look for any subtle changes occurring in the liposomes during incubation at 40°C. A liposome preparation was divided into two portions. One portion was incubated in air in a sealed vial at 40°C and the other portion incubated under nitrogen in a sealed vial at the same temperature. The samples were examined prior to oxidation, after 3 days and after 6 days. Significant changes were observed between the samples after oxidation for 3 and 6 days. The sample stored under nitrogen, Figure (3.7), showed an increase in phospholipid stacks with increased incubation time. These were absent from the oxidised sample, Figure (3.8). Van Duijn et al<sup>(52)</sup> studied the influence of peroxidation on the phase behaviour of PC and PE. He used an amino indicating probe to study the effect of oxidation on the free amino groups of PE. The probe lost 33% reactivity towards PE

after a 24 hour incubation at 40°C. The authors suggest the formation of a Schiff base (between PE and malondialdehyde, a secondary oxidation product) which was also confirmed by  $^{13}\text{C}$  NMR. Both  $^{31}\text{P}$  NMR and x-ray diffraction studies revealed a transformation from a lamella organization at 25°C to a hexagonal  $\text{H}_{11}$  phase at 40°C in a fresh PE dispersion. After 6 hours of peroxidation the bilayer was stable up to 70°C. At this degree of oxidation there was a 26% drop in the free amino group of PE. It may be possible that the phospholipid stacks were unsaturated phospholipids probably PE whose bilayer to hexagonal ( $\text{H}_{11}$ ) transitions occur at a lower temperature and were therefore able to migrate from the membrane. Where oxidation had occurred the formation of schiff-base cross links may have restricted this migration.

Another experiment set out to establish whether any structural changes occurred in liposomes when oxidised under frozen storage at -22°C. The liposome suspension was stored frozen on a copper stub to prevent having to thaw the liposomes with the possibility of phase transitions occurring. However as can be seen from Figure (3.9) all the liposomes become clustered together as the ice front was formed. Because of this it was extremely difficult to obtain any useful information as oxidation progressed.

**FIGURE 3.3**

**MAG x40 000**



**FIGURE 3.4**

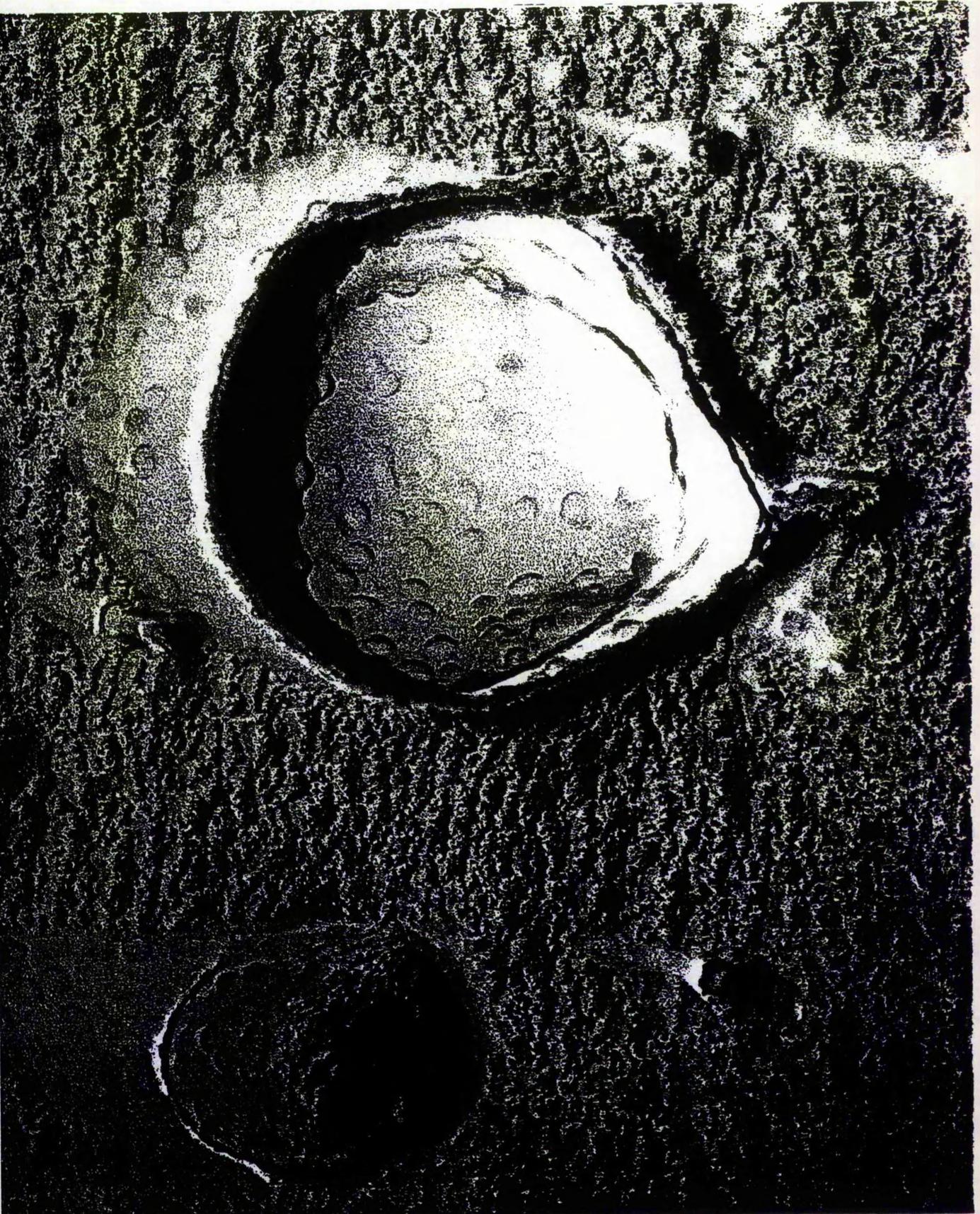
MAC  $\times 40\ 000$



**FIGURE 3.5**MAC  $\times 40\ 000$ 

**FIGURE 3.6**

MAG x 200 000



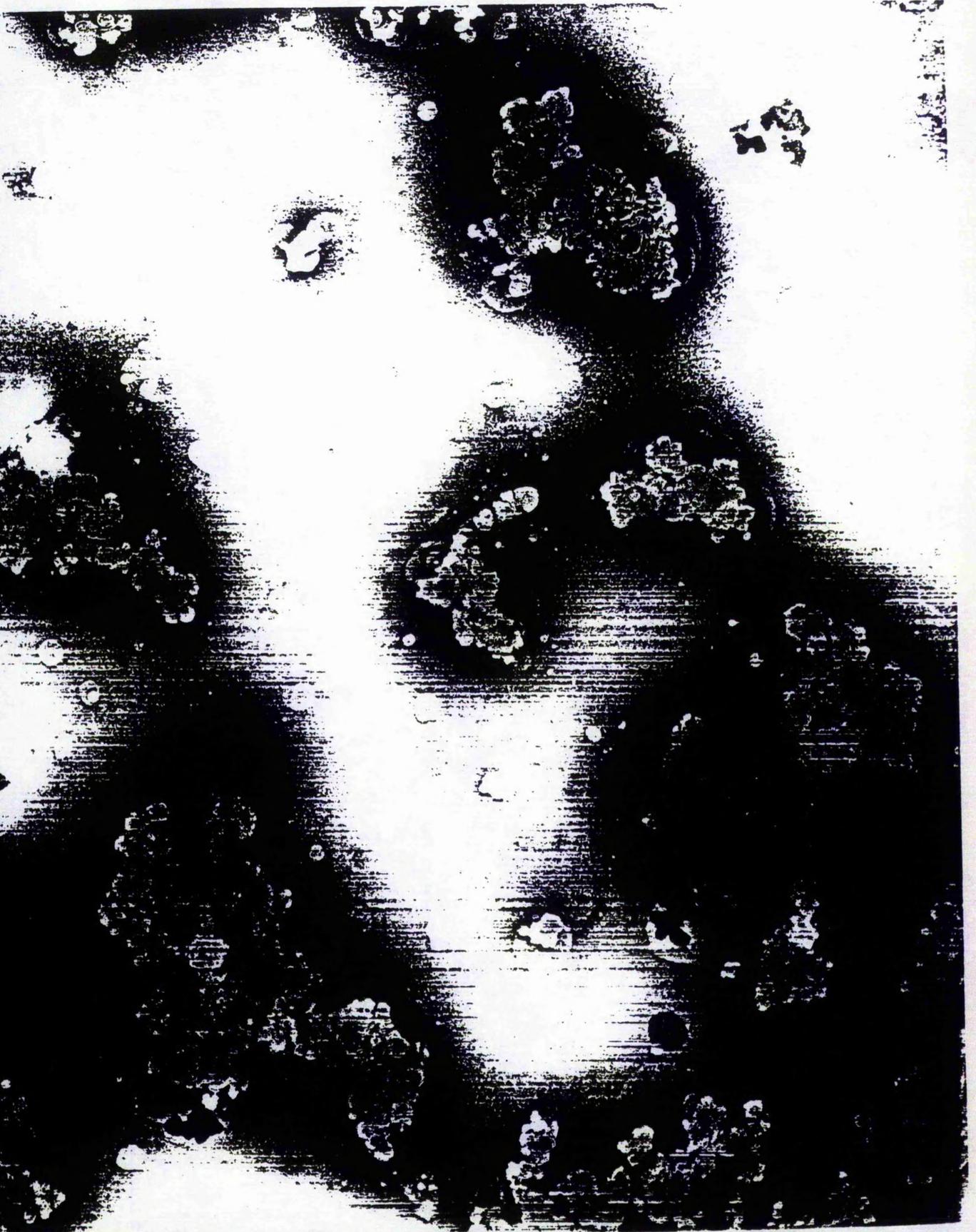
**FIGURE 3.7**

MAC x 40 000



**FIGURE 3.8**

**MAG x 40 000**



**FIGURE 3.9**

MAG x 40 000



## CHAPTER 4

#### 4.1 EFFECT OF ADDITIVES ON LIPID OXIDATION IN MEAT

Oxidation of phospholipid fatty acyl chains in membranes has become an important issue due to the realisation of its connection with a variety of pathological conditions<sup>(53-56)</sup> and its deteriorative effect in foodstuffs.<sup>(57-59)</sup> There exist many reports in the literature on lipid autoxidation in model systems including liposomes.<sup>(60-68)</sup> Several studies investigated the kinetics of membrane oxidation while others investigated the products of oxidation.

This chapter deals with the effect of various additives on the rate of oxidation of pork phospholipid liposomes. Oxidation was followed by the decrease in phospholipid FAME's as indicated by an oxidation index, OI, as described in chapter two. The additives investigated in the study were substances either found naturally in meat or added to meat to enhance characteristics such as taste, stability and handling. A brief outline of their occurrence, use and function is provided below.

##### 4.1.1 Effect of Transition Metal Ions

Several research groups have published literature covering the occurrence and pro-oxidant activity of transition metal ions in meat and other food products.<sup>(69-71)</sup> Their mode of action is believed to involve the decomposition of trace

amounts of lipid hydroperoxides. However, there still exists some uncertainty as to whether these metals are active as free ions or in chelated form like iron in proteins. Both copper and iron have been investigated as catalysts in lipid peroxidation.<sup>(72,75)</sup> Experiments described in this chapter (in model systems) have chiefly been concerned with copper (II) induced lipid peroxidation both in aqueous and non-aqueous environments.

#### 4.1.2 Effect of Sodium Nitrite

Sodium nitrite is added to meat because of its ability to impart flavour, colour and bacterial stability. There is also evidence in the literature concerning the antioxidant behaviour of nitrites<sup>(76-77)</sup> although the mechanism by which nitrite prevents lipid oxidation is still unclear. Pearson et al<sup>(76)</sup> suggested that nitrite may act on the lipid components of membranes or denature natural pro-oxidants present in muscle. Experiments were designed to investigate the effect of sodium nitrite alone and in the presence of copper (II) on the oxidative stability of liposomes.

#### 4.1.3 Sodium Chloride and Polyphosphates

Sodium chloride is added to meat for several reasons. Firstly salt is believed to extract membrane proteins

resulting in better binding properties of the meat. Salt also promotes water uptake which can result in the swelling of meat to a level of 30-40% in massaged ham.<sup>(78)</sup> The consumer also demands salt to be present in meat products. Phosphates are added to meat to reduce the amount of sodium chloride required and also to create conditions where meat can bind together. Several studies have produced different conclusions as to the effect of sodium chloride on lipid oxidation.<sup>(79-81)</sup> Research on fatty fish showed sodium chloride to be pro-oxidant at low concentrations and antioxidant at high concentrations. The pro-oxidant behaviour correlated with the production of free fatty acids which are suspected to promote lipid peroxidation.<sup>(30)</sup> However the exact mechanism of interaction still remains unclear. Experiments were undertaken to investigate the action of sodium chloride and polyphosphate on the rate of oxidation of liposomes both with and without additives.

#### 4.1.4 Effect of Ascorbic Acid

Ascorbic acid is used in the curing of meat. It has been found to prevent the build up of nitrosamines and to enhance the flavour of the product. The literature suggests that ascorbic acid may act in several different ways. It chelates metal ions, effectively removing them from solution. It may also reduce metal ions such as ferric to ferrous - a more

effective peroxide splitter.<sup>(83)</sup> Ascorbic acid may also be oxidised by molecular oxygen in the presence of transition metal ions.<sup>(84)</sup> It has been shown to act as pro-oxidant at low levels and as anti-oxidant at high levels. The anti-oxidant behaviour has been attributed to a radical chain breaking mechanism.<sup>(85)</sup> It was decided to look at the effect of low concentrations of ascorbic acid on Cu(II) induced peroxidation.

#### 4.2 PRESENTATION OF RESULTS

All results presented in this chapter are in the form of an oxidation index (OI) as represented by 16:0+18:0/x. At the bottom of each column in tables of results is a number in brackets. This is the sum of the percentage of 16:0 and 18:0 in the chromatogram for each sample. Therefore it is simple procedure to obtain the percentages of unsaturated esters in the chromatogram.

For example:-

$$\text{OI} = \frac{(\text{number in brackets})}{\% \text{ ester}}$$

$$\text{ester}\% = \frac{(\text{number in brackets})}{\text{OI}}$$

#### 4.3 REAGENTS

All chemicals used in the oxidation experiments were of AR grade. Sodium chloride was of ARISTAR grade and water was both distilled and deionised and showed no trace of the metals Cu, Co and Fe by atomic absorption spectroscopy.

#### 4.4 Fe(II) - ASCORBIC ACID LIPOSOME PEROXIDATION

This experiment was carried out to investigate the catalytic activity of Fe(II)/ascorbic acid on the autoxidation of phospholipid liposomes at 42°C.

Liposomes were prepared at a concentration of 9.17 mg/ml. They were then divided into two portions and placed in 10 ml glass vials in 1 ml aliquotes. Each aliquot was added to one of the solutions indicated below, then the vials closed and the experiment started immediately.

S1 - liposomes (1ml), water (1.5 ml)

S2 - liposomes (1ml), FeSO<sub>4</sub> ( $2.667 \times 10^{-4}$  M 1 ml), ascorbic acid ( $1.33 \times 10^{-2}$  M, 0.5 ml)

Results from the FAME analysis are provided in Appendix (4.1), Figure (4.1) and discussed in section (4.10).

#### 4.5 NaNO<sub>2</sub> LIPOSOME PEROXIDATION

Liposomes were prepared at a concentration of 9.65 mg/ml. The liposome preparation was divided in two and to each half was added one of the solutions below. Peroxidation was carried out at 42°C in 10 ml glass vials in darkness.

S1 - liposomes (1 ml), water (1 ml)

S2 - liposomes (1 ml), NaNO<sub>2</sub> (1.365 x 10<sup>-4</sup> M, 1 ml)

Results are presented in Appendix (4.2), Figure (4.2) and discussed in section (4.10)

#### 4.6 Cu(II)/NaNO<sub>2</sub>/ASCORBIC ACID LIPOSOME PEROXIDATION

Liposomes were prepared at a concentration of 8.75 mg/ml. The liposomes were divided into four portion and each portion treated under one of the experimental conditions below. Peroxidation was carried out at 42°C (10 ml vials) in darkness.

S1 - water (2 mls)

S2 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $4.712 \times 10^{-4}$  M, 1 ml = 29.9 ppm CuII)  $\text{H}_2\text{O}$   
(1ml)

S3 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $4.712 \times 10^{-4}$  M, 1 ml = 29.9 ppm CuII) Sodium  
Nitrite ( $1.365 \times 10^{-4}$  M 1 ml)

S4 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $4.712 \times 10^{-4}$  M, 1 ml = 29.9 ppm Cu(II))  
ascorbic acid ( $1.365 \times 10^{-4}$  M, 1 ml)

Results are presented in Appendix (4.3), Figure (4.3) and discussed in section (4.10).

#### 4.7 Cu(II)/NaCl LIPOSOME PEROXIDATION

The following experiment was concerned with the action of Cu(II), sodium chloride and Cu(II)/sodium chloride on the rate of autoxidation of phospholipid liposomes.

Liposomes were prepared at a concentration of 8.75 mg/ml. Aliquots (1 ml) of the liposome suspension were added to one of the solutions below. Peroxidation was carried out at 42°C (10 ml vials) in darkness.

S1 - water (1 ml)

S2 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $5.128 \times 10^{-4}$  M, 1 ml)

S3 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $4.712 \times 10^{-4}$  M, 1 ml = 29.9 ppm Cu(II))

S4 - Sodium Chloride ( $1.026 \times 10^{-3}$  M, 1 ml)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $4.712 \times 10^{-4}$  M, 1 ml = 29.9 ppm Cu(II))

The results are tabulated in Appendix (4.4), graphically presented in Figure (4.4) and discussed in section (4.10).

#### 4.8 Cu(II)/POLYPHOSPHATE LIPOSOME PEROXIDATION

This experiment investigated the rate of peroxidation of liposomes in the presence of copper (II) and polyphosphate. Liposomes were prepared at a concentration of 10.2 mg/ml and to 1 ml portions was added one of the following solutions. Peroxidation was performed at 42°C in 10 ml vials in darkness.

S1 - water (2 mls)

S2 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $5.712 \times 10^{-4}$  M, 1 ml = 36 ppm Cu(II)),  $\text{H}_2\text{O}$   
(1ml)

S3 -  $\text{Na}_5\text{P}_3\text{O}_{10}$  ( $1.430 \times 10^{-3}$  M, 1 ml)  $\text{H}_2\text{O}$  (1 ml)

S4 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $5.712 \times 10^{-4}$  M, 1 ml = 36 ppm Cu(II)),  
 $\text{Na}_5\text{P}_3\text{O}_{10}$  ( $1.430 \times 10^{-3}$  M, 1 ml)

The results are tabulated in Appendix (4.5), presented in Figures (4.5 and 4.6) and discussed in section 4.10.

#### 4.9 THE EFFECT OF SONICATION AND DILUTION ON THE RATE OF PEROXIDATION

This experiment was carried out to study the effect of sonication and liposome dilution on the rate of peroxidation. Phospholipids were dispersed in water at a concentration of 12 mg/ml, divided into two portions and each portion added to one of the solutions below. Peroxidation was carried out at 42°C in darkness in 10 ml vials.

Portion 1 - S1 - unsonicated (hand shaken liposomes) (1 ml)

Portion 2 - S2 - Sonicated (until translucent) (1 ml)

S3 - S2 (1 ml) diluted with water (2 ml)

S4 - S2 (1 ml) diluted with water (4 ml)

The results are set out in Appendix (4.6), graphically presented in Figure (4.7) and discussed in section (4.10).

#### 4.10 DISCUSSION ON EXPERIMENTS 4.4 TO 4.9

During the course of these experiments it was noticed that variation in the rate of oxidation of the control samples (liposomes oxidised without additives) occurred. In some experiments little or no induction period was observed (experiment 4.6) while in others long induction periods occurred (experiment 4.7). The exponential region of the rate profile was analysed (as discussed in chapter two) for several control samples and demonstrated that the relative rates of oxidation of individual fatty acyl chains was not consistent. For example in experiment 4.5 (Appendix 4.2) the relative rates of oxidation between 18:2, 20:4 and 20:5 were 1:3.02:4.24 which were in reasonable agreement with Cosgrove et al.<sup>(42)</sup> However in experiment 4.6 (Appendix 4.3) the relative rates of oxidation of the same FAME's were 1:1.96:0.84. However it may be argued that the accuracy of these figures is low as too small a number of data points were obtained and no external standard was used. Below is a list of factors which may have had an affect on the observed rates.

1. Trace amounts of phospholipid hydroperoxide.
2. The exact phospholipid composition and fatty acid profile.
3. The morphology of the liposomes.

1. In fresh meat it is believed that trace amounts of hydroperoxides already exist which can decompose promoting oxidation. No measurement of the hydroperoxide content of extracted phospholipids was made which in retrospect should have been carried out. The purity of the phospholipids was checked by TLC (Chapter 2). It may have been possible that trace amounts of hydroperoxides were present at the beginning of the experiment.
2. In all experiments no control was kept on the content of each phospholipid class and of the fatty acid profile of the total phospholipids. However it has been shown by other researchers that these two factors may play an important role in the rate of oxidation.<sup>(86,87)</sup> Mowri et al <sup>(86)</sup> showed that the composition of liposomes has a major influence on the rate of peroxidation. Liposomes composed of 1-16:0-2-20:4-PC (substrate) and 18:0-PC (molar ratio 1:9) and incubated for 30 minutes at 37°C with ferrous ions and ascorbate showed an 80% loss in the content of arachidonate. In contrast no appreciable change of arachidonate was observed in liposomes composed of substrate and 1-16:0-2-18:1-PC (molar ratio 1:9) under the same experimental conditions. In case one above the rate of peroxidation in liposomes containing substrate and 18:0-PC was independent of substrate content. The group suggests that in this case the substrate will be clustered on the 18:0-PC membrane at 37°C

since the substrate ( $T_c=0$ ) is in the liquid-crystalline state and the latter ( $T_c=57^\circ\text{C}$ ) is in the gel state. The local density of the substrate in a cluster on the membrane may be high enough for propagation of the radical-chain reaction, even though the apparent content of the substrate is low. In case two both substrate and 1-16:0-2-18:0-PC will be in the liquid-crystalline state above  $37^\circ\text{C}$  and therefore homogeneously distributed within the membrane. The rate of peroxidation of these liposomes decreased almost linearly with decrease in substrate concentration until at a substrate content of 10% no oxidation occurs. This may well show that the importance of substrate density within the membrane in promoting the chain reaction.

Liposomes consisting of substrate and 14:0-PC (molar ratio 1:9) were insensitive to peroxidation at  $37^\circ\text{C}$  but oxidised rapidly below  $10^\circ\text{C}$ . This is thought to be due to the temperature dependence of lateral distribution of the substrate on a 14:0-PC membrane. Above  $20^\circ\text{C}$  the substrate is located homogeneously in the matrix of the 14:0 PC in the liquid crystalline state. Again the density of the substrate becomes low under these conditions and peroxidation may be retarded. However below  $10^\circ\text{C}$  the substrate will be clustered since  $T_c=17.2^\circ\text{C}$  for 14:0 PC.

Sunamoto et al.<sup>(87)</sup> studied the autoxidation of arachidonic acid at 25°C after addition to liposomes composed of either 14:0-PC or 16:0-PC. They found that arachidonic acid was more easily oxidised in 14:0-PC than in 16:0-PC where the gel to liquid-crystal phase transition temperatures are 23 and 41°C respectively. From this they suggest that membrane fluidity may be an important factor in autoxidation. These studies show that phospholipid composition may play a significant role in the rate of oxidation although it is difficult to assess to what degree it contributes to the observed variation.

3. The autoxidation experiment involving sonicated and hand shaken phospholipid dispersions dramatically showed how structure may influence the rate of oxidation. In the preparation of liposomes the phospholipids were sonicated from a milky suspension to a translucent one. This transformation was taken to be due to the formation of small unilamella liposomes. It cannot however, be assumed that identical liposomal structures would have been formed with different relative amounts of individual phospholipid classes. Variation in phospholipid class may also have had an effect on molecular mobility within the liposome as discussed in Chapter 3. Again it is extremely difficult to determine to what extent small changes in composition affect the rate of oxidation.

4. Recently it has been shown that free fatty acids can promote the decomposition of lipid hydroperoxides. Mryashita<sup>(88)</sup> demonstrated that oleic and linoleic acid were oxidised faster than their corresponding methyl esters and that stearic acid decomposed methyl linoleate hydroperoxides. The proposed mechanism, although not proven involves the homolytic decomposition of the hydroperoxide by the free carboxy group. Liposomes were checked periodically for free fatty acids by TLC both immediately after sonication and after extensive oxidation and non were found to be present. Therefore, it is unlikely that this factor is contributing to the observed variations.

Experiments 4.4 and 4.6 (Figures 4.1-4.3) demonstrated that Pork phospholipid liposomes were sensitive to Fe(II)-ascorbic acid, Cu(II) and Cu(II)-ascorbic acid induced peroxidation. As we would expect the more unsaturated the fatty acyl chains the more susceptible they were to oxidation. Garner-Suillerst<sup>(75)</sup> suggests that Fe(II) induced peroxidation occurs via the formation of a Fe(II)-phospholipid complex which involves fast fixation of iron to the phospholipid at the membrane surface. They suggest that it is unlikely for iron to move passively through the membrane therefore the hydroperoxides accumulate at the membrane surface where hydroperoxide decomposition occurs. From these ideas they proposed the following two step reaction:-



PL = phospholipid

ROOH = phospholipid hydroperoxide

For chain propagation to occur the radicals formed by the decomposition of the hydroperoxides must then react via the molecular movements described in chapter three, ie, bobbing, flip-flop and lateral movement or by lateral propagation. Transition metal ions are believed to decompose hydroperoxides although some evidence has recently cast doubt over this theory.<sup>(89)</sup>



The control sample in experiment 4.4 did not follow the expected rate profile for an autoxidation reaction. It was not known why this occurred and why oxidation was greater than in the Fe(II)/Ascorbic acid samples in the early stages of oxidation. This did not occur in control samples in the other experiments. Experiment (4.8) provided interesting results on the activity of polyphosphate in copper (II) induced peroxidation. From Figure

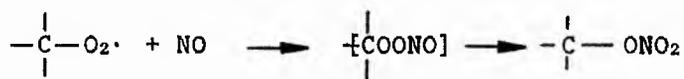
(4.6) we can see that Cu(II) induced peroxidation could be presented as a straight line.

Liposomes oxidised with both copper (II) and phosphate initially had a similar rate of peroxidation as copper only, until a certain level of oxidation was attained when the rate fell markedly. As discussed later in this chapter liposome peroxidation is sensitive to the copper ion concentration which suggests that the copper concentration in both sets of samples were similar. We could have expected the phosphate in solution to compete with the phosphate of the phospholipid for copper ions which would have reduced the ion concentration at the membrane surface and altered the rate. However this was unlikely to have occurred which raises questions regarding the mechanism of phosphates action. It is obvious from Figure (4.6) that some drastic event occurred after 5 to 6 hours which was dependent on the phosphate being present. It is difficult to speculate further on the precise reason behind this observation without having carried out further experiments.

As illustrated in experiments 4.5 and 4.6 sodium nitrite was shown to act as an antioxidant in the oxidation of liposomes with and without copper ions present. The precise mechanism of nitrites antioxidant action is not known but a mechanism which involves a decrease in chain propagating radicals is likely. Sodium nitrite under acidic conditions forms nitrous acid (HONO). Nitrous acid can readily disproportionate at room temperature<sup>(90)</sup>.



Nitric oxide has been shown to add to peroxy radicals producing nitrites.



This reaction sequence may result in a decrease in rate. Although the pH of the water used in the oxidation experiments was not measured it was likely to have been slightly acidic (dissolved  $\text{CO}_2$ ) which may give more substance to the mechanism. If non-detectable amounts of metal ions were present in experiment 4.5 nitrite or nitrate may have co-ordinated with them which may have reduced the rate of oxidation. It is however, difficult to state the precise reason for nitrites antioxidant behaviour without having performed further experiments.

Small concentrations of sodium chloride, Figure (4.4) did not appear to alter the rate of peroxidation substantially. The activity of higher concentrations of sodium chloride is provided in greater detail later in this chapter.

The dramatic changes in rate between sonicated and hand-shaken liposomes is presented in Figure (4.7). As discussed in chapter two, sonicated liposomes exist as small unilamella structures and hand shaken liposomes as large multilamella structures. Therefore, the difference in the rate was probably due to the differences in structure. The small sonicated liposomes were probably highly curved which would mean that the outer phospholipids of the bilayer were further apart than in a linear bilayer structure and the inner phospholipids would have been closer together. This may have affected both the diffusion of oxygen into the bilayer and the closeness of neighbouring fatty chains which may have had a considerable impact on radical propagation.

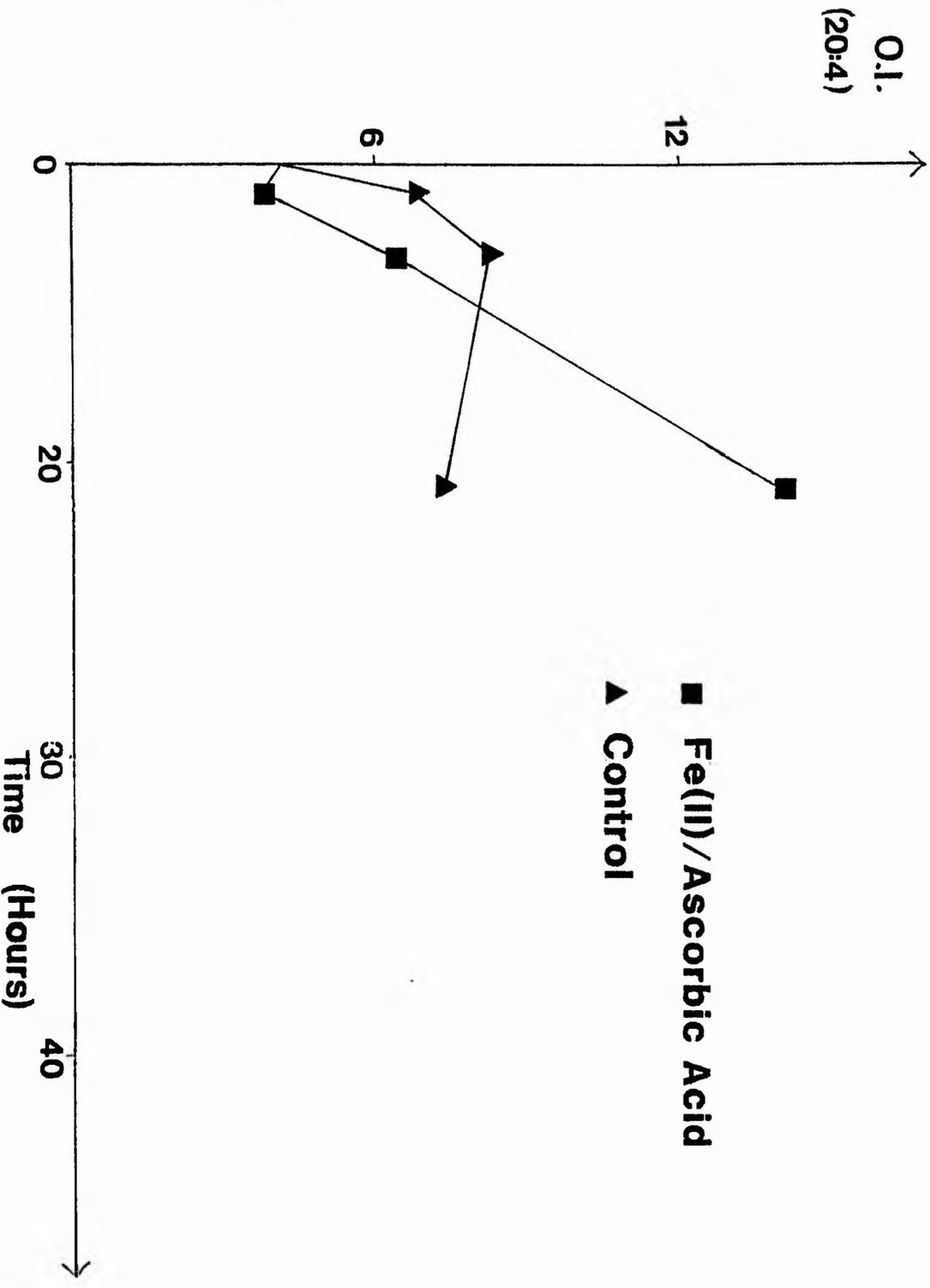


FIGURE 4.1

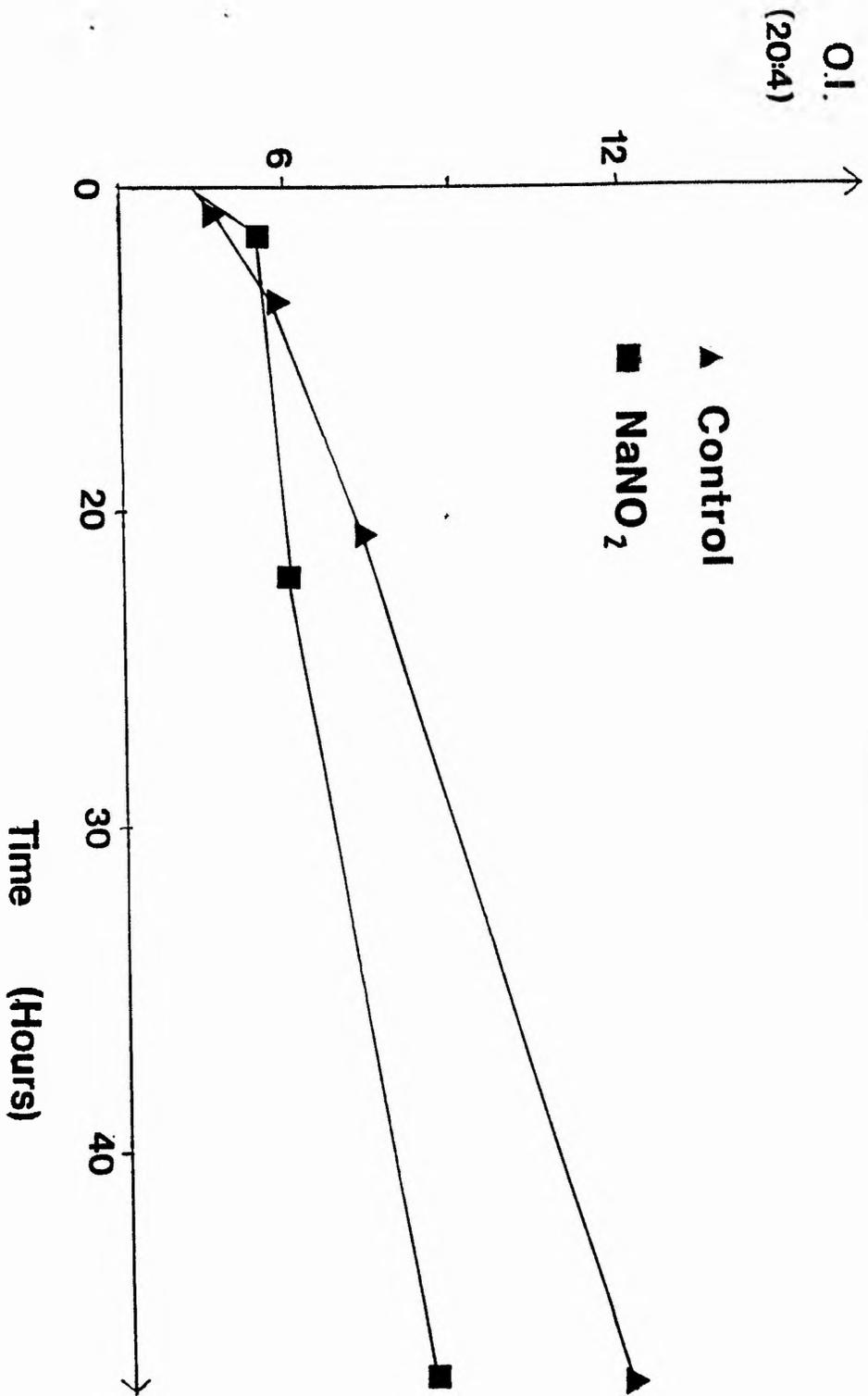


FIGURE 4.2

FIGURE 4.3

- Cu(II)/Ascorbic Acid
- Cu(II)
- ▲ Cu(II)/NaNO<sub>2</sub>
- \* Control

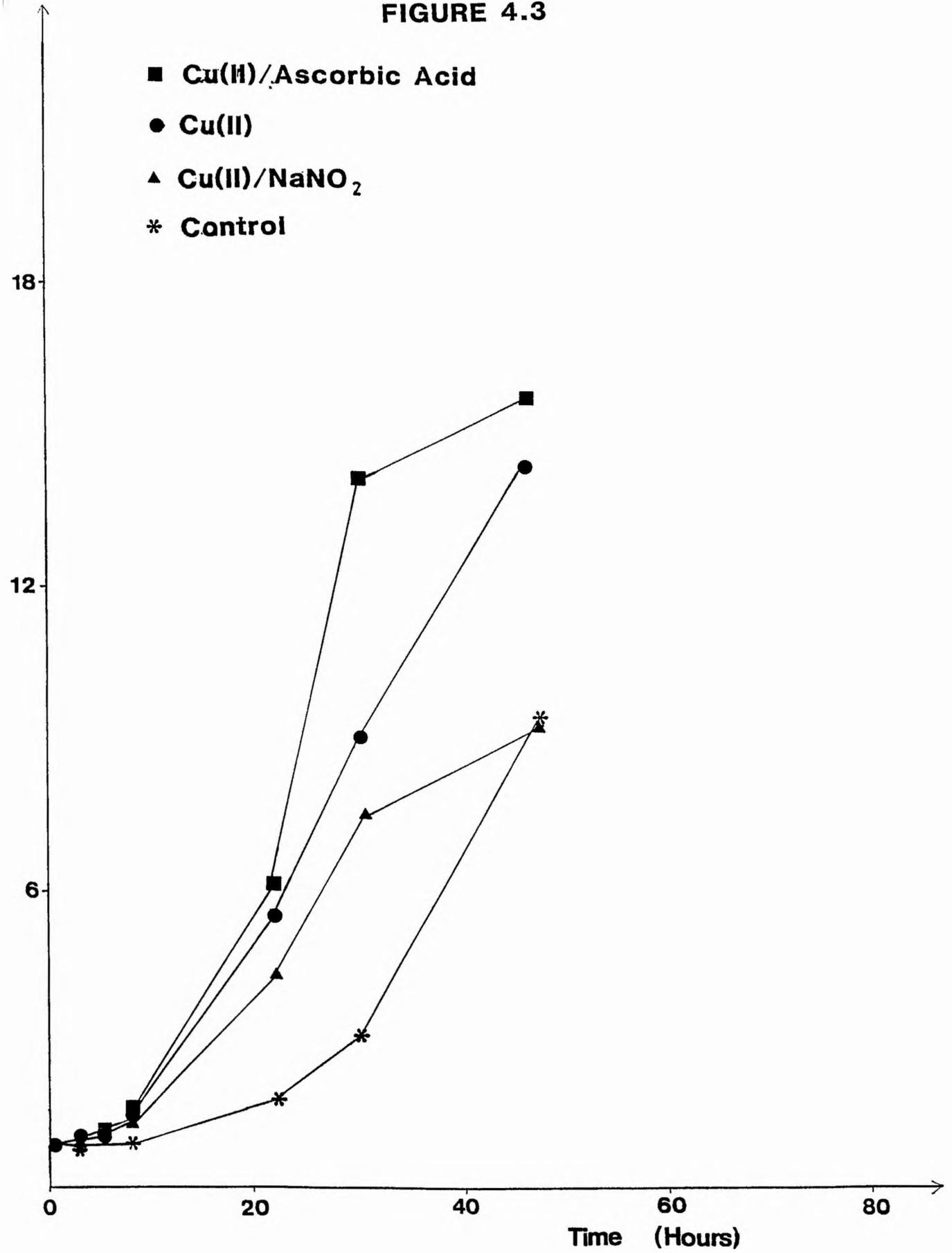
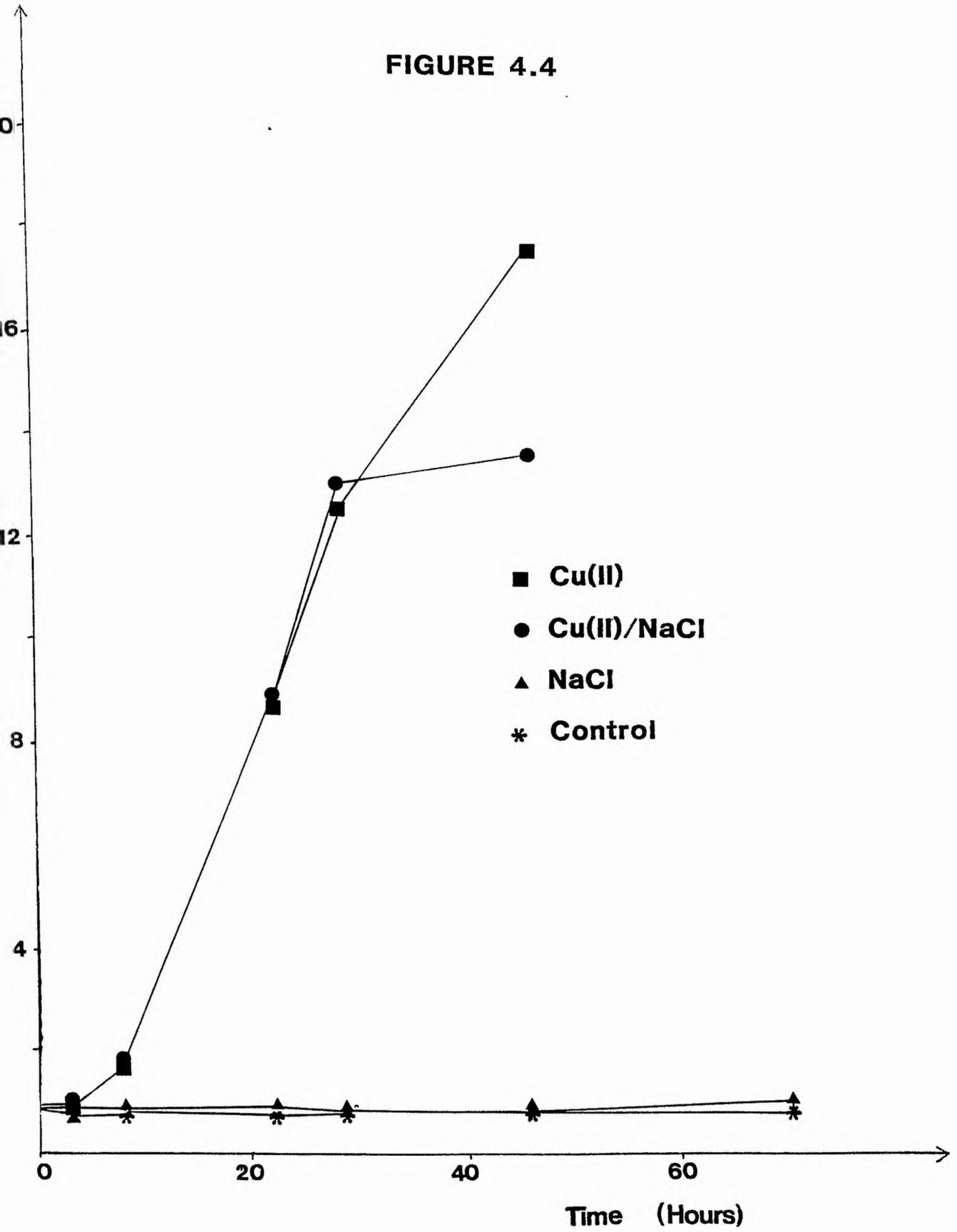
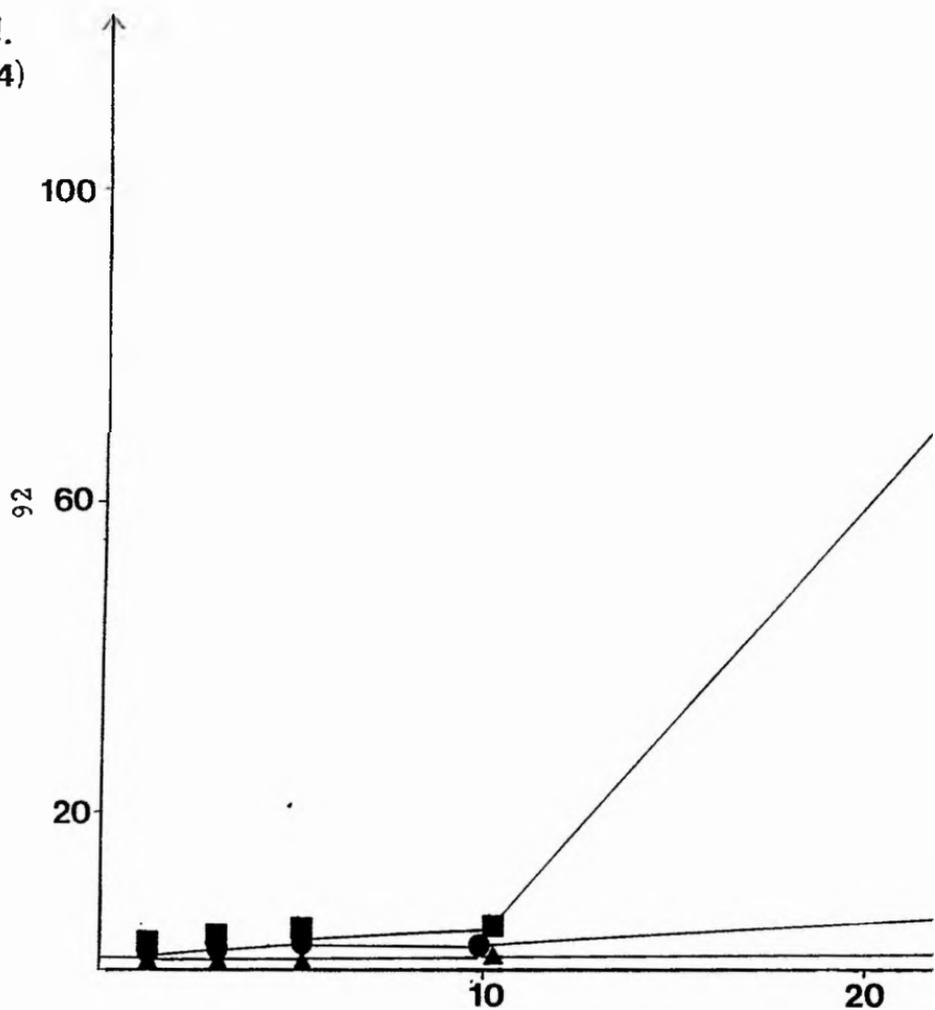


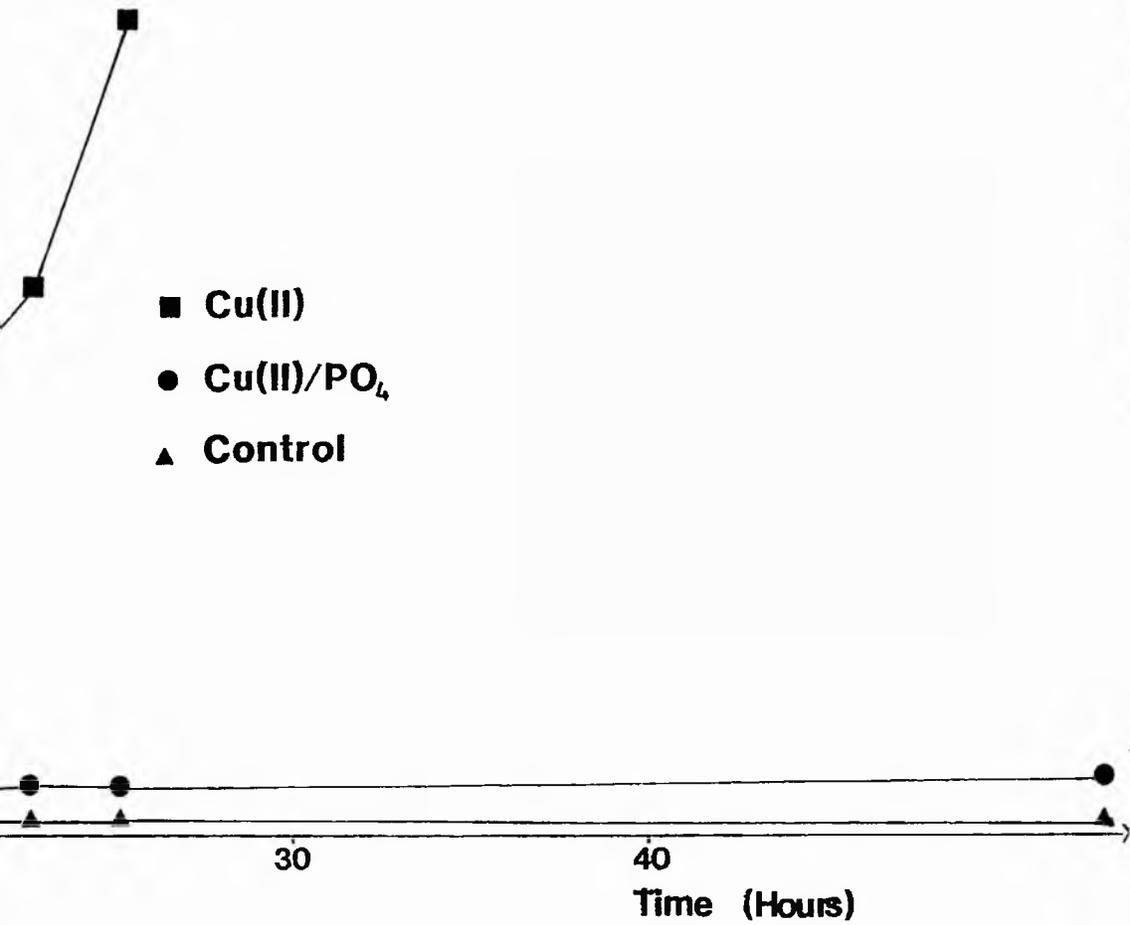
FIGURE 4.4

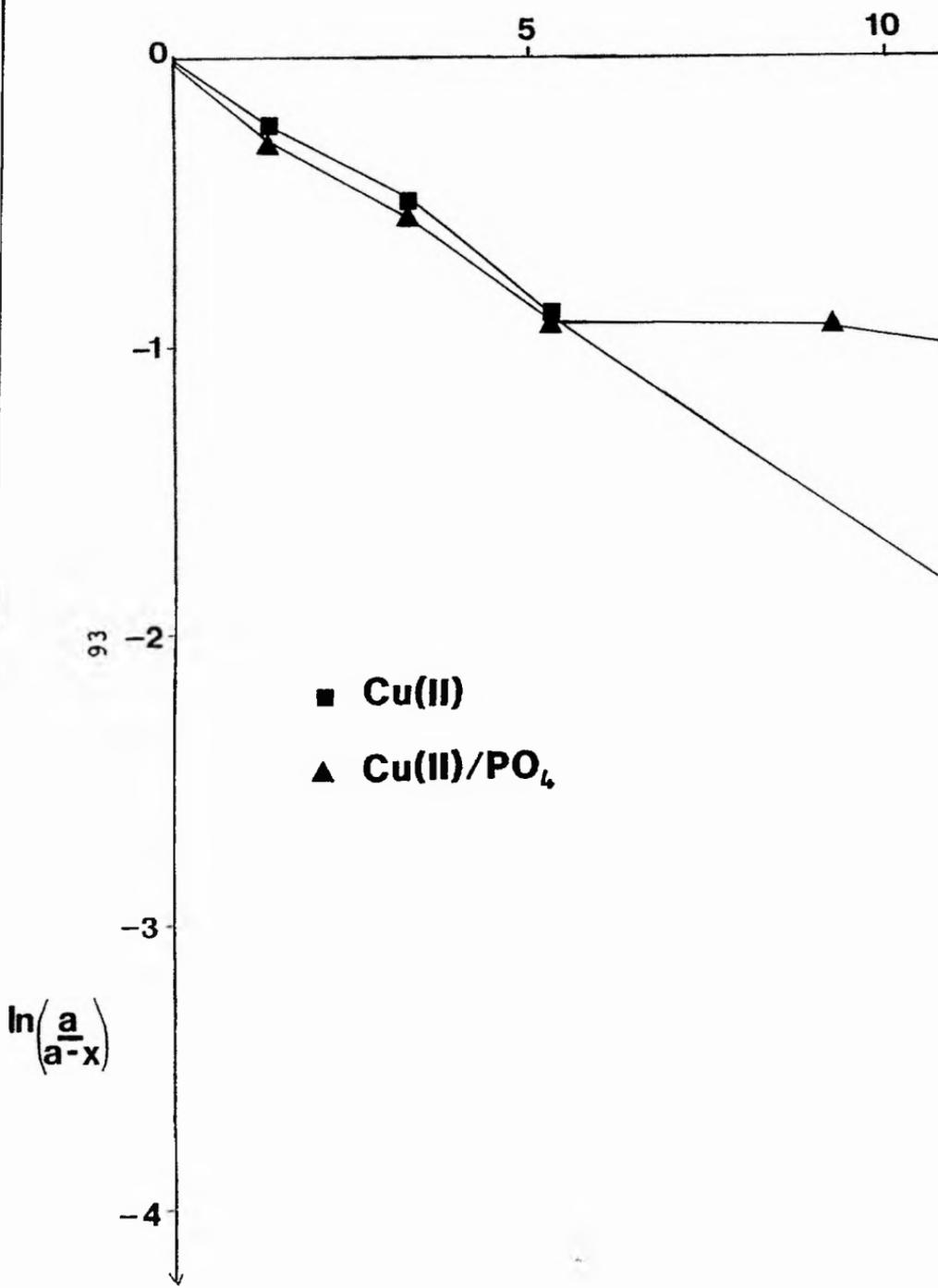


O.I.  
(20:4)



**FIGURE 4.5**





93

**FIGURE 4.6**

**Time (Hours)**

15

20

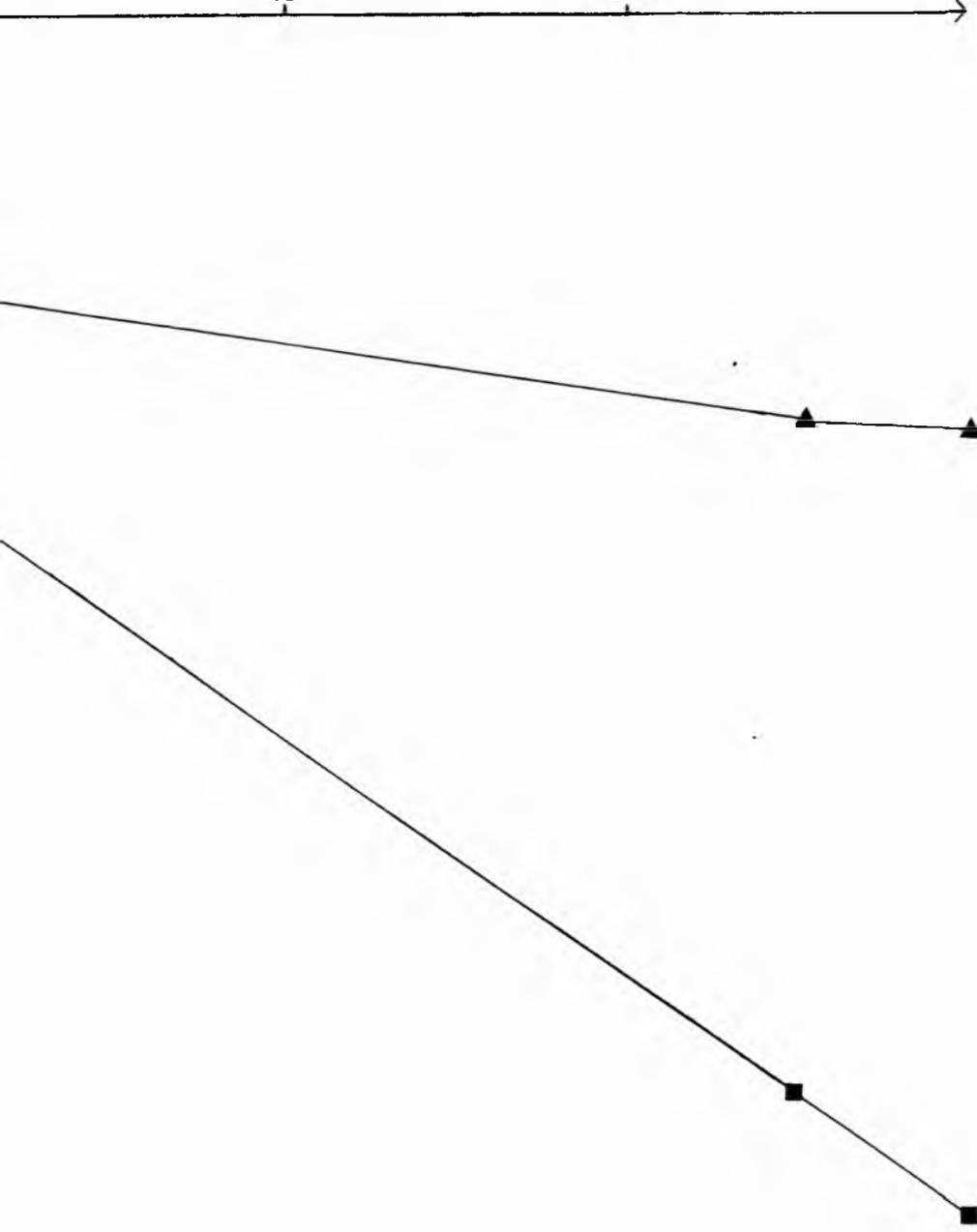
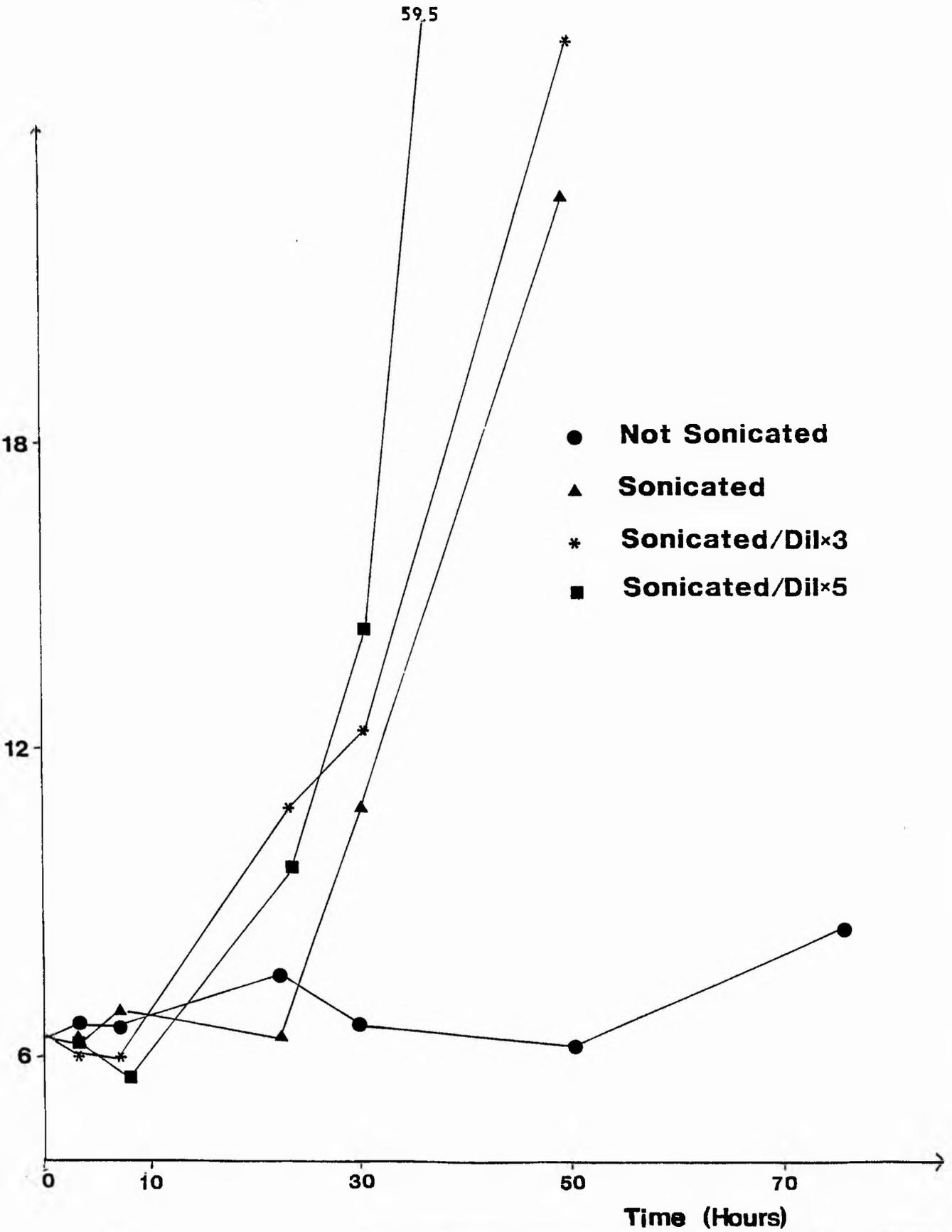


FIGURE 4.7



#### 4.11 VARIATION OF Cu(II) INDUCED PEROXIDATION WITH TEMPERATURE

In previous experiments it was shown that pork phospholipid liposomes were sensitive to copper (II) induced peroxidation. In investigating the pro-oxidant activity of copper (II) further an experiment was carried out which studied the effect of temperature on the rate of Cu(II) peroxidation.

Liposomes were prepared at a concentration of 10 mg/ml. The liposomes were divided into portions (1 ml) and placed in 10 ml vials. To each portion was added  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  ( $4.7116 \times 10^{-4}$  M 1 ml, 30 ppm Cu(II)). The vials were sealed and incubated in darkness at one of the following five temperatures.

S1 - 42°C

S2 - 24°C

S3 - 4°C

S4 - -13°C

S5 - -22°C

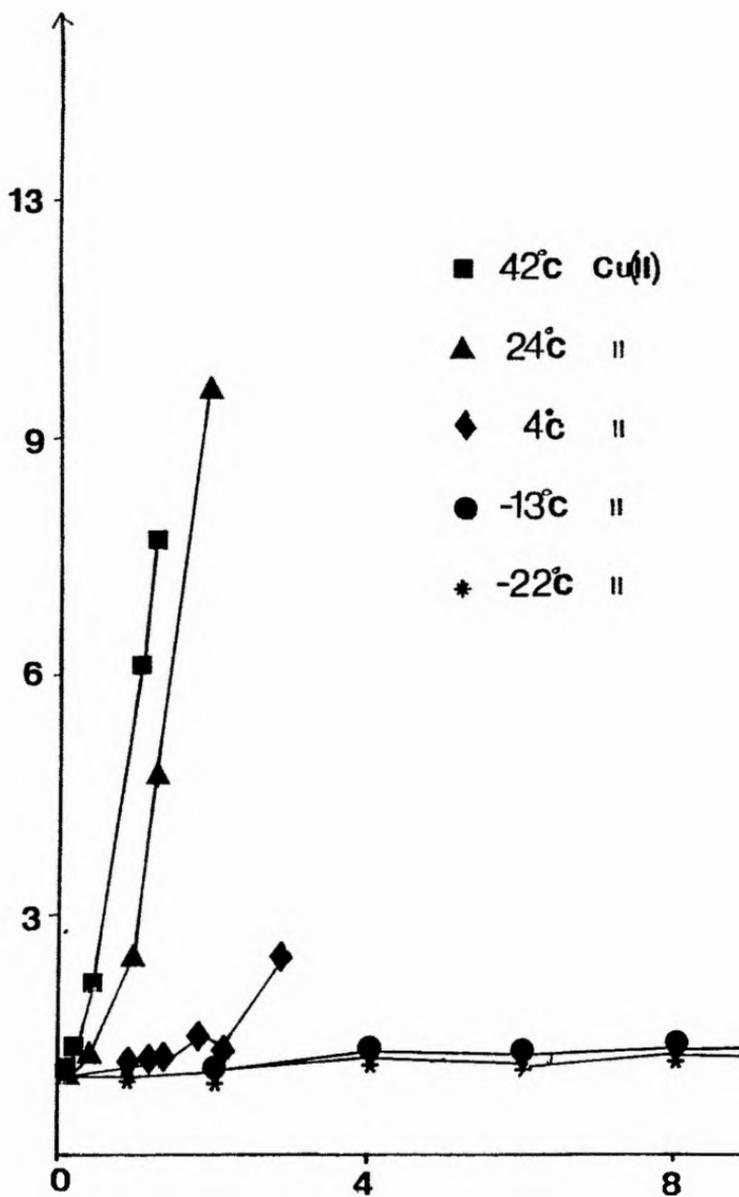
Samples were removed at appropriate times for analysis. The results are presented in Appendices (4.7) and Figure (4.8).

The rate of a chemical reaction is related to temperature by the Arrhenius equation. The rate of reaction is believed to double for every 10° rise in temperature. Unfortunately it was difficult

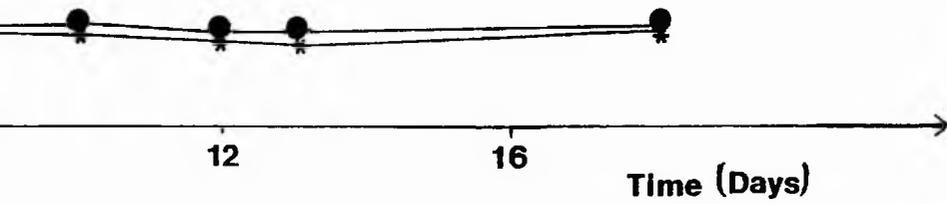
to try and quantify the change in rate over the temperature range studied. However from a qualitative standpoint the rate of reaction was found to decrease dramatically on lowering the temperature. Liposomes stored frozen showed very little change in their fatty acid profile over the extent of the experiment. Qualitatively, there appeared to be a larger decrease in the rate between 42 and 24°C. The phospholipid gel-liquid crystal transition is one factor which may have contributed to the observed differences. At the lower temperature more of the phospholipids would have been in the gel state. This may have reduced the molecular movements associated with the phospholipids and resulted in a reduction in chain propagation. At both -13°C and -22°C the copper ions would have been expected to be concentrated on the liposome surface. A high concentration of copper would be expected to increase the rate, but as discussed in section (4.15) copper becomes antioxidant at high concentrations. Ice formation could also restrict molecular motions of the phospholipids which may be crucial for chain propagation. These results clearly demonstrate that the rate of Cu(II) peroxidation of liposomes is lowered by both temperature and a change from an aqueous to non-aqueous environment.

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**FIGURE 4.8**



#### 4.12 VARIATION IN THE RATE OF LIPOSOME PEROXIDATION WITH ADDITIVES AND TEMPERATURE

This study investigated differences in the rate of oxidation at storage temperatures of +4, -8 and -20°C in the presence of various additives. Liposomes were prepared at a concentration of 11.85 mg/ml and aliquots (1 ml) placed in glass vials. To each vial was added one of the sets of solution below. The vials were then sealed and stored in darkness at either +4, -8 or -20°C.

S1 - water (4 mls).

S2 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 ml,  $2.39 \times 10^{-4}$  M 15.08 ppm Cu(II)) and  $\text{H}_2\text{O}$  (3 ml).

S3 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 ml,  $2.39 \times 10^{-4}$  M 15.08 ppm Cu(II)), NaCl (5% soln, 1 ml) and water (2 ml).

S4 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 ml,  $2.39 \times 10^{-4}$  M 15.08 ppm Cu(II)), NaCl (5% soln, 1 ml), ascorbic acid (1 ml,  $7.01 \times 10^{-4}$  M) and water (2 ml).

S5 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 ml,  $2.39 \times 10^{-4}$  M 15.08 ppm Cu(II)), NaCl (5% soln, 1 ml),  $\text{NaNO}_2$  (1 ml,  $4.46 \times 10^{-2}$  M) and water (1 ml).

S5 -  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (1 ml,  $2.39 \times 10^{-4}$  M 15.08 ppm Cu(II)), NaCl (5% soln, 1 ml),  $\text{NaNO}_2$  (1 ml,  $4.46 \times 10^{-2}$  M) and ascorbic acid (1 ml,  $7.01 \times 10^{-4}$  M).

The results are provided in Appendices (4.8-4.10) and Figures (4.9-4.12).

The results of samples S2, S3 and S4 stored at the three temperatures are presently graphically in Figures (4.9-4.11). It can be seen that liposomes stored at  $+4^\circ\text{C}$  with Cu(II) only (S2)

showed a higher rate of oxidation than those stored with Cu(II)/NaCl (S4) and Cu(II)/NaCl/ascorbic acid (S3). However when the above samples were stored frozen (-8 and -20°C) there was a change in the relative rate of oxidation. Both samples (S3) and (S4) exhibited a higher rate of oxidation than sample (S2). It was likely that liposomes incubated with NaCl at sub zero temperatures contained a considerable amount of unfrozen water. This would have diluted metal catalysts relative to the frozen state (assuming metal ions stayed in the aqueous phase) and also allowed water to exist unfrozen around the liposomes. The mobility of the copper ions which is probably an important factor in their pro-oxidant activity would have been restricted in an ice lattice which may also have prevented molecular movement of the phospholipids. A more detailed account of salts pro-oxidant effect is provided in section 4.13.

It is unclear why salt exhibited antioxidant character with liposomes oxidised at +4°C. Osmotic differences between the inside of the vesicle wall and the bulk aqueous phase on the outside may have been one factor. A further experiment would have been to prepare the liposomes in salt solution and therefore remove any osmotic effect. Another factor to consider is that of lower oxygen solubility which occurs in salt solutions. A paper by Mabrook et al<sup>(91)</sup> studied the oxidation of linoleic acid emulsions in the presence of sodium chloride. They found that salt decreased the rate of oxidation of linoleic acid and

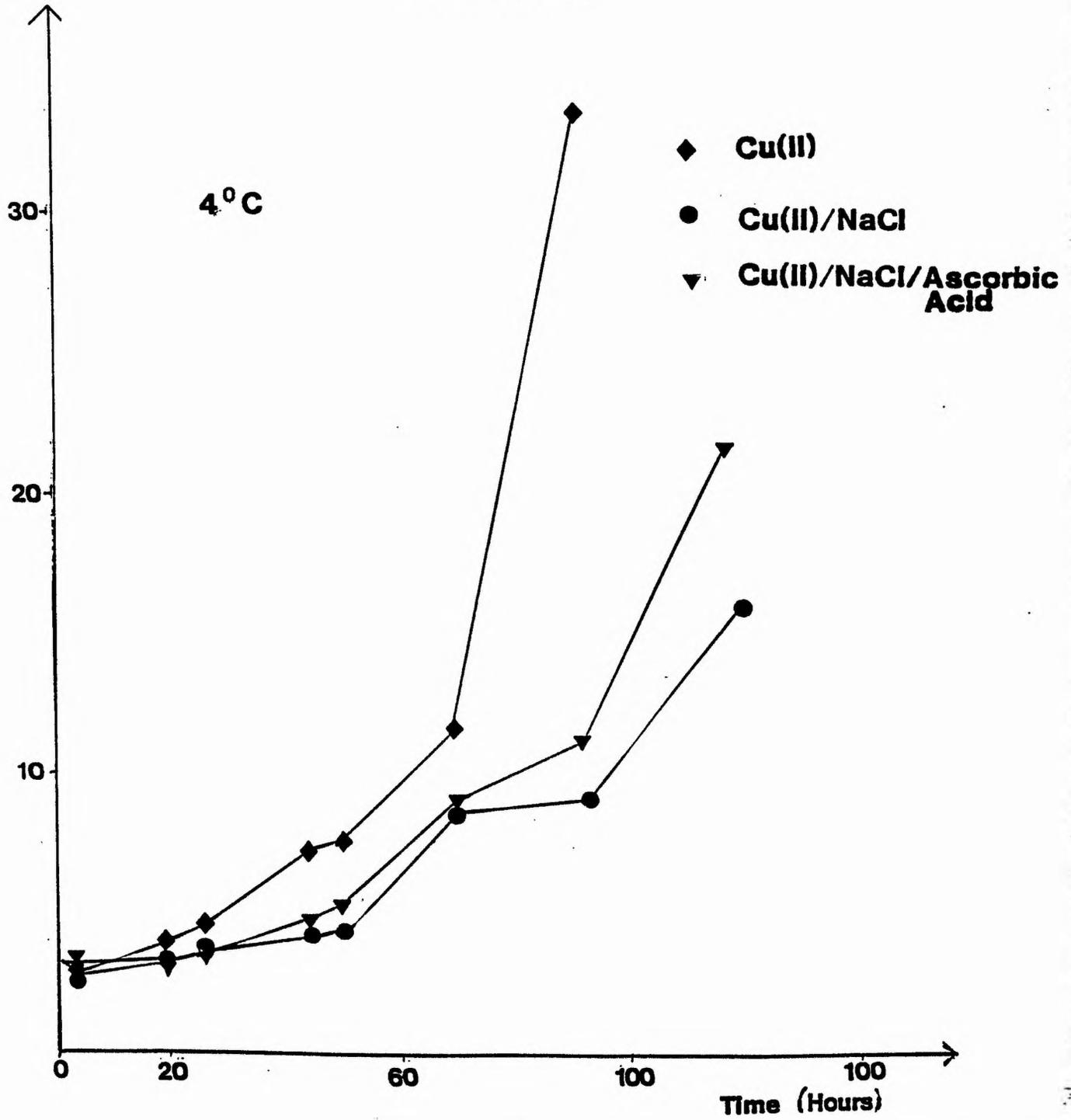
explained the observation by the lower oxygen solubility in the emulsion. If this was an important factor in the liposome system then the observed pro-oxidant effect exhibited by salt at sub-zero temperatures must also take into account this observation. Other than the changes in relative rates between samples S2, S3 and S4 the rate of peroxidation also decreased on decreased temperature. For example sample (S4) took approximately 110 hours to reach an oxidation index of 20 at +4°C, 40 days at -8°C and 140 days at -20°C. Taking into account the above factors the overall rate of oxidation may have been dependant on oxygen solubility, temperature and copper ion concentration.

Samples (S5) and (S6) stored at +4°C with the addition of NaNO<sub>2</sub> showed considerable stability to oxidation. This also confirmed work carried out in earlier experiments. There were interesting differences between samples (S5) and (S6) stored at -8°C and -20°C. Figure (4.12). Samples containing Cu(II), NaCl and NaNO<sub>2</sub> (S5) showed a lower rate of oxidation than samples containing Cu(II), NaCl, NaNO<sub>2</sub> and ascorbic acid (S6) at -8°C. However at -20°C samples (S6) oxidised more slowly than samples (S5). It is difficult to speculate on a reason for this change in relative rates without having performed further experiments. However, if the interaction of the various additives with oxidising lipids is a multistep process then there shall be corresponding activation energy parameters associated with them which are temperature dependent. Therefore the change in relative rates of oxidation

may have been a result of the dependence of the thermodynamic parameters with temperature under the two experimental conditions studied. We can also say that the copper ion concentration would have been greater at  $-20^{\circ}\text{C}$  than  $-8^{\circ}\text{C}$  and the same at one particular temperature. It is known that ascorbic acid exhibits antioxidant activity by chain termination of peroxy radicals<sup>(85)</sup> and that copper (II) exhibits a lower pro-oxidant effect of the rate of peroxidation at higher concentrations section (4.15) which suggests that there was a lower concentration of radicals present. Therefore the reason ascorbic acid appeared to be more effective at chain termination at the higher concentration of copper ions may have been because there were fewer radicals present in the liposomes. These observations may be useful in determining the stability of cured products at different temperatures.

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FIGURE 4.9



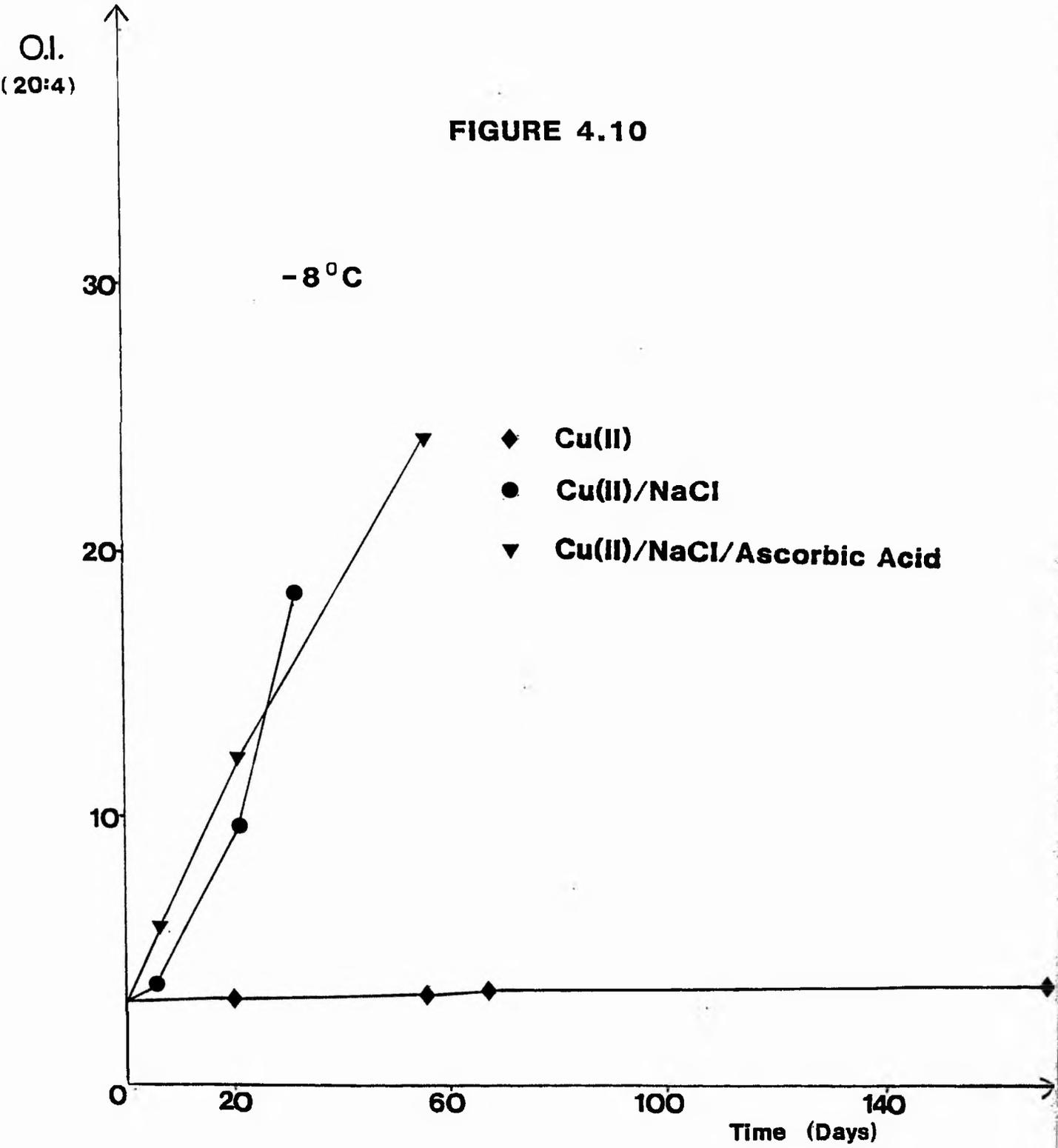


FIGURE 4.11

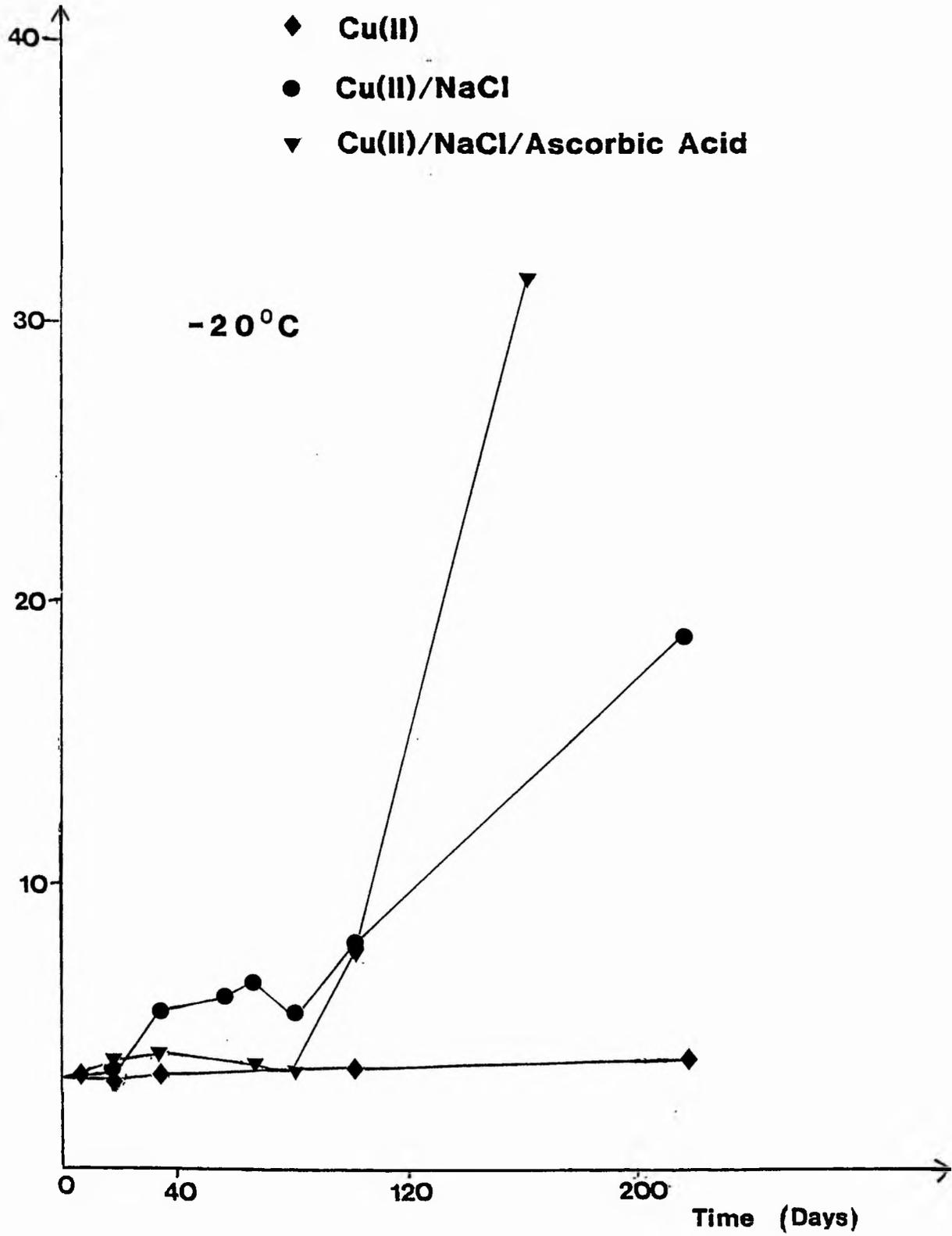
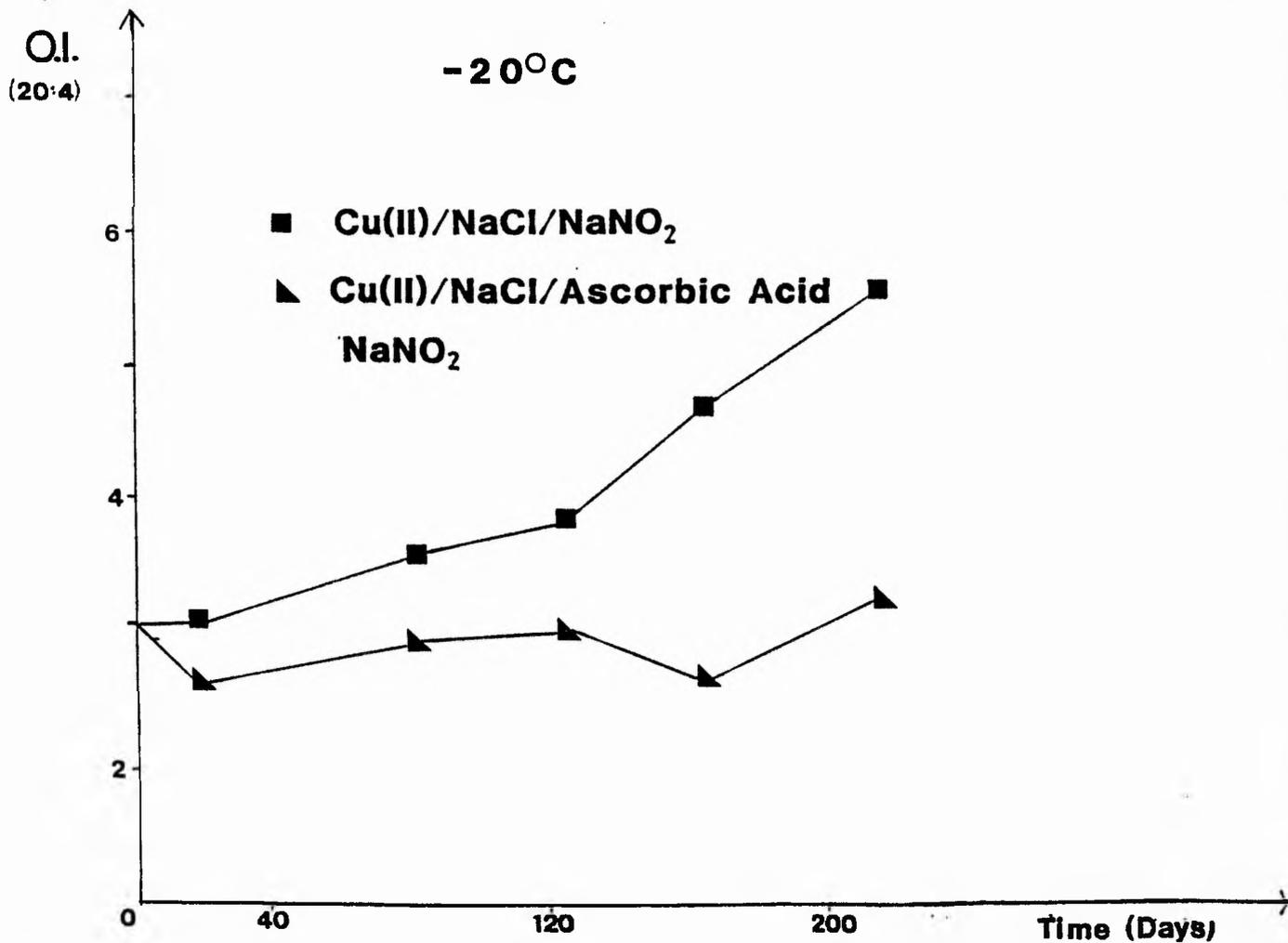
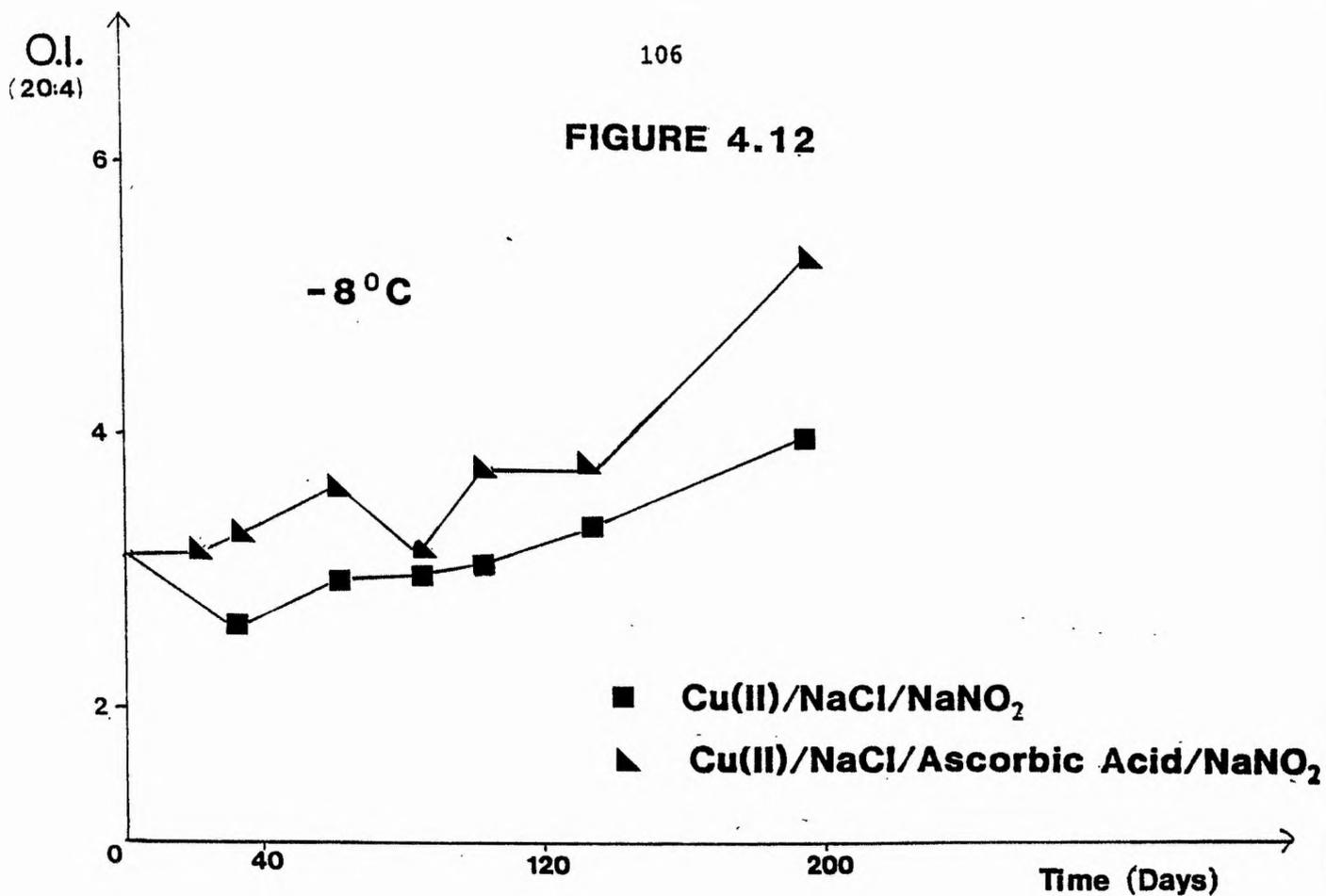


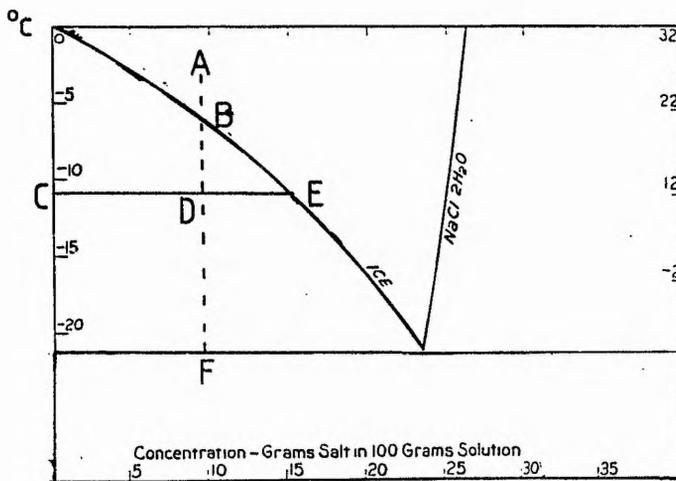
FIGURE 4.12



#### 4.13 Cu(II)-NaCl INDUCED LIPOSOME PEROXIDATION AT -8.5°C

This experiment was designed to look more closely at Cu(II)-NaCl induced peroxidation of liposomes at a fixed sub-zero temperature. When a salt solution is cooled below 0°C pure water freezes out and an equilibrium is established between the amount of aqueous phase to ice. This can best be illustrated by considering the NaCl-H<sub>2</sub>O phase diagram, Figure (4.13).

FIGURE 4.13



If the temperature of a salt solution of composition A is lowered to temperature C, a depression in freezing point will result. Ice formation of the salt solution starts at point B and the ratio of aqueous salt solution to ice is given by CD:DE at temperature C.

Therefore at a constant temperature and by varying the composition of initial salt solution we can alter the ratio of salt solution to ice. The concentration of the salt solution at temperature C is given by E and is independent of the initial salt concentration. If the copper ions stay in the aqueous phase then their concentration will be dependant on the salt concentration at a particular sub zero temperature above the NaCl-H<sub>2</sub>O eutectic (-21.3°C).

Liposomes were prepared at a concentration of 8.05 mg/ml placed in 10 ml glass vials with Cu(II) SO<sub>4</sub> 5H<sub>2</sub>O ( $5.7114 \times 10^{-4}$  M, 1 ml, = 36 ppm CuII) and of the salt solutions below added.

S1 - NaCl (30g/100 ml) 1 ml

S2 - NaCl (15g/100 ml) 1 ml

S3 - NaCl (7.5g/100 ml) 1 ml

S4 - NaCl (3.75g/100 ml) 1 ml

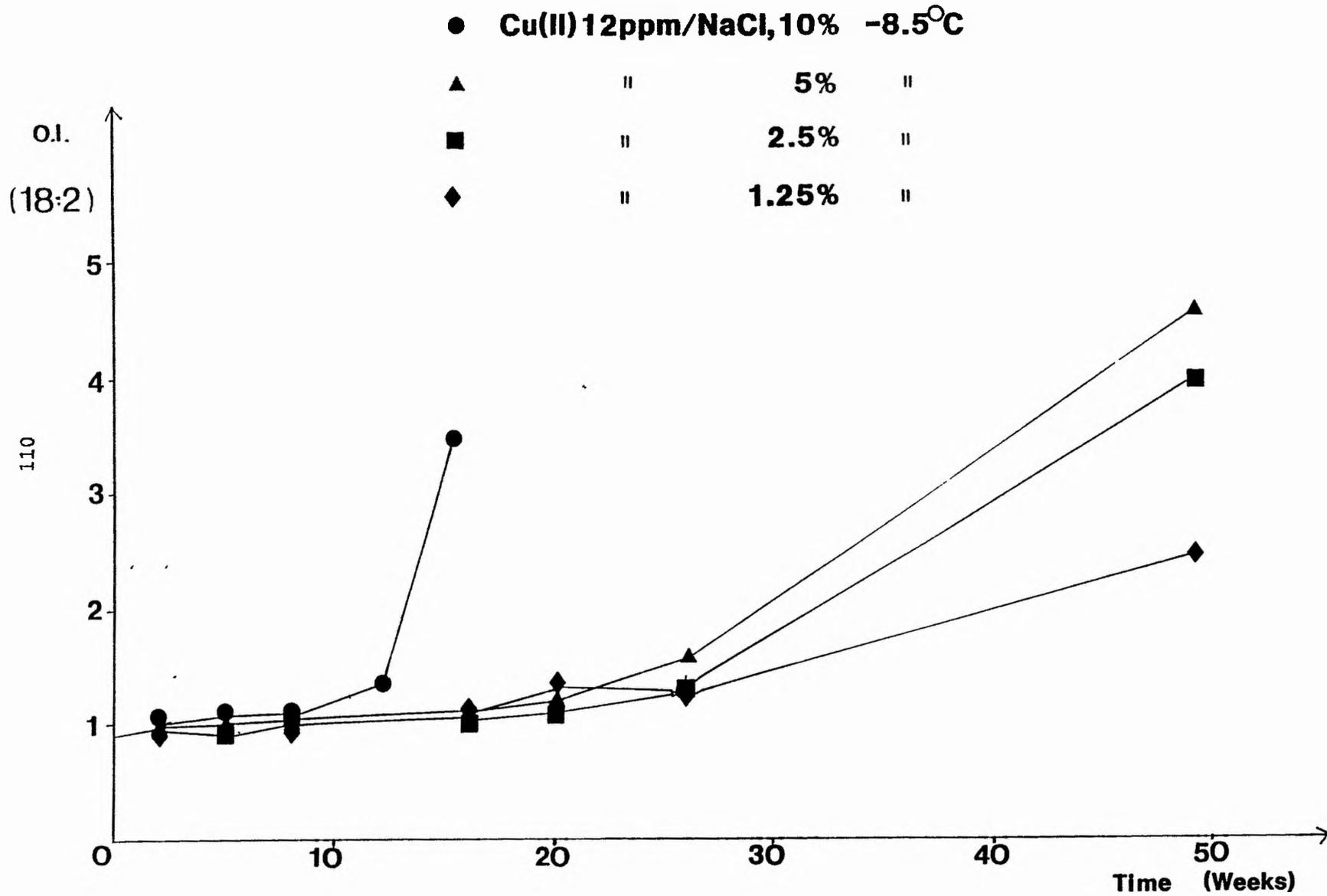
The samples were stored in the dark at -8.5°C. The results are presented in Appendices (4.11) and Figures (4.13-4.14). With reference to the International Critical Tables<sup>(92)</sup> a rough estimate of the concentration of metal ions occurring in the different samples was obtained. By measuring the relative amounts

of aqueous phase to ice the following concentration factors were applied to the samples.

<u>Sample</u>	<u>Factor</u>	<u>Effective Cu(II) (ppm)</u>
S1	1.25	15
S2	2.75	33
S3	5.50	66
S4	11.00	132

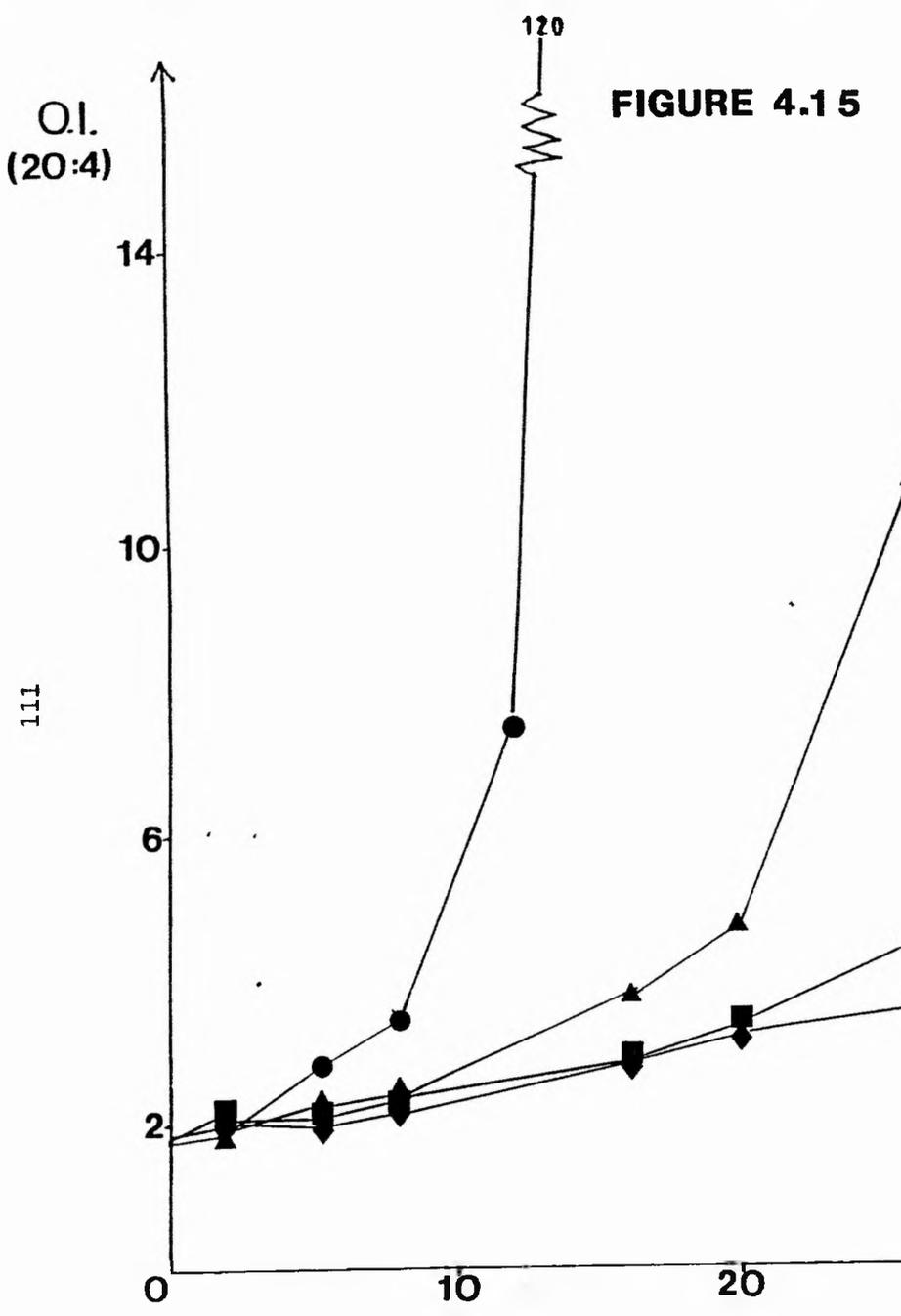
The rate of peroxidation (20:4) of the four samples are plotted in Figure 4.15. It appeared that liposomes stored with increasing levels of salt oxidised more rapidly than those stored with low levels of salt. If copper (II) remained in the aqueous phase upon freezing then the results suggest that copper (II) is more pro-oxidant at low concentration than at high concentrations. In addition there appeared to be a dramatic reduction in rate between copper concentration of 15 and 33 ppm (sample S1 and S2 respectively). This is discussed in greater detail in experiment 4.14.

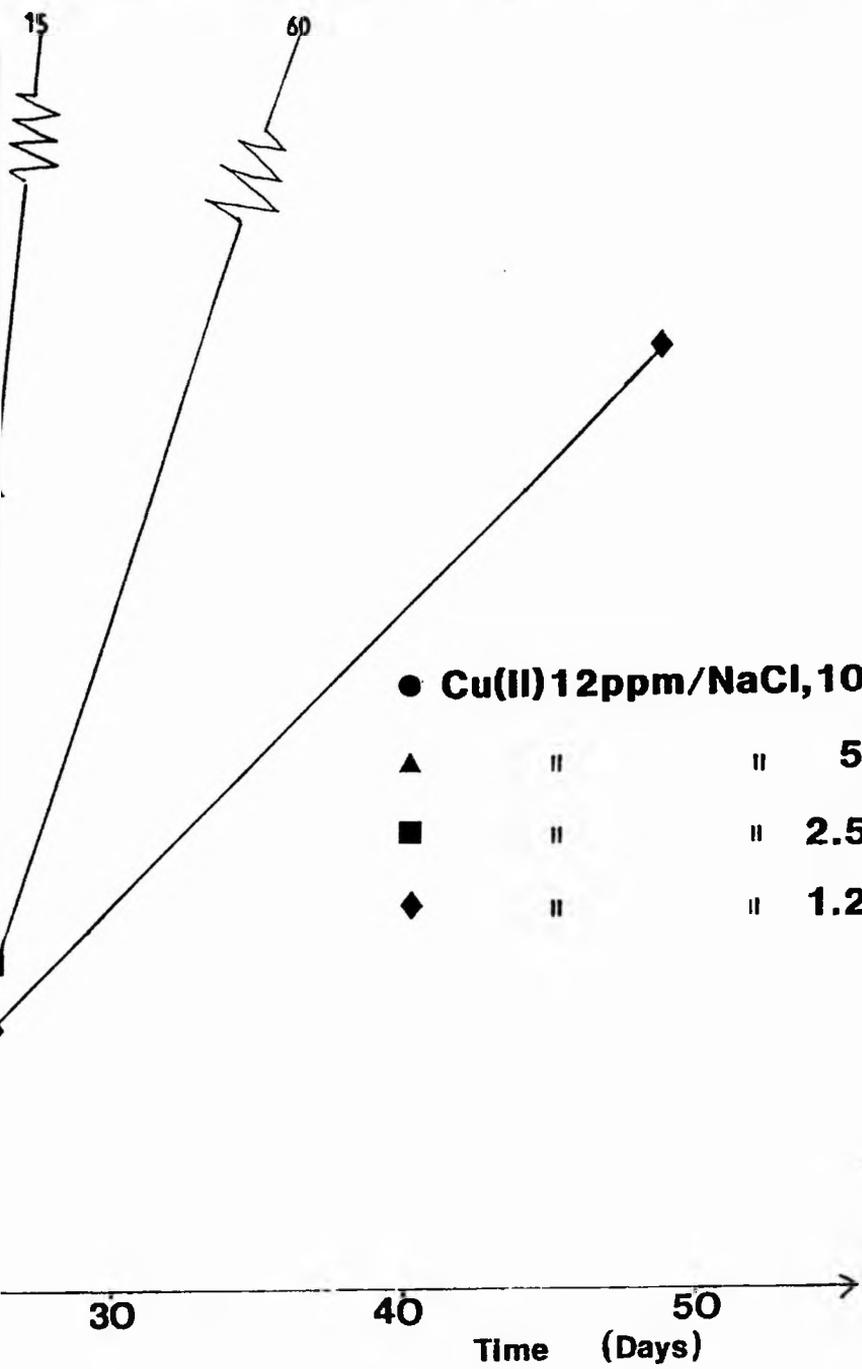
FIGURE 4.14



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FIGURE 4.15





#### 4.14 VARIATION OF Cu(II) CONCENTRATION ON PEROXIDATION

To investigate the theory that an increase in concentration of copper (II) results in a lowering in pro-oxidant activity an experiment was performed on liposomes at 42°C in the presence of different concentrations of copper. Liposomes were prepared at a concentration of 11.06 mg/ml. 1 ml aliquotes were placed in 10 ml glass vials and one of the following solutions added.

Control - Water (1 ml).

S1 -  $2.89 \times 10^{-5}$  M,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1 ml (1.82 ppm Cu(II)).

S2 -  $1.44 \times 10^{-4}$  M,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1 ml (9.1 ppm Cu(II)).

S3 -  $2.89 \times 10^{-4}$  M,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1 ml (18.82 ppm Cu(II)).

S4 -  $1.44 \times 10^{-3}$  M,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1 ml (91 ppm Cu(II)).

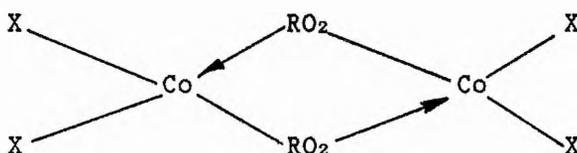
S5 -  $2.89 \times 10^{-3}$  M,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1 ml (182 ppm Cu(II)).

The vials were sealed and incubated at 42°C in darkness. The results are provided in Appendix (4.12) and Figure (4.16).

#### Discussion

Figure (4.16) showed that Cu(II) exhibited antioxidant behaviour on increased concentration. It is difficult to speculate why this

occurred but this phenomenon has been observed previously. Betts et al (93) showed that cobalt (II) acts a catalyst at low concentration and as inhibitor at high concentration in the oxidation of hydrocarbons and methyl linoleate. They explained this observation by the formation of a bidentate chelate  $(CoX_2)_2 2RO_2$  in which  $RO_2$  radicals act as bridges.



They state that bidentate chelates of this structure are well known and peroxy bridges are not infrequent. However there is also evidence for the reaction of peroxy radicals with metal ions(94).

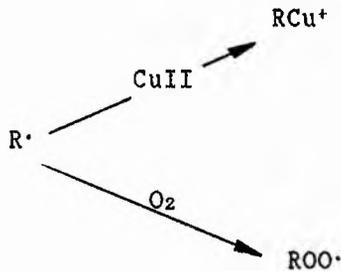


Another reaction that may be important is the radical-radical combination producing a peroxide anion(95).



Superoxide radical can be produced from the interaction of the metal ion and oxygen. Therefore at high concentration of copper there may be high concentrations of  $O_2^-$  which may cause chain

termination to occur. Within the polymer industry Bagheri et al<sup>(96)</sup> demonstrated that cupric stearate is an effective processing stabilizer. This was said to be surprisingly due to the known reaction of copper (II) with hydroperoxides. They suggested that at low oxygen partial pressures which are encountered in mixers, copper (II) competes for alkyl radicals with oxygen.

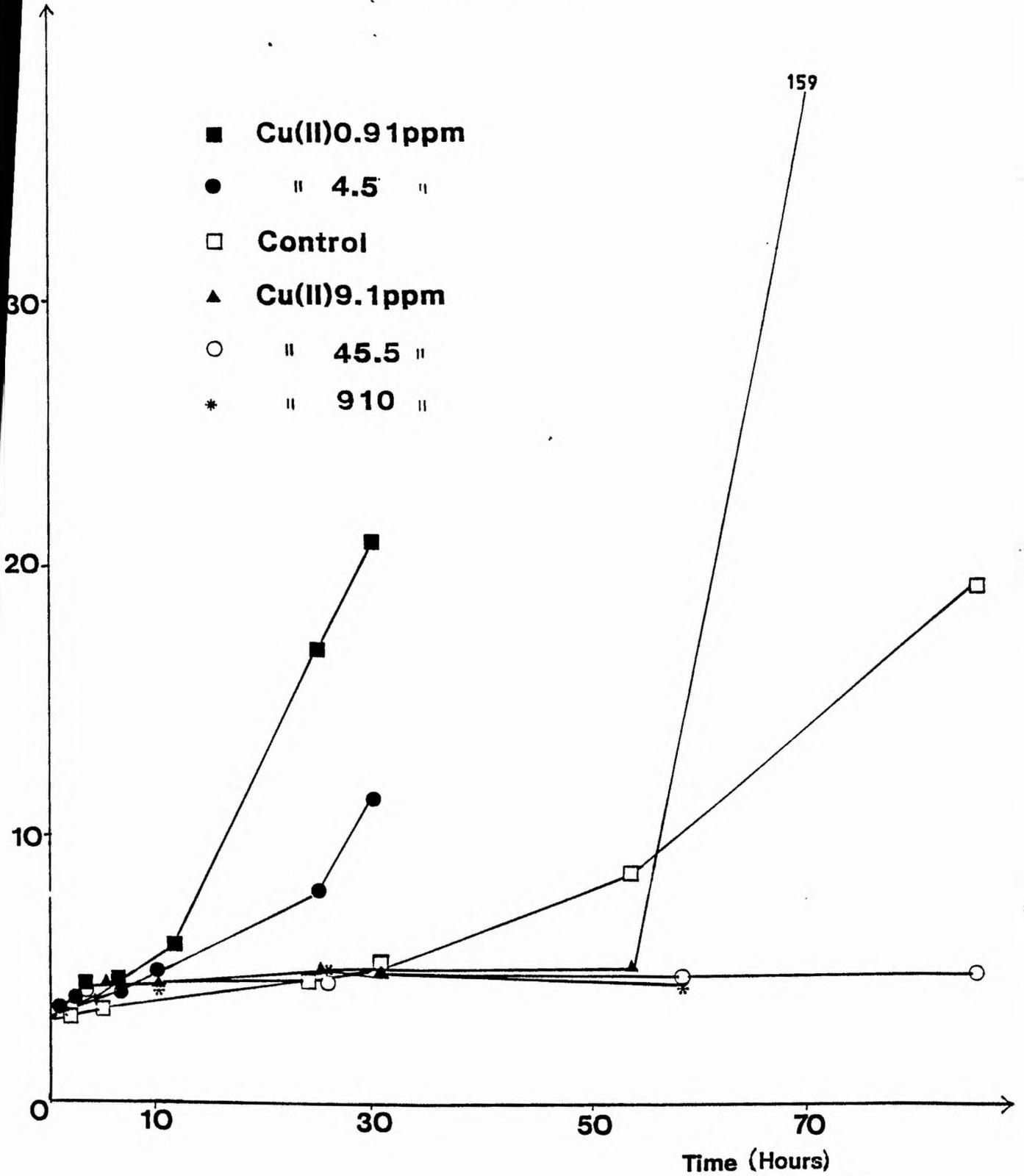


In liposomes it is difficult to speculate on the exact mechanism by which copper is acting without having performed further experiments.

These results raise questions regarding the conclusions obtained in earlier experiments concerning the lack of pro-oxidant activity exhibited by copper (II) in the absence of salt. It may be the case that very high concentrations of copper on the membrane surface is the reason for the lower rate and not the lack of mobility of the ions. However, water may allow phospholipid movement within the bilayer to occur more freely which may in turn promote oxidation.

The results may be of great significance for lipid peroxidation in meat and other food systems. Copper (II) may be present in very small amounts and at these low concentrations may not have a major influence on peroxidation. However, if the copper is concentrated as it would be by freezing it may come into a range where it is highly pro-oxidant. Alternatively, if copper (II) is present at a significant level, 1-10 ppm and then concentrated an inhibitory effect on lipid oxidation may be observed. The addition of salt or other electrolyte may decrease the copper concentration back into a more pro-oxidant range. It may be necessary in food products to closely monitor the level of copper (II) and other metal ions to aid in the prediction of shelf-life.

FIGURE 4.1 6



**CHAPTER 5**

## 5.1 LIPID OXIDATION IN MEAT

### 5.1.1 Introduction

Lipid oxidation in meat is a considerably more complex process than in model systems. This is due to the occurrence of other molecular species which may interact with lipids and their oxidation products affecting reaction pathways. The effects of natural phenolic antioxidants, synergists and trace metal ions are well known but their interactions with various food components may have a considerable impact on the development of rancidity. Within the meat industry, process, storage and packing conditions may also play an important part in lipid oxidation.

### 5.1.2 Effect of Transition Metal Ions

Many researchers have demonstrated the presence of transition metal ions and their catalytic role in lipid oxidation. (72,97,98) The interaction of metal ions with lipid hydroperoxides has been discussed in detail in chapter 1. Ke and Ackman<sup>(72)</sup> found Fe(II) and Cu(II) to be strong catalysts when added to mackerel skin and meat. The meat lipids were particularly sensitive to oxidation below 5 ppm (Cu(II) and Fe(II)) but increments above this level did not result in a further increase in catalytic activity. Igene et al<sup>(99)</sup> have

shown that the addition of EDTA to meat effectively removes nonheme iron thus significantly reducing lipid oxidation.

### 5.1.3 Effect of Lipid Enzymes

In plants and animals there exists a wide range of enzymes which can interact with lipid components causing their oxidation and breakdown. Lipolytic enzymes catalyze the release of free fatty acids from glycerides. Then the unsaturated free fatty acids can be oxidised to hydroperoxides and endoperoxides by lipoxygenase (LOX) and cyclooxygenase respectively. A variety of lipolytic enzymes are known which are specific in their ability to hydrolyse the acyl bond of a defined lipid substrate. For example, true lipases hydrolyse only triglyceride, phospholipases act on phospholipids and glycosyldiglyceride lipases hydrolyse glycolipids. Enzyme nomenclature can be further subdivided to describe the position at which hydrolysis occurs, for example, phospholipase - A hydrolyses the fatty acid in the 2 - B - position of phospholipids. LOX oxidises polyunsaturated fatty acids in a similar manner to autoxidation except that it is selective in both the substrate and products formed. The fatty chain has to have a cis - cis - 1, 4 - pentadiene moiety before oxidation is observed.<sup>(100)</sup> Oxidation of linoleic acid by LOX's from different sources give different products and are also

dependent on pH. For example, the lipoxygenase enzyme from human and pig platelets give 100% 12 - hydroperoxylinoleic acid at pH 7 <sup>(101)</sup>. Flax seed LOX produces a mixture of 9- and 13 - hydroperoxylinoleic acid in the ratio 20:80 at pH 6.5<sup>(102)</sup> and lentil seed LOX gives the 9 - and 13-hydroperoxides in the ratio 26.1:73.9 <sup>(103)</sup>. In addition to the production of fatty acid hydroperoxides LOX can catalyze their decomposition. It is generally believed that this property of LOX is due to the redox nature of its iron active - site; the ferrous form of LOX causes homolytic cleavage of the hydroperoxide to an alkoxy radical which is transformed into secondary products. Platelet cyclooxygenase has been shown to oxidise arachidonic acid to 15 - hydroperoxy - 9 , 11 - peroxidoprostanoic acid (PGG<sub>2</sub>). PGG<sub>2</sub> is the precursor for various prostaglandins and thromboxanes.<sup>(104)</sup>

Gardner et al <sup>(105,106)</sup> used FeCl<sub>3</sub> - cysteine as a model for the enzymic decomposition of lipid hydroperoxides. They found that under aerobic conditions a mixture of oxodienes, hydroxydienes, oxoepoxyenes, hydroxyepoxyenes, oxyhydroxyene, trihydroxyene, and dihydroxyethoxyene were produced from the hydroperoxides of linoleic acid. Under anaerobic conditions the same model system produced oxooctadecadienoic acid as an additional lipid product.<sup>(106)</sup>

In addition to volatiles being produced from the B - scission of alkoxy radicals it has now been shown that enzymes may also cause hydroperoxide decomposition into volatile products.<sup>(107)</sup> For example, pea LOX preparations have been reported to oxidise linoleic acid to trans 2, cis -4-heptadienal, propanal, 2- pentenal, acetaldehyde, crotonaldehyde and hexenal.<sup>(107)</sup>

It is apparent from these studies that enzymes play an important role in the oxidation of lipids. They partly control the stereochemistry of the hydroperoxide isomers produced and their concentration which ultimately affects the nature of flavours and odours detected in foods.

#### 5.1.4 The Effect of Water Activity

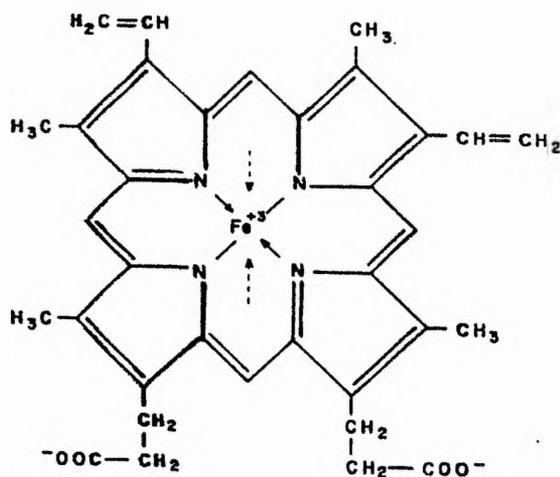
Water activity has been recognised as a major factor affecting lipid oxidation in foods. At very low levels water acts as an antioxidant by decreasing the pro-oxidant activity of metal ions and by preventing the decomposition of hydroperoxides (stabilization by hydrogen-bonding with water). As water increases, water acts as a pro-oxidant by mobilization of reactants such as metal ions and by exposing additional catalytic sites in proteins. However at high water activities dilution of metal ions may reduce oxidation. The importance of water in lipid oxidation was demonstrated

by Karel.<sup>(108)</sup> He freeze-dried unsaturated fatty acids from a solution containing carbohydrates. After drying the fatty acids they were present in two states, as surface lipid and entrapped lipid. When the surface lipid was washed with hexane oxidation stopped entirely. When the lipid was then plasticised with increasing water activities oxidation resumed immediately, illustrating water's key role in promoting access to reactants.

#### 5.1.5 Effect of Haemes, Proteins and Free Fatty Acids

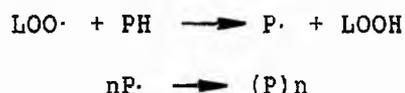
One of the major group of catalysts of lipid oxidation in meats are the iron porphyrins or heme compounds. The basic structure of a compound is illustrated in Figure (5.1).

FIGURE 5.1



Labuza<sup>(3)</sup> suggested that the protein portion of heme molecules may cause steric hindrance of the iron preventing it from catalyzing oxidation. When meat is heated denaturation of the protein portion of the molecule may facilitate exposure of iron to unsaturated fatty acids. Erikson<sup>(109)</sup> showed that protein denaturation increased the ability of the heme-containing proteins, peroxidase and catalase to promote lipid oxidation. While heme compounds are generally regarded as pro-oxidants of lipid peroxidation some researchers have shown that their action is concentration dependant and at certain ratios of lipid to heme their function changes to that of an antioxidant. Kendrick<sup>(110)</sup> using a model system, showed that linoleate to heme ratios for maximum catalysis were 100 for hemin and catalase, 400 for cytochrome C and 500 for methmyoglobin. When heme concentrations were 2 or 4 times the optimum catalytic concentrations no oxidation occurred.

Several authors have demonstrated that lipids and their oxidation products interact with proteins.<sup>(111-113)</sup> Kanazawa et al<sup>(19)</sup> showed that linoleic acid, linoleic acid hydroperoxides and their oxidation products all interact with the protein casein. Prolonged incubation of casein with the hydroperoxide oxidation products resulted in polymerisation of casein. Karel<sup>(114)</sup> suggested the mechanism of polymerisation to be:-



PH = protein

LOO = lipid peroxy radical

A study by Miyashita et al<sup>(88)</sup> showed that the level of free fatty acids may be an important factor in the development of rancidity in meat. They demonstrated that free fatty acids such as stearic acid accelerates the rate of autoxidation of methyl linoleate and the decomposition of methyl linoleate hydroperoxide. Also, oleic acid oxidised more rapidly than the corresponding methyl esters. They suggest that the carboxyl group promotes the decomposition of the hydroperoxide since increased rates of autoxidation were observed. Heterolytic cleavage which occur with H<sub>2</sub>SO<sub>4</sub> and HCl produces non radical products and would therefore not increase the rate.

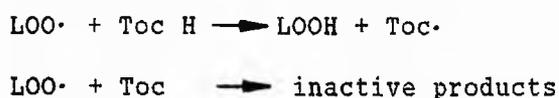
#### 5.1.6 Effect of Antioxidants

The use of antioxidants in the food industry is necessary to increase both the quality and shelf-life of products through processing, packaging and storage. The use of natural antioxidants in food is becoming more popular as the cost for testing the safety of new synthetic antioxidants is

prohibitory. The public are also becoming more concerned at the volume of artificial substances which are added to foods and the risk to health this may bring.

The tocopherols (TocH) are naturally occurring antioxidants and known to act by donating a hydrogen atom to lipid peroxy radicals, (115, 116) Figure (5.2).

FIGURE 5.2



Tocopherols are consumed by the reaction of chromoxy radicals (Toc.) with other peroxy radicals or with each other to form dimers. By determining the rate constant for the above reaction Burton and Ingold (115) concluded that  $\alpha$ -tocopherol is the most effective chain-breaking antioxidant among the tocopherols. However, at high concentrations  $\alpha$ -tocopherol does not exert an antioxidant effect but allows oxidation to occur smoothly and controls the stereochemistry of the hydroperoxide products. (117) Terao et al (118) observed a similar prooxidant effect by  $\alpha$ -tocopherol and explained their results by proposing that the chromoxy radicals can compete with peroxy radicals at hydrogen atom abstraction from the lipid substrate. Ascorbic acid, another

natural antioxidant is generally believed to function as an oxygen scavenger but Packer et al (119) has demonstrated that ascorbic acid can regenerate  $\alpha$ -tocopherol by donating a hydrogen atom to the chromoxy radical. Therefore the lipid soluble  $\alpha$ -tocopherol and water soluble ascorbic acid can act synergistically together in preventing peroxidation.

Water and ethanol soluble extracts from various foods have been used as natural antioxidants, although the precise molecular composition of the antioxidant moiety has been difficult to establish in some cases. Chipault et al (120) analysed over thirty spices and herb extracts and found rosemary and sage to be the most effective in preventing the oxidation of lard. Brand et al (121) reviewed the pro- and antioxidant properties of phospholipids and ascribed their antioxidant activity to the regeneration of phenolic antioxidants and complexation of pro-oxidant metals. The role of carotene in stabilising edible oils has been reviewed by Kroschel.(122) It is believed that carotene and carotenoids in general are singlet oxygen quenchers although their oxidation products may act as pro-oxidants.

## 5.2 METHODOLOGY FOR FOLLOWING LIPID OXIDATION IN MUSCLE FOODS

At present there are numerous methods available for following lipid oxidation in meat. In deciding which method to use one must bear in mind several points. What does the method measure and is the method specific to what we want to measure? Another important factor which may help to decide the method employed is how well does it correlate to sensory analysis.<sup>(123)</sup> The following methods represent what researchers are currently employing to follow lipid oxidation.

### 5.2.1 Peroxide Value

This method measures the total peroxide content of the extracted fat. However, no information on the secondary breakdown products is provided. The most common methods for measuring peroxide value (PV) use iodometric techniques similar to the AOCS (1973) method and PV is reported in meq of iodine/kg of fat. Bailey et al<sup>(124)</sup> found that PV in fat from pork carcasses was less than 1 meq of I/kg of fat after 9 months storage at -20°C. Another research group<sup>(125)</sup> found that peroxides in porcine muscle stored for 10 weeks at -10°C ranged from about 10 to as high as 70 meq of I/kg of fat with 70% of this increase occurring within the first four weeks. Jeremiah<sup>(126)</sup> used PV to investigate the lipid deterioration in frozen pork in different types of wrap. He found that PV

increased for up to 140 days of frozen storage for fresh pork cuts but only 56 days for cured meat products. He reported significant relationships between PV and flavour rancidity score for fresh pork samples but not for cured pork samples. Noble<sup>(127)</sup> found that PV of ground deboned chicken meat stored at  $-23^{\circ}\text{C}$  for 12 weeks increased from 1.5 meq of I/kg of fat at 0 weeks to a maximum of 3.70 at 6 weeks of storage then decreased to 1.9 after 12 weeks. Noble did not propose any cause for the significantly higher PV in the 6 week samples. Palmer et al<sup>(128)</sup> did not find any apparent decrease in PV in ground turkey meat stored at  $-12.8$  to  $-29.9^{\circ}\text{C}$  for up to eight months.

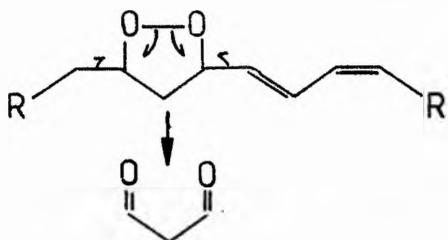
From these several studies it seems that peroxide value is capable of observing oxidation in muscle foods. However, it seems difficult to directly correlate increasing PV to organoleptic changes. This may be due to the breakdown of these peroxides giving a complex array of secondary products.

#### 5.2.2 The TBA Test

This is the most widely used method for following lipid oxidation in meat. The 2-thiobarbituric acid test or TBA test expresses lipid oxidation as mg of malonaldehyde per kg of sample. Initially the test was thought only to measure malonaldehyde<sup>(129)</sup> which is a secondary oxidation product of

unsaturated fatty acids with three or more double bonds,<sup>(130)</sup>  
Figure (5.3).

FIGURE 5.3



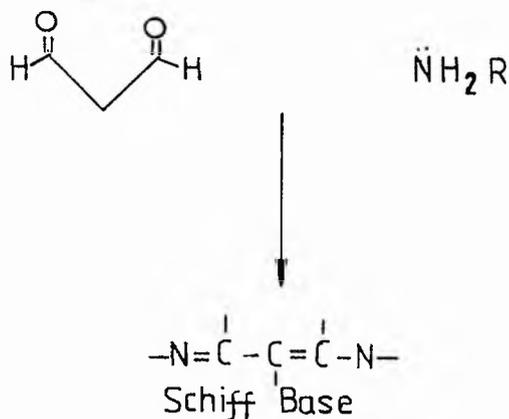
However, other researchers have shown that lipid oxidation products such as alka -2, 4- dienals also react with TBA to form a red complex with the same absorption maximum as the malonaldehyde - TBA complex at 532 nm<sup>(131)</sup>. There are three ways the TBA test can be carried out. One method involves direct reaction on the food product, followed by extraction of the coloured complex. Another procedure forms the complex in a food extract while the third method involves complex formation on an extract of the steam distillate of the food. This third method is the most popular for measuring the TBA number in muscle foods.<sup>(132)</sup> This procedure has been used by many research workers to follow oxidation in meat and meat products.<sup>(133-135)</sup>

Some researchers have modified the method and used an antioxidant mixture containing 20% BHA, 6% propyl gallate and

4% citric acid in propylene glycol at the distillation stage to prevent oxidation of chicken meat.<sup>(136)</sup>

Although malanaldehyde is a secondary reaction product there is evidence for its decrease on increased storage time in muscle foods. TBA values have been observed to decline during frozen storage of cooked meat and fishery products<sup>(132)</sup> and more recently in freeze-dried meats.<sup>(137)</sup> The reason for this lowering of TBA values has been attributed to reaction of malonaldehyde with amino groups in phospholipids and proteins,<sup>(138,139)</sup> Figure (5.4).

FIGURE 5.4



### 5.2.3 Carbonyl Compounds

This method for following lipid oxidation is a colorimetric technique which can determine total carbonyl compounds, total monocarbonyl compounds or individual classes of

monocarboxyls, for example, alka-2-ones, alka-2-ones, etc. The most frequently used determination is that of either total carbonyls or total monocarboxyls. Two methods have been used in the separation of total carbonyls from muscle foods. One method (140) extracts lipid and carbonyls from meat with hexane followed by derivatisation to the 2, 4-dinitrophenyl - hydrazones on a celite 545 column impregnated with 2, 4 - dinitrophenylhydrazine and measured at 340 nm. In the method of Lawrence(141) the total carbonyls are converted to their 2, 4 - dinitrophenylhydrazones in an aqueous medium, then extracted with hexane prior to their measurement at 340 nm. Kunsman et al(142) found that the total monocarboxyls were a better indicator of lipid oxidation in mechanically deboned red meat than the total carbonyl content. Overall, changes in carbonyl content tend to be erratic and do not give a good indication of oxidative deterioration.(143,144)

#### 5.2.4 Changes in Fatty Acid Composition

This method measures the decrease of individual fatty acyl chains fo lipids. Several workers have shown that the decrease in PUFA of phospholipids can be used in following lipid oxidation.(118,135,136) Dimick et al(144) and Moerck et al(136) showed that PUFA of phospholipids in poultry decreased rapidly during frozen storage. However, Kunsman et al(142) found little change in PUFA of phospholipids of beef

during frozen storage. Overall, this method should prove very useful as all oxidation products measured by other techniques originate from the oxidation of unsaturated fatty acids.

#### 5.2.5 Aldehyde Measurement by GLC

Frankel et al.<sup>(145)</sup> showed that hexanal is one of the major secondary products formed during the oxidation of linoleic acid. This and other aldehydes have been successfully identified and used in following lipid oxidation in oils and foods.<sup>(146,147)</sup> Besides determination of their 2, 4 dinitrophenylhydrazine derivatives, aldehydes can be analysed by gas liquid chromatography (GLC). The aldehydes can either be analysed by a head space gas sampling technique followed by GLC<sup>(147)</sup> or by directly applying the food sample into a liner which is inserted into the inlet assembly of a GC and heated to 100-160°C.<sup>(148)</sup> Few researchers use this technique and more research is required to determine its usefulness in following lipid oxidation in foods.

#### 5.2.6 Oxygen Absorption

Oxygen absorption has been successfully used by several research groups<sup>(148-150)</sup> to follow oxidation in muscle foods. However oxygen absorption is not specific to lipids as

proteins may also react with oxygen. No information is obtained on the products of oxidation.

#### 5.2.7 Pentane Measurement

Pentane and other short chain hydrocarbons are known products of lipid oxidation - Pentane has been measured by many workers. The method of Seo et al<sup>(150)</sup> using a head space analyzer gave results which were in good agreement with rancid odour scores. This method has received little attention but may prove to be highly successful in following lipid oxidation.

#### 5.2.8 Fluorescent Products

The fluorescent products called schiff bases are formed from the interaction of malondialdehyde with amino groups of proteins and amino containing phospholipids. The method has been used analytically to quantify peroxidation in chloroform/methanol extractables from biological tissue.<sup>(151, 152)</sup> However, the method requires further investigation to determine its usefulness in following lipid oxidation in muscle foods.

### 5.2.9 Overview of Oxidation Methodology

It is apparent from the wide range of methods that are available to follow lipid oxidation in meat that no single one is satisfactory. Each technique has advantages and disadvantages.

As described in chapter 1 hydroperoxides are the primary products of autoxidation and may be determined by measuring the peroxide value. However, this method only measures hydroperoxides and gives no information on secondary oxidation products. Oxygen absorption is a simple and useful technique for following lipid oxidation but unfortunately gives no structural information. The TBA test, carbonyl analysis, fluorescent product analysis and the determination of hydrocarbons are also of limited value since they detect specific compounds or groups of compounds which result from secondary oxidation of hydroperoxides. The decrease in the fatty acyl chains of lipids as measured by GLC of the lipid FAME's offers direct evidence for peroxidation and of the susceptibility of individual fatty acids to oxidation. However, as with oxygen absorption no structural information is obtained. Although these methods have limitations they all show some correlation between the degree of oxidation and the decrease in quality as determined by organoleptic assessment.

### 5.3 THE CHEMISTRY OF MEAT FLAVOUR

It has only been in the past twenty years that substantial advances have been made in the chemistry of meat flavour. Modern instrumentation techniques have helped uncover the complex reaction pathways leading to a vast array of products which make up the chemical profile we recognise as meat. The chemistry of meat flavour is an enormous area and therefore only a brief outline of the subject is presented here.

#### 5.3.1 Taste Components in Meat

The taste components in meat are water - soluble, non-volatile and influence the taste sensations of saltiness, sweetness, sourness, bitterness and umami (succulence). The umami effect in meat has been attributed to glutamic acid, its sodium salt and the 5 - ribonucleotides, inosine - 5 - monophosphate (IMP) and guanosine - 5 - monophosphate (GMP). Researchers (153,154) have found IMP present in meats at levels well above its threshold and therefore plays an important role in taste appreciation, whereas GMP is present at levels on the verge of its recorded threshold range. Meat taste is strongly influenced by the fat content and recently a relationship between total lipids and fatty taste and between free fatty acids and the pleasant taste of beef broth has been established. It will also be the case that

lipid oxidation products when decomposed on cooking contribute to the complex but characteristic taste associated with rancidity.

### 5.3.2 Volatile Aroma Components in Meat

Literally hundreds of volatile compounds which contribute to the chemical profile we associate with meat have now been identified. This introduction shall consider classes of compounds and their contribution to meat aroma.

#### 5.3.2.1 Fatty Acids, Esters and Lactones

Free fatty acids are naturally found in meat but may also be derived from triglycerides and phospholipids by either the action of bacterial enzymes or by hydrolysis and thermal oxidation during the cooking process. Early researchers (155,156) found that there could be significant differences in the concentration of unsaturated free fatty acids between various types of meat. Wong et al<sup>(157)</sup> attributed the low consumer acceptability of sheep meat in many counties to 4-methyloctanoic acid and to a lesser extent 4-methylnonanoic acid. Aliphatic and aromatic esters have been shown to be produced from the interaction of free fatty acids and alcohols in the adipose tissue of

meat.<sup>(158)</sup> In pork and lamb the esters are believed to impart a fruity sweet aroma while in beef a fatty aroma. Lactones have also been identified and have been described as buttery, fatty and fruity.

#### 5.3.2.2 Aldehydes and Ketones

The major source of aldehydes and ketones is from the decomposition of lipid oxidation products. A full description of their formation has been given in chapter 1. It is not thought that they contribute significantly to meat aroma.

#### 5.3.2.3 Furans and Thiophenes

Furans and thiophenes are generally regarded as the most important classes of compounds contributing to meat flavour. The furans are believed to derive from the interaction of carbohydrates with amino acids. 2-Pentylfuran is normally found in the highest concentration and Chang et al<sup>(159)</sup> suggested that it was produced from the cyclisation of 4-ketnonanol derived from linoleic acid. Furans seem to impart sickly flavours while thiophenes give burnt or rubbery aromas.

#### 5.3.2.4 Pyrazines

It is generally believed that pyrazine formation only occurs with cooking. However, recently Kinderlerer et al<sup>(160)</sup> showed that alkylpyrazines were not formed as a result of heating but by bacterial metabolism in the case of desiccated coconut. Pyrazines are described as having a nutty/roasted aroma and are thought to contribute significantly to meat aroma.

#### 5.3.2.5 Sulphur Compounds

It is thought that sulphur containing compounds are major contributors to the aroma of cooked meat flavours but most possess non-meaty objectionable odours. Wasserman<sup>(161)</sup> found that when sulphur compounds were removed from meat samples a loss of meatiness resulted. They have been described as having oniony, burnt, fatty and meaty aromas.

#### 5.3.2.6 Miscellaneous

Hydrocarbons, alcohols, pyrroles and others have all been identified in meat aroma although much work is still required to establish their contribution.

**CHAPTER 6**

## 6.1 INTRODUCTION

Four storage trials were undertaken to investigate the effect of salt content, temperature, cooking and curing on the oxidative stability and shelf-life of pork burgers.

Pork back fat was freshly obtained from Mathessons Walls prior to each experiment. Batches of pork were minced and mixed using a Hobart mincer and mixing attachments respectively. The burgers were produced on a pattie former with an average burger weight of 72g.

### 6.1.1 The Stability of Uncooked Pork Burgers Containing 1% Salt at Different Temperatures

This storage trial was undertaken to investigate the effect of sub-zero temperatures on the oxidative stability of burgers containing 1% salt.

#### Method

Pork burgers were produced containing 1 w/w% salt, added as 50 mls of 20% salt per soln kg of meat (ignores weight of water). The burgers were separated with waxed paper interleaves and frozen to -30°C in a Foster blast freezer.

divided into five batches which after packaging were stored at either -4, -8, -13, -20 or -30°C. Burgers were also prepared with added water but no salt and stored at -20°C. Burgers (25) were stored in polythene bags. Six packages were normally produced for each sample treatment.

6.1.2 The Stability of Cooked Pork Burgers Containing Different Amounts of Salt at -20°C

This experiment investigated the effect of salt content on the oxidative stability of cooked burgers stored at -20°C.

Method

Pork burgers were produced with 1, 2 or 3 w/w% salt. The salt (added dry) was carefully and slowly mixed with comminuted pork before cooking. The burgers were cooked in a Rapidair steam cooker for 25 minutes at 100°C. This resulted in a burger centre temperature of 70-75°C. Samples were allowed to cool to room temperature and then frozen to -30°C in a Foster blast freezer (2 hours). Burgers were packaged as described in experiment 6.1.2 and stored at -20°C.

6.1.3 The Stability of Uncooked Pork Burgers  
Containing Different Amounts of Salt at -20°C

This experiment was undertaken to consider the effect of salt content of the oxidative stability of raw pork burgers held at -20°C.

Method

Salt (added dry) was carefully added with mixed to comminuted pork to produce burgers with a salt content of 1, 2, 3 and 4 w/w%. Batches of burgers were also prepared without added salt and with 1 w/w% KCL. The raw pork burgers were then frozen to -30°C in a Foster blast freezer (2 hours) and packaged as described in experiment 6.1.2 and stored at -20°C.

6.1.4 The Stability of Uncooked Pork Burgers Containing  
Sodium Nitrite and Different Amounts of Salt at-  
20°C

This experiment investigated the salt content of a salt/nitrite cure on the oxidative stability of raw pork burgers stored at -20°C.

### Method

The procedure described in experiment 6.1.4 was carried out with the modification that 0.250 g of sodium nitrite was added per kg of communicated pork. Burgers were also prepared containing 1 w/w% KCL.

#### 6.1.5 Sample Analysis

At each sample take-off date the burgers were analysed by the following methods:-

1. 1 burger - FAME profile on total lipid and phospholipid fraction.
2. 1 burger - Colour analysis using reflectance spectroscopy.
3. 1 burger - HPLC on oxidised neutral lipid.
4. The remaining burgers - organoleptic assessment using a trained panel of food assessors.

Burgers sent to St Andrews for FAME analysis, were

hopefully preventing any chemical deterioration. Samples examined at Colworth House were vacuum packed and stored at  $-30^{\circ}\text{C}$  if not analysed immediately.

Samples in all four storage trials have been coded A to V according to treatment and conditions of storage, Table (6.1). The lettered codes have been used throughout the various analysis and Table (6.1) should be referred to for burger treatments and conditions of storage. The beginning of the storage trial was taken to be the same in all four experiments even though a few days separated the start of individual experiments.

TABLE 6.1

Storage Trial 1 (Sect 6.1.2) - burgers uncooked

<u>Sample</u>	<u>Treatment</u>	<u>Storage Temp °C</u>
A	1% sodium chloride	-4
B	1% sodium chloride	-8
C	1% sodium chloride	-13
D	1% sodium chloride	-20
E	1% sodium chloride	-30
F	no sodium chloride	-20

## Storage Trial 2 (Sect 6.1.3) - burgers cooked

<u>Sample</u>	<u>Treatment</u>	<u>Storage Temp °C</u>
G	no sodium chloride	-20
H	1% sodium chloride	-20
I	2% sodium chloride	-20
J	3% sodium chloride	-20

## Storage Trial 3 (Sect 6.1.4) - burgers uncooked

<u>Sample</u>	<u>Treatment</u>	<u>Storage Temp °C</u>
K	no sodium chloride	-20
L	1% sodium chloride	-20
M	2% sodium chloride	-20
N	3% sodium chloride	-20
O	4% sodium chloride	-20
P	1% potassium chloride	-20

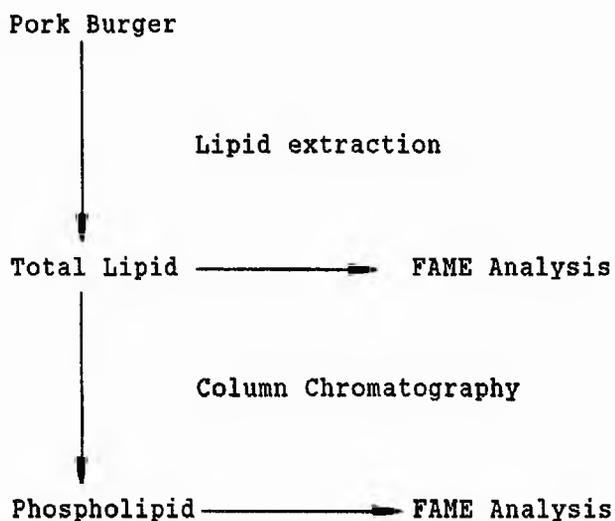
## Storage Trial 4 (Sect 6.1.5) - burgers uncooked

<u>Sample</u>	<u>Treatment</u>	<u>Storage Temp °C</u>
Q	nitrite, no sodium chloride	-20
R	nitrite, 1% sodium chloride	-20
S	nitrite, 2% sodium chloride	-20
T	nitrite, 3% sodium chloride	-20
U	nitrite, 4% sodium chloride	-20
V	nitrite, 1% potassium chloride	-20

**6.2 FAME ANALYSIS**

At each take off point total lipid was extracted from a burger. FAME analysis was carried out on the total lipid and the phospholipid fractions, Figure (6.1).

FIGURE 6.1



#### 6.2.1 Lipid Extraction

The procedure was based on the method of Maxwell et al.<sup>(162)</sup> The frozen burger was divided into sixteen portions using a razor blade. One portion (~4.5g) was partially thawed and ground in a 750 ml porcelain mortar with 20 g granular anhydrous sodium sulphate. After efficient mixing, 15 g Celite 545 was added and the mixture ground to a fine powder. This was packed above a CaHPO<sub>4</sub>/Celite 545 (1:9w/w) trap (10 g) in a glass chromatography column (id 35 mm). The total lipid was then eluted with 230 ml of dichloromethane-methanol (9:1 w/w). The solvent was removed at a temperature not exceeding 30°C on a rotary film evaporator and any

remaining water removed by the addition of small quantities of acetone and subsequent evaporation. The lipid was stored in chloroform under a nitrogen atmosphere at  $-20^{\circ}\text{C}$ . Lipids were normally analysed within 2 days of extraction.

#### 6.2.2 Isolation of Phospholipid

The method of Juaneda et al<sup>(163)</sup> was slightly modified and used to isolate pork phospholipids. Phospholipids were separated from total lipids using small silica cartridges 25 mm x 10 mm (Waters, Millipore UK). Approximately 100 mg of lipid was dissolved in 0.5 ml chloroform and loaded onto the column. Before the solution was fully absorbed 40 mls of chloroform was pushed through the column using a gas tight syringe with leur tip. This removed all neutral lipids as detected by tlc (developing solvent-hexane/diethylether/methanol/acetic acid 90:20:5:2 v/v/v/v). Phospholipids were removed from the column with methanol. The methanol was evaporated from the phospholipids by blowing nitrogen over the surface and gently heating. The dry phospholipid was then esterified immediately.

### 6.2.3 Esterification

#### Phospholipids

Phospholipids were transesterified using sodium methoxide as described in chapter 2.

#### Total Lipids

Total lipids were esterified by the following method. Approximately 50 mg of total lipid was added to a test tube containing 2 mls of 2% H<sub>2</sub>SO<sub>4</sub> in dry methanol and 1 ml tetrahydrofuran. The mixture was refluxed for 2 hours in a heating block then cooled and 5 mls of saturated salt solution added before finally extracting with petrol 40-60 (2 x 5 mls). The extract was stored over anhydrous sodium sulphate and under nitrogen.

### 6.2.4 Gas-Liquid Chromatography

Total lipid FAME's were analysed on a Pye Unicam 4500 gas chromatograph and phospholipid FAME's on the Hewlett Packard 5890A gas chromatograph. The conditions of analysis were as described in chapter 2.

### 6.2.5 Results and Discussion

The results of the FAME analysis of the total lipids and phospholipids are presented graphically in Figures (6.2-6.9) and tabulated in Appendices (6.1 - 6.8). As discussed in chapter 2 the results of the FAME analysis are presented as an oxidation index. This is the ratio of the percentage composition of the saturated FAME's palmitate and stearate divided by the percentage composition of an unsaturated FAME. Therefore, an increase in the oxidation index (OI) represents an increase in oxidation.

#### 6.2.5.1 The Stability of Uncooked Pork Burgers containing 1 w/w% Salt at Different Temperatures

It is apparent from the analysis of the total lipid FAME's of all samples, Figure (6.2), Appendix (6.1) that an increase in oxidation occurred as the storage trial progressed. Additionally, increasing the storage temperature from  $-30^{\circ}\text{C}$  to  $-4^{\circ}\text{C}$  resulted in an increase in oxidation. Sample F which was stored at  $-20^{\circ}\text{C}$  in the absence of salt appeared to be more stable to oxidation than sample D which contained 1% salt and stored at the same temperature.

The analysis of the phospholipid FAME's are presented in Figure (6.3) and Appendix (6.2). A significant increase in the OI of burgers stored at  $-8^{\circ}\text{C}$  (sample B) occurred after 8 weeks. The OI then remained relatively constant until the end of the trial. This was in contrast of what was observed in the total lipid FAME analysis where there was a general increase in OI on increased storage time. One reason that may help explain this observation is that the phospholipids may have been hydrolysed releasing free fatty acids. The alkali-catalyzed esterification procedure which was used to determine the pork phospholipid FAME's does not esterify free acids and therefore loss of unsaturated fatty acyl chains through hydrolysis is not accounted for. In the analysis of total lipid the acid-catalyzed esterification method does esterify free fatty acids and therefore the FAME profile of the total lipid should not have been affected. Another possibility may be the interaction of oxidising lipids with protein forming cross-linked materials which are not recovered by the extraction procedures used.<sup>(111)</sup> Phospholipids normally have one saturated and one unsaturated hydrocarbon tail. If an oxidation product from the unsaturated moiety forms a cross link it has the effect of also removing the unreacted saturated acyl group which would alter the

obtained on burgers stored at  $-4^{\circ}\text{C}$  (Sample A) as they had become rancid after 10 weeks. Samples C-F showed very little change in their phospholipid FAME OI until after 20 weeks. After 38 weeks there were significant increases in all samples. Apart from sample B, oxidation indices increased on increased temperature ie  $-30^{\circ}\text{C}$  to  $-4^{\circ}\text{C}$ . If hydrolysis is an important factor in interpreting the results it is not known to what extent it occurred and if it did to what degree at different temperatures.

The influence of temperature on the rate of 'simple' chemical processes is well documented.<sup>(164)</sup> As a rough guide, the rate of reaction doubles for every  $10^{\circ}$  rise in temperature. However in meat and other food systems this explanation is insufficient due to the complex nature of meat chemistry. As discussed in chapter 5, water content and water activity have been shown to be important factors which can affect the rate of lipid peroxidation. Although these parameters were not measured during the study they would have been expected to decrease at lower storage temperatures. Poulsen and Lindelov<sup>(165)</sup> found that the water activity of fresh meat fell for 0.981 at  $1^{\circ}\text{C}$  to 0.784 at  $-25^{\circ}\text{C}$ . Within this range enzymic reactions would be expect to decrease with lowering water activity and metal ion concentration

with lowering water activity and metal ion concentration increase. A lowering in temperature would also have increased the ionic strength of unfrozen water. Higher ionic strengths have been reported to increase protein extraction from membranes.<sup>(165)</sup> Therefore, protein catalyzed peroxidation may have occurred to different extents at the different storage temperatures. A decrease in temperature would have been expected to increase the concentration of 'free' metal ions since the amount of aqueous phase is reduced. If copper (II) was an important catalyst of lipid peroxidation in pork then its activity would be concentration dependant (chapter 4). The observed rates of peroxidation were therefore a result of numerous factors, each of which could have affected peroxidation to different extents over the temperature range studied. The results also showed that the higher the level of unsaturation of the fatty acyl chain the more prone to peroxidation. This was in agreement with other research groups who investigated the stability of a variety of meats held under frozen storage.<sup>(124, 136, 166, 167)</sup>

#### 6.2.5.2 The Stability of Cooked Pork Burgers containing Different Amounts of Salt at -20°C

The results from this experiment are presented in

Figures (6.4 - 6.5) and Appendices (6.3 - 6.4). From the total lipid analysis we can see that a general increase in oxidation occurred on increased storage time. Due to the variation in experimental results no definite conclusions can be made about the effect salt content had on total lipid oxidation. However, in the analysis of phospholipid FAME's large differences were observed in samples stored with different concentrations of salt. There was very little change in the OI of burgers stored with no salt (sample G) but as the salt content increased from 1 to 3% there was a corresponding increase in the OI. Several authors have found a pro-oxidant activity exhibited by salt while others an antioxidant effect but very few speculate on the exact mechanism by which it acts.<sup>(3, 168-170)</sup> Drerup et al<sup>(168)</sup> demonstrated that prerigor grinding and salting of pork reduced the rate of oxidation during storage of pork sausage at 0°C which contrasted with an increased rate of oxidation when pork was ground and salted postrigor. Mabrook et al<sup>(169)</sup> studied the autoxidation of methyl linoleate and linoleic acid emulsions in the presence of sodium chloride at 40°C. They found that the rate of oxidation decreased with increased salt content and found no evidence for pro-oxidant activity. They suggested that the inhibitory effect exhibited by sodium chloride was due to the lower solubility of

oxygen in the emulsion.

The cooking of meat is believed to denature iron-containing proteins which may result in an increased exposure of iron to oxidising lipids.<sup>(3,123)</sup> Igene et al<sup>(123)</sup> demonstrated that by heating meat extracts to 70°C an increase in the nonhaem iron concentration occurred. MacDonald et al<sup>(171)</sup> showed that pork muscle extract was pro-oxidant when added to linoleate emulsions and attributed this effect to metal ion catalysis. The cooking process itself may have caused additional peroxidation. When the burgers were placed into frozen storage a higher concentration of lipid hydroperoxides may have been present which with an increase in the nonhaem iron concentration may have caused an acceleration in the deterioration of the pork. The findings in chapter 4 of this thesis demonstrated that by lowering the copper (II) concentration an increase in the rate of peroxidation can occur. If a similar process occurs in meat then the addition of salt would have increased the unfrozen water content, reducing the copper (II) concentration, which may have increased the rate of peroxidation. The copper content of the pork was not determined therefore it is not known how much this finding would have contributed to the observed salt effect. The ability of salt to extract protein from cell membranes is another important factor

which may have altered the susceptibility of membrane lipids to protein induced peroxidation.<sup>(165)</sup> As expected the more unsaturated the fatty acyl chains of the pork lipids the greater was their susceptibility to peroxidation. No attempt has been made to quantify the rates of peroxidation of the different acyl chains as no internal standard was used.

#### 6.2.5.3 The Stability of Uncooked Pork Burgers Containing Different Amounts of Salt at -20°C

The results from this storage trial are presented in Figure (6.6 - 6.7) and Appendices (6.5 - 6.6). From Figure (6.6) we can see that there were erratic changes in the OI of total lipid FAME's with increased storage time. However, there was very little difference between samples analysed at a particular take-off date. Burgers stored in the absence of salt generally had a lower OI than those stored with salt. In contrast, Figure (6.7), large changes in the OI of the phospholipid FAME's occurred with increased storage. However, there was not a consistent increase in the rate of peroxidation with increased salt concentration. Burgers containing 4% salt (sample O) had a lower rate of peroxidation than samples M and N which contained 2 and 3% salt respectively. The decrease in pro-oxidant activity at

high concentrations of salt may have been indirectly due to the proportion of aqueous phase to ice. With reference to the International Critical Tables (92) an approximation of the ratio of aqueous phase to ice at 20°C can be made. With no added salt all water would exist as ice. At salt concentrations of 1, 2, 3 and 4%, the aqueous phase would represent 4.2, 8.4, 12.6 and 16.8% of the mixture respectively. Therefore free metal ions would be diluted (x4) between salt concentrations of 1 and 4%. This is however, a simplified picture as meat contains other dissolved solutes which shall also affect the water content at temperatures below 0°C. As discussed in section 6.2.5.2, if copper (II) catalyzed peroxidation may have been an important process in pork and its pro-oxidant activity was concentration dependant (chapter 4) and its contribution to lipid oxidation may have been controlled by the salt content of the burgers. Therefore, in burgers containing up to 3% salt, copper (II) may have been reduced in concentration to a level where it became more pro-oxidant. However, with further dilution (burgers containing 4% salt) the copper (II) concentration may have been reduced to a level where its pro-oxidant activity was significantly reduced. The use of 1% potassium chloride (sample P) in place of 1% sodium chloride (sample L) caused a reduction in the rate of peroxidation. This could have been due to an

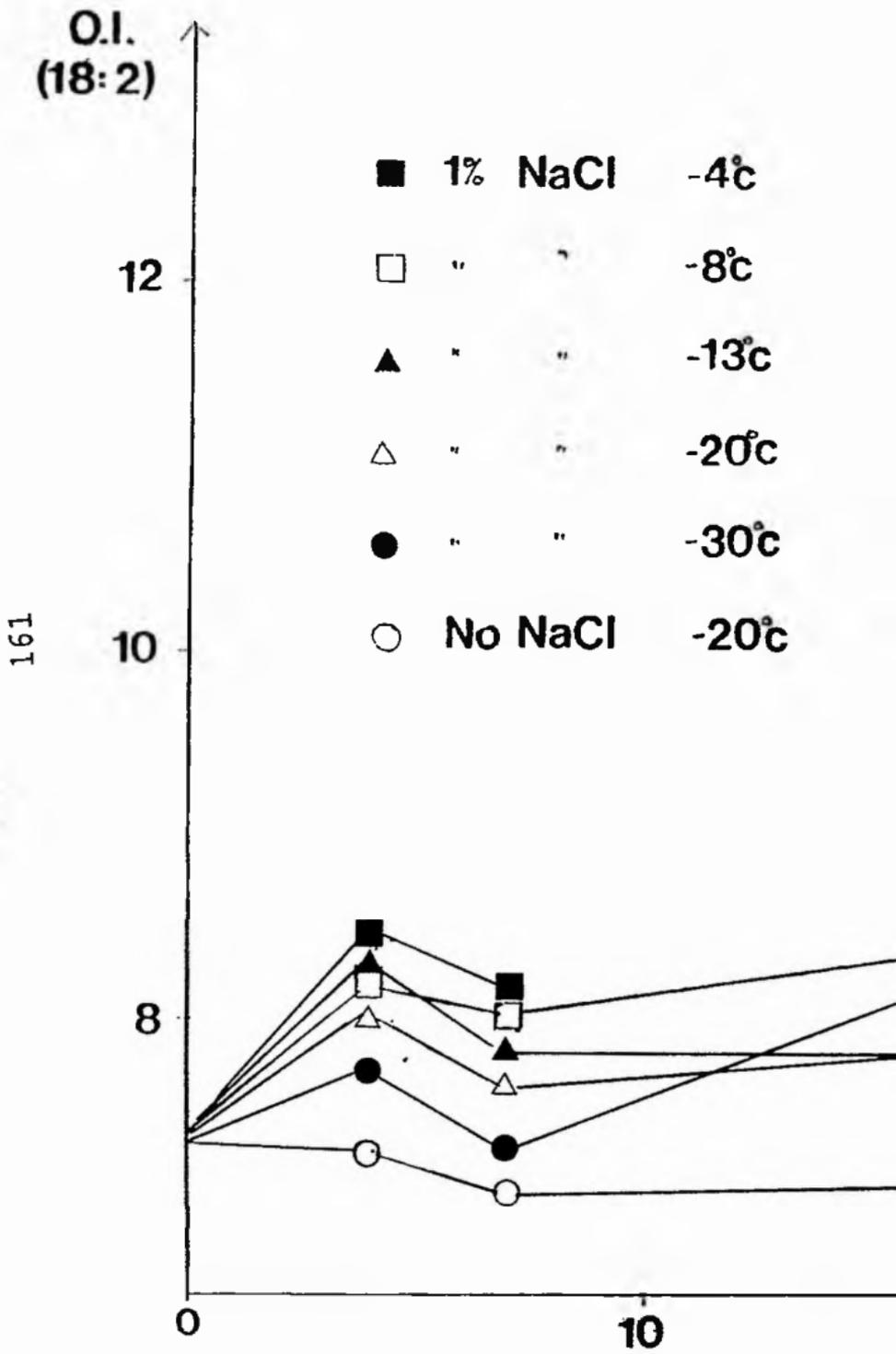
increased concentration of metal ions (copper (II)) in burgers containing potassium chloride. Potassium chloride has a eutectic temperature of  $-12.5^{\circ}\text{C}$  and would have reduced the amount of aqueous phase in the meat. As discussed for cooked burgers, factors such as reduced oxygen solubility at high salt concentrations and the ability of salt to extract membrane proteins would also have contributed to the observed rates. In agreement with the preceding two storage trials the more unsaturated the fatty acyl chain of the phospholipids the greater was its susceptibility to peroxidation. The reason for salts activity in pork burgers has been discussed in terms of metal ion concentration and its effect on peroxidation. However, since the chemistry of meat is extremely complex and the interactions between food components not well understood it is possible that there may be other reasons for salts behaviour which are not discussed here.

#### 6.2.5.4 The Stability of Uncooked Pork Burgers Containing Sodium Nitrite and Different Amounts of Salt at $-20^{\circ}\text{C}$

The results are presented in Figures (6.8 - 6.9) and Appendices (6.7 - 6.8). From Figure (6.8) we can see that there were erratic changes in the OI of the total

lipid FAME's with increased storage. After 16 to 20 weeks there was a significant decrease in the OI which is difficult to explain. As mentioned previously a decrease in OI would not be expected to occur as a direct result of peroxidation since the saturated fatty acyl chains are considerably more stable to oxidation than the polyunsaturated chains. The hydrolysis of glycerides producing free fatty acids is also unlikely to have been the reason as free acids are esterified by the acid-catalyzed procedure used in the preparation of the total lipid FAME's. The erratic changes may have been partly due to experimental error in the extraction, esterification and chromatographic analysis. Overall, samples stored with high levels of salt appeared to have slightly lower oxidation indexes. This was in stark contrast to the other three storage trials where salt caused an increase in the rate of peroxidation. Therefore nitrite exhibited greater antioxidant activity at higher salt levels. This agrees with many other researchers who also found nitrite to behave as an antioxidant.<sup>(172-174)</sup> Hadden et al<sup>(172)</sup> reported that nitrite alone and in the presence of salt exhibited an antioxidant effect on lipid oxidation in meat compared to a control (no nitrite or salt). They further showed that salt was pro-oxidant on its own when added to meat. The authors suggest the antioxidant activity of nitrite

is due to its interaction with haemes, converting the ferric (III) haemes to the ferrous (II) haemes. The formation of the ferrous nitrosylmyoglobin in burgers was confirmed by the colour analysis (chapter 6.3). Several authors also confirm that the ferric haemes are more pro-oxidant than the ferrous haemes.<sup>(175-176)</sup> Therefore, this seems to suggest that the haemes are important catalysts of lipid oxidation in stored cured pork. However MacDonald et al<sup>(171)</sup> showed that nitrite reduced the rate of peroxidation of unsaturated fatty acids containing added Fe(II). This and the ability of nitrite to inhibit the rate of peroxidation of phospholipid liposomes both with and without added copper (II) suggests that the mechanism is more complex.



**FIGURE 6.2**

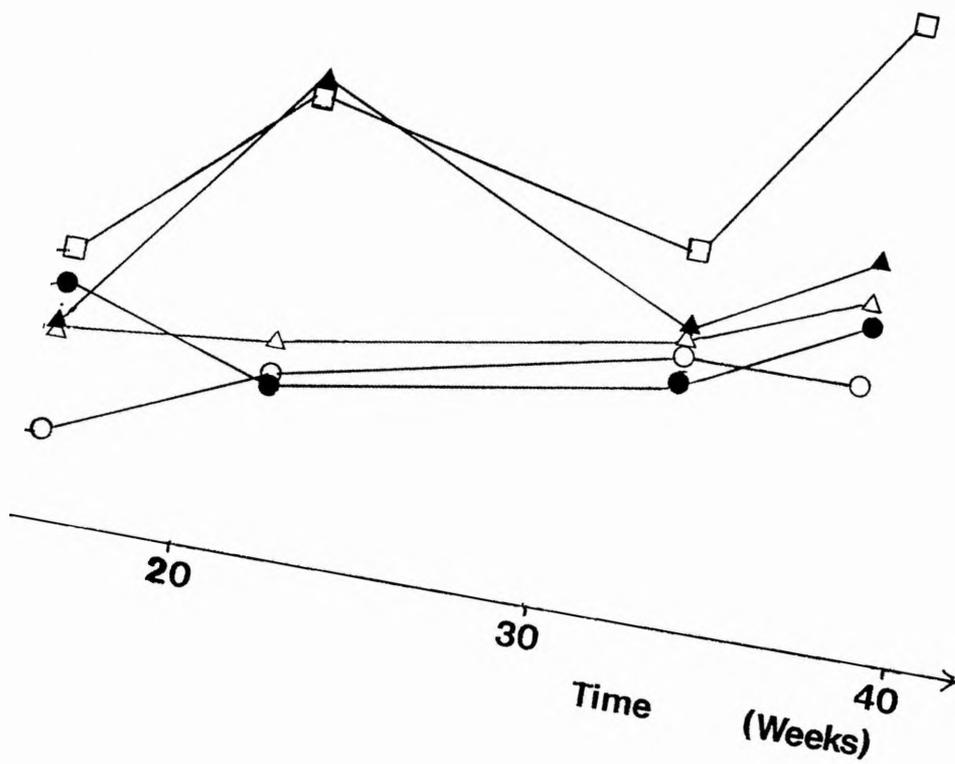


FIGURE 6.3

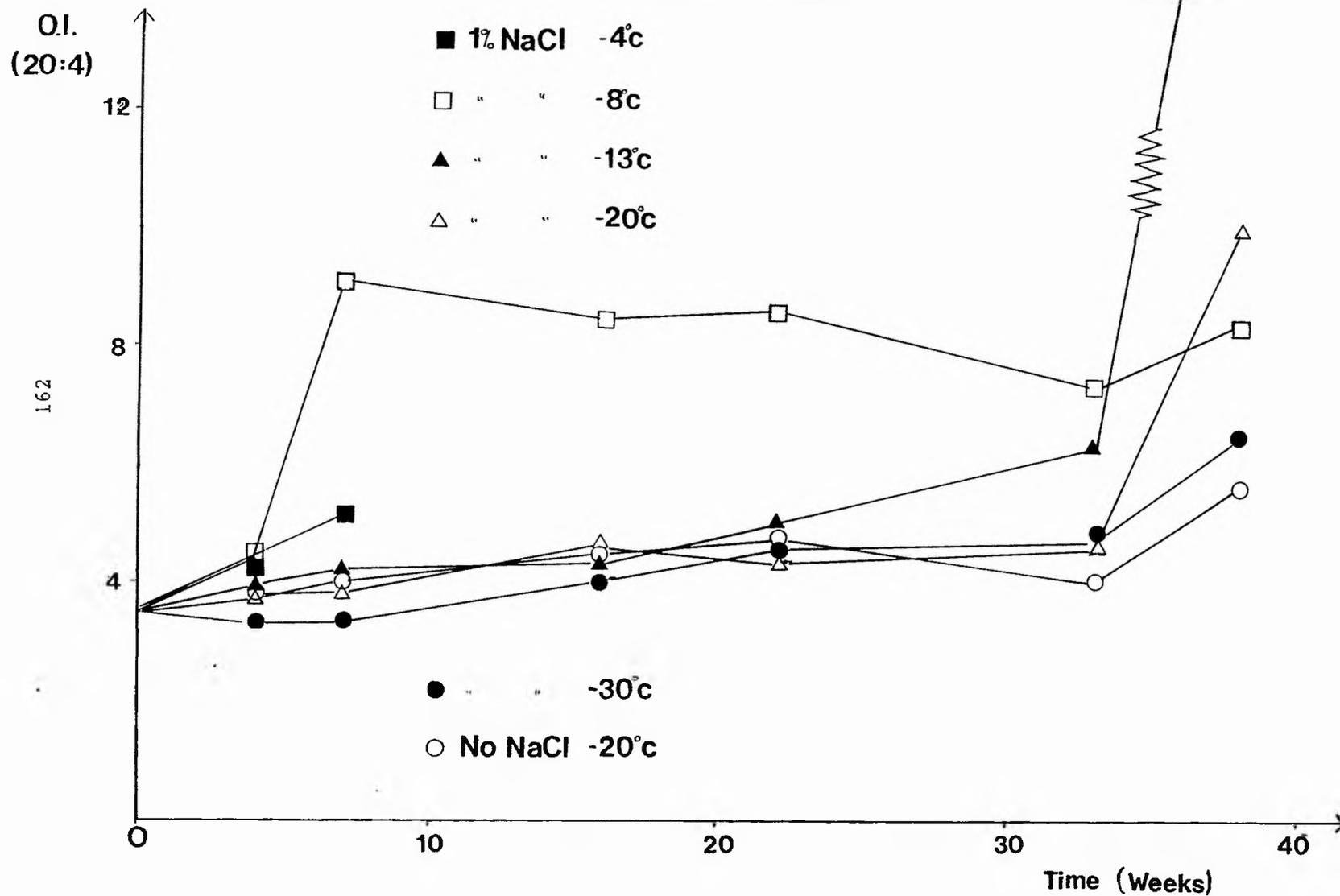
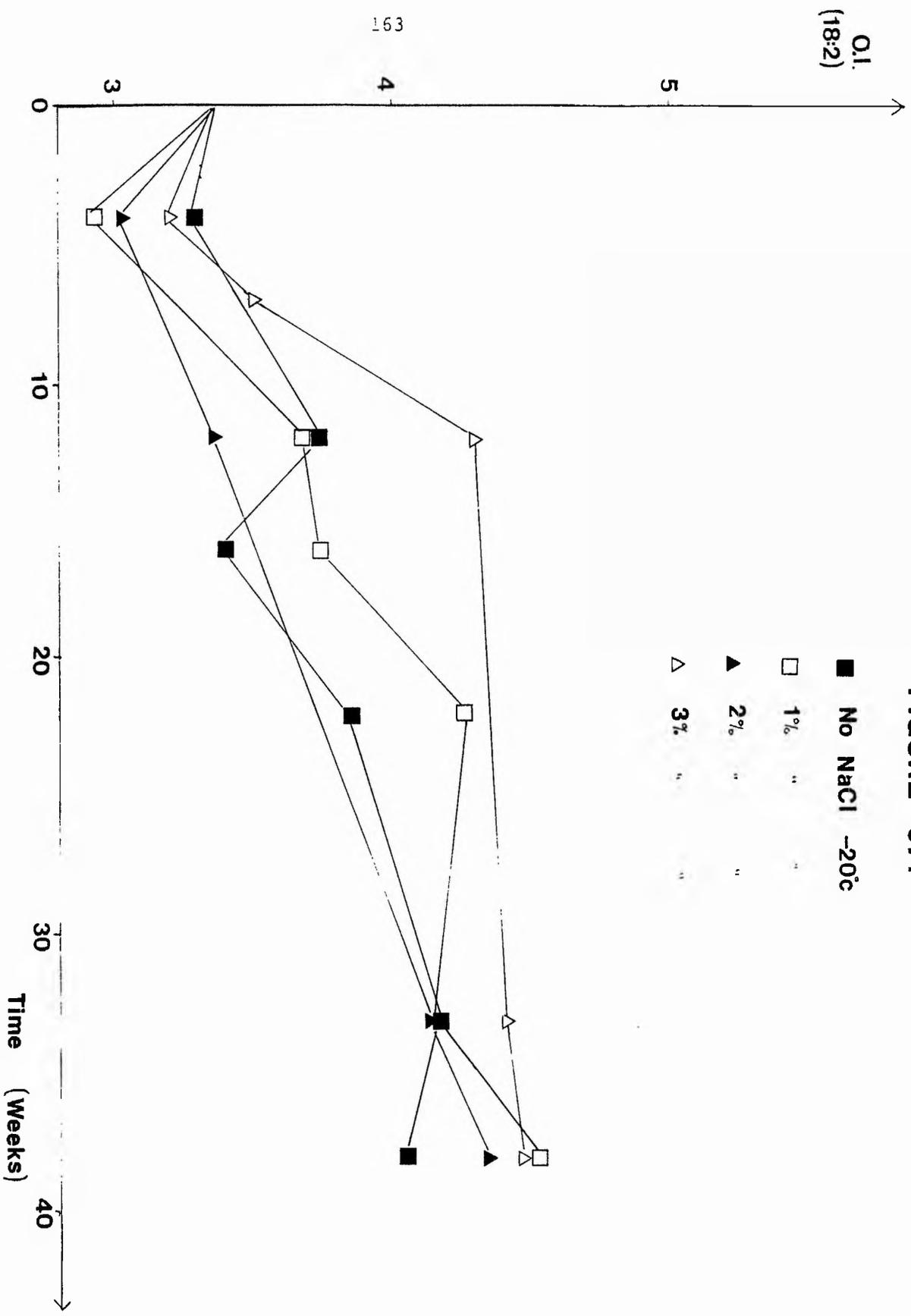
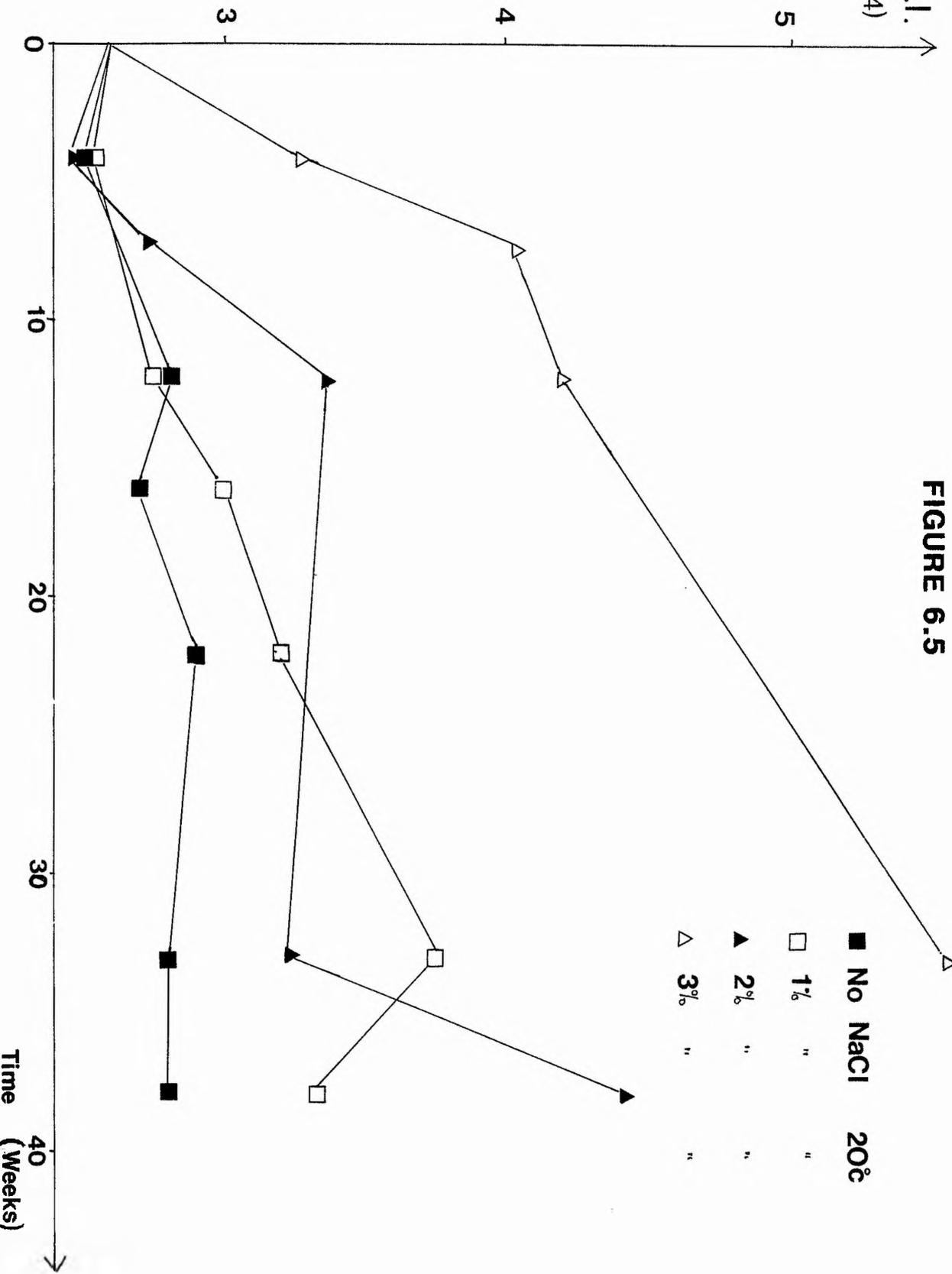


FIGURE 6.4



O.I.  
(20:4)

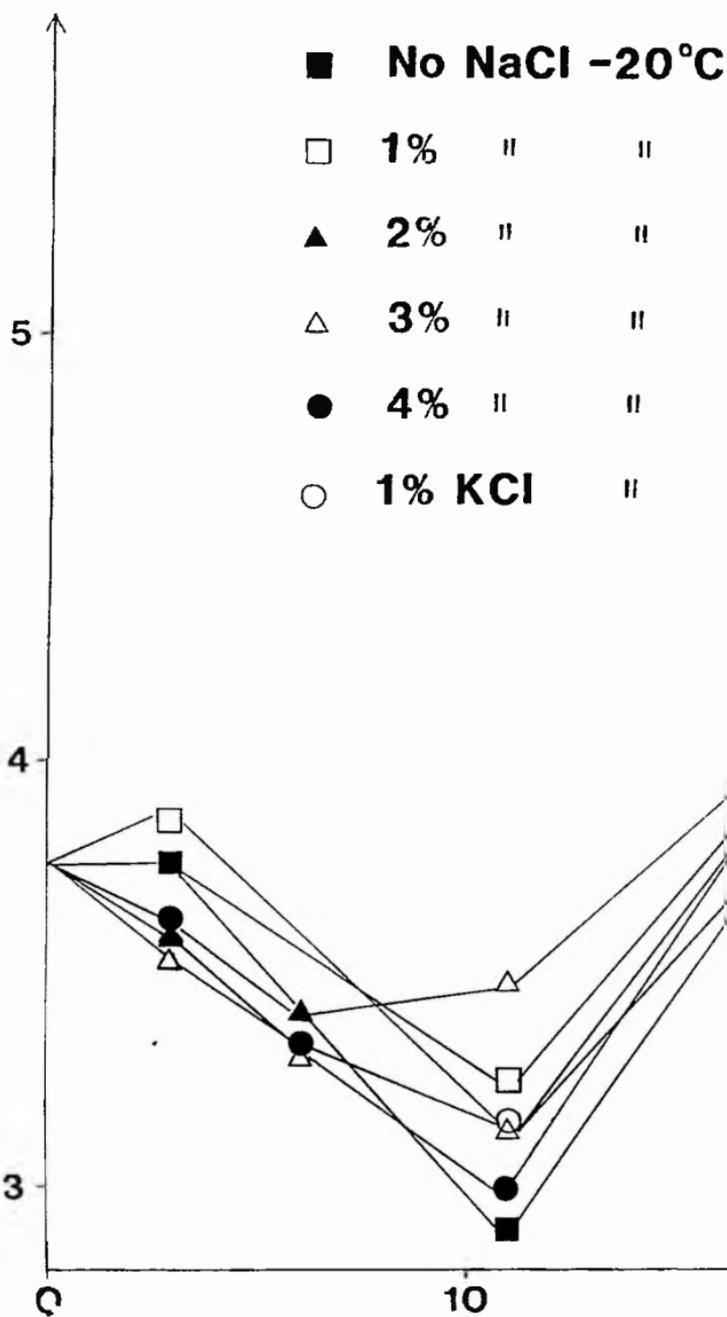
FIGURE 6.5



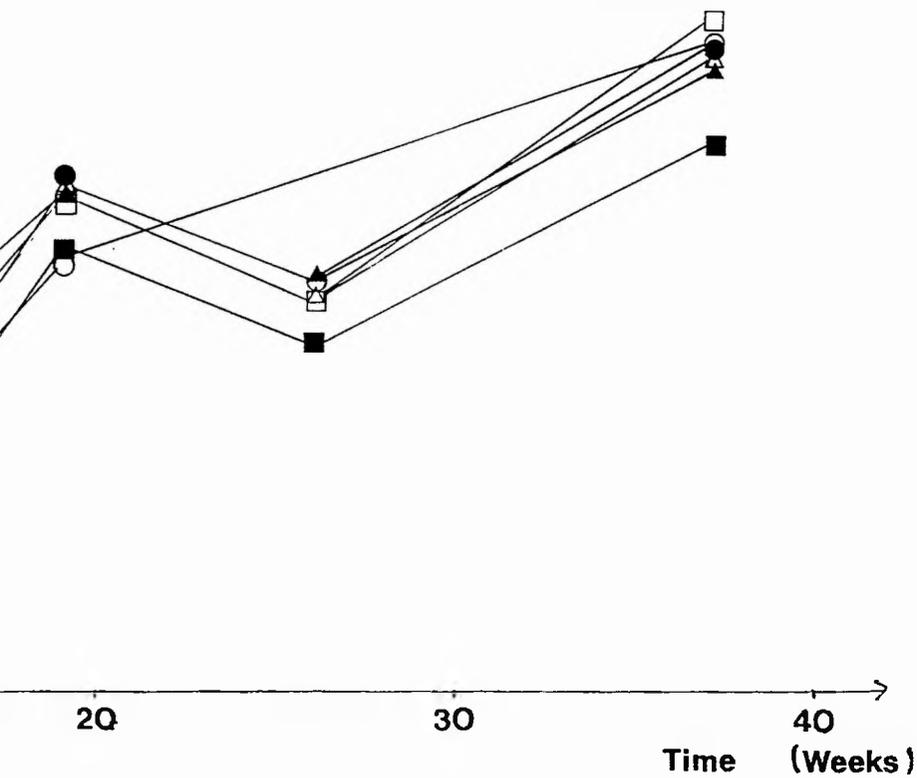
4.0.03

O.I.  
(18:2)

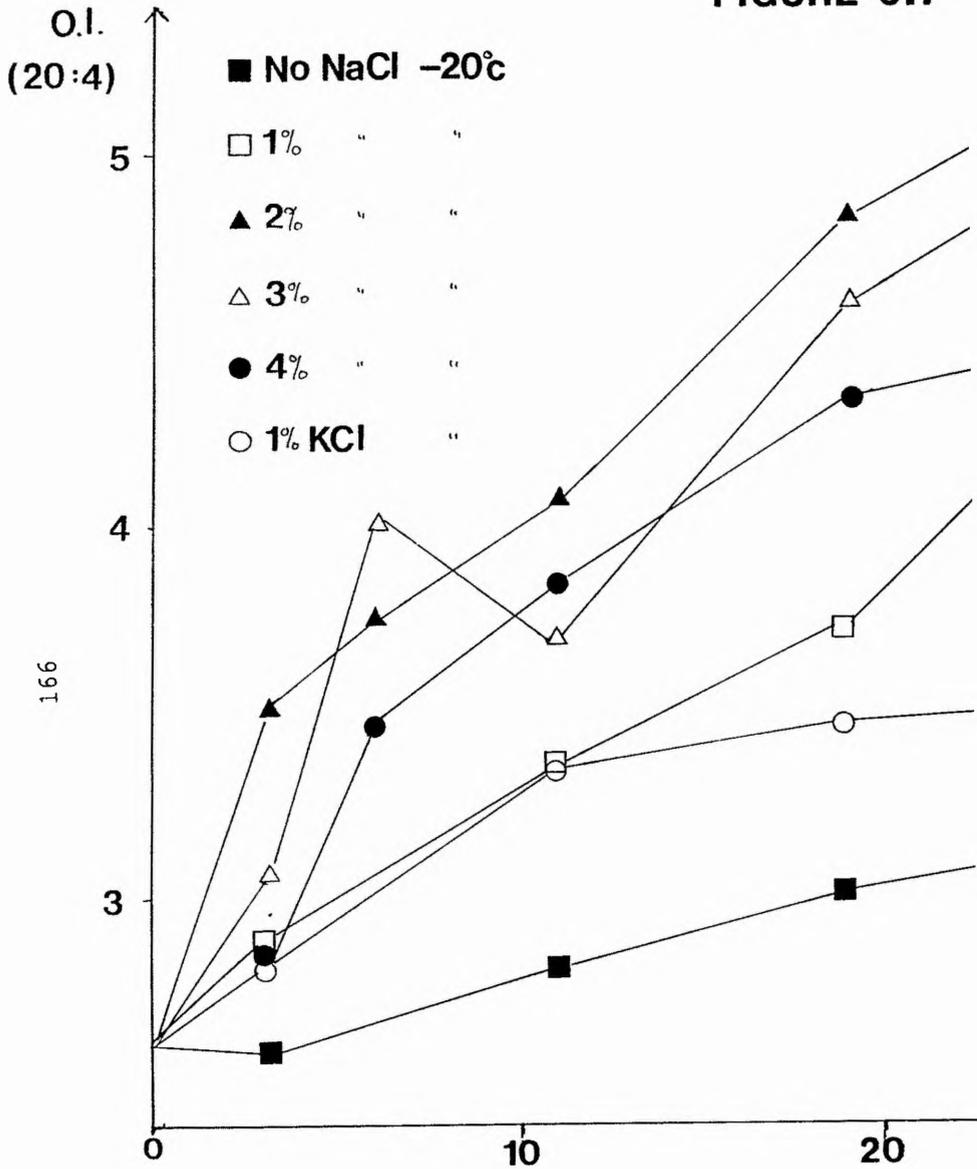
1.65

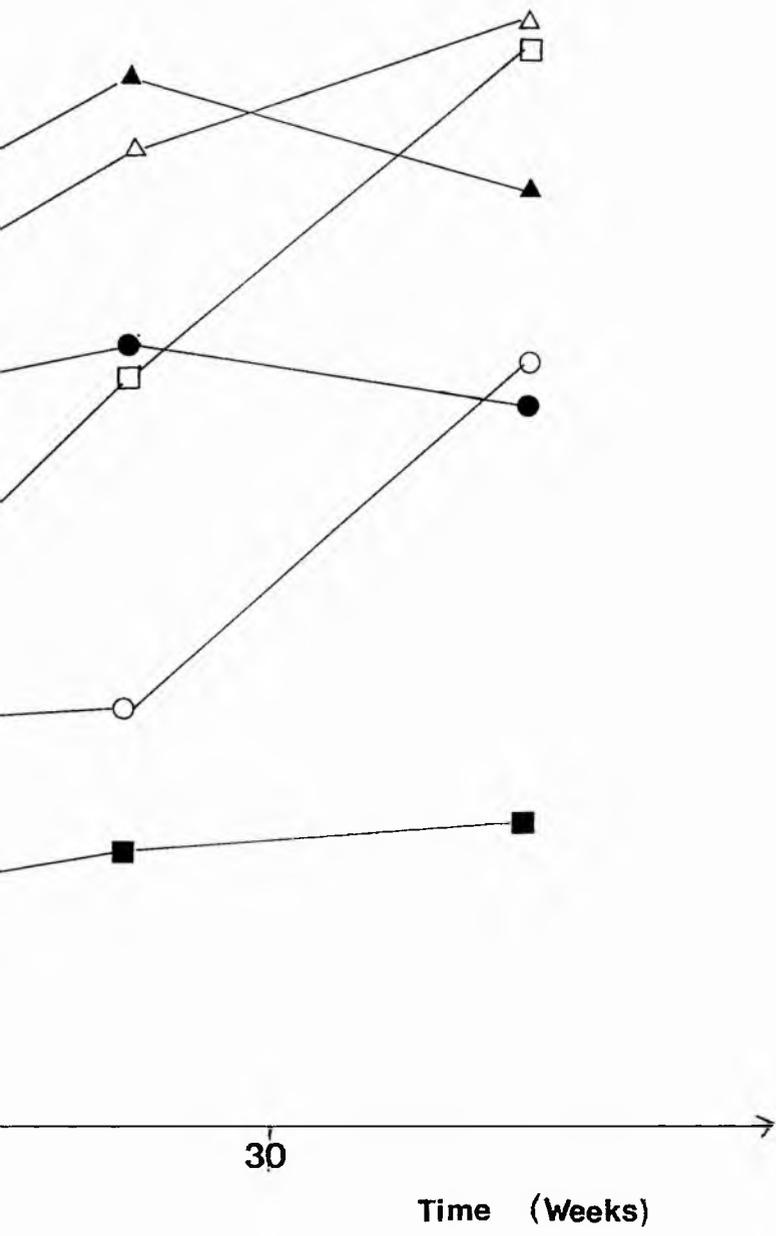


**FIGURE 6.6**



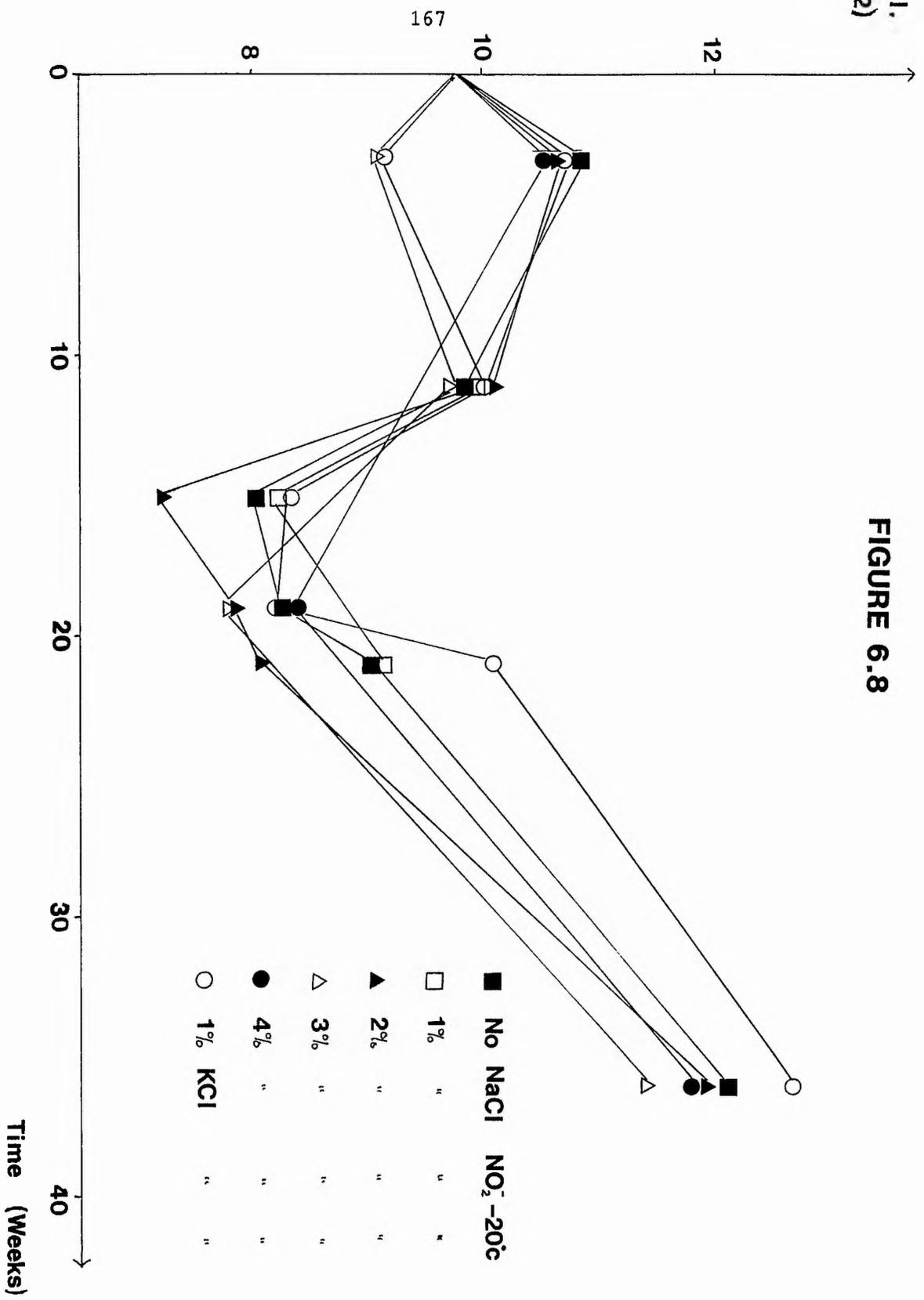
**FIGURE 6.7**



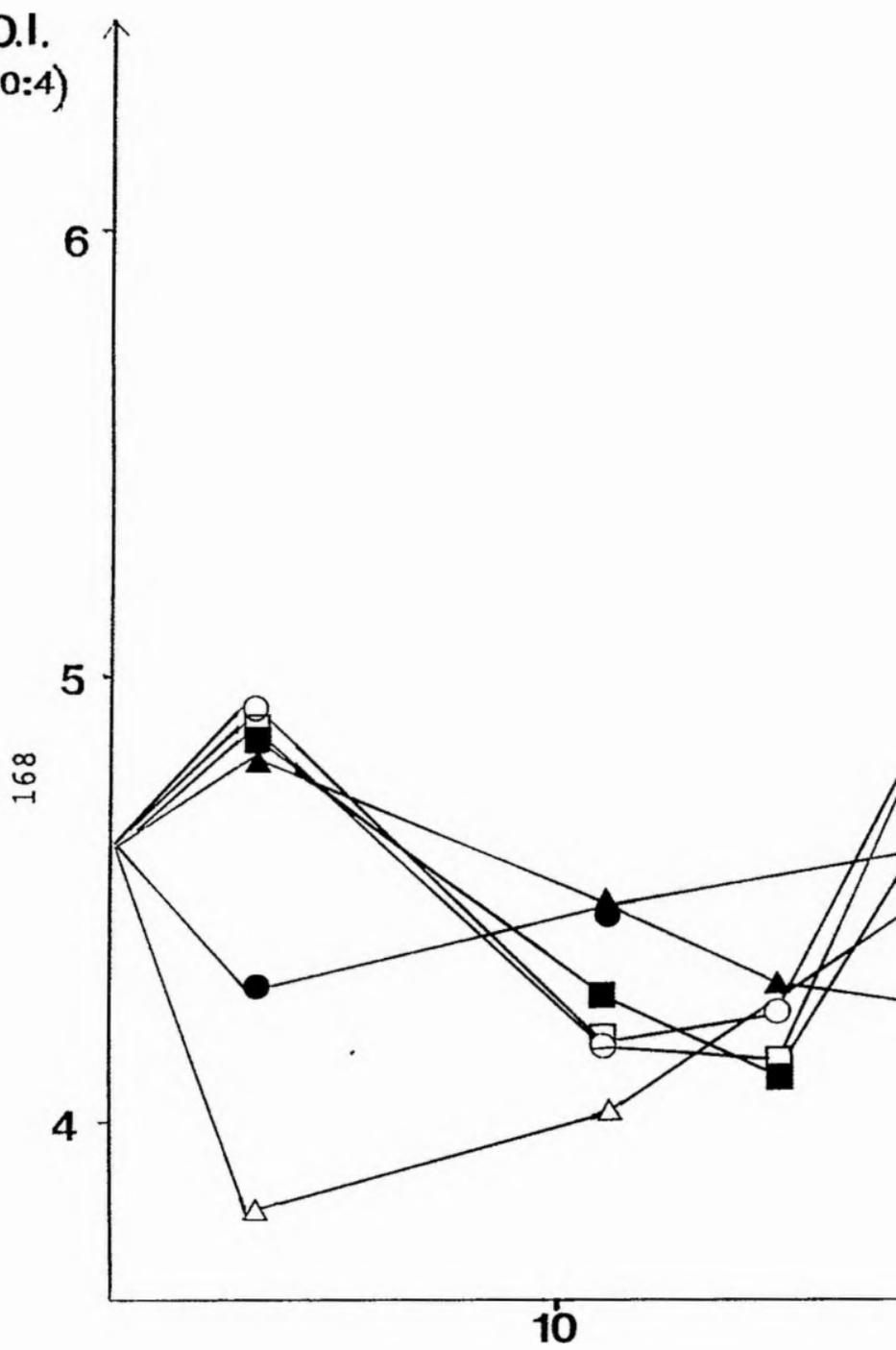


0.1.  
(18:2)

FIGURE 6.8



O.I.  
(20:4)



**FIGURE 6.9**

■ No NaCl NO<sub>2</sub><sup>-</sup> -20°C

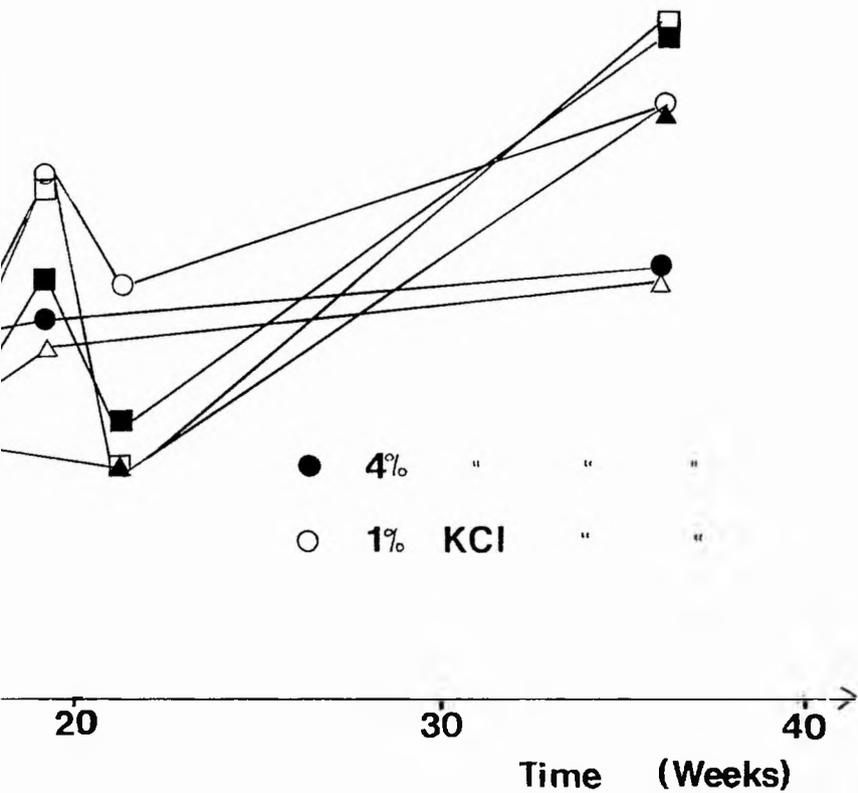
□ 1% " " "

▲ 2% " " "

△ 3% " " "

● 4% " " "

○ 1% KCl " " "



### 6.3 COLOUR STUDIES

From a consumers point of view the colour of meat is an important factor in deciding whether to purchase, because it provides an indication of the freshness of the product. Nitrites and ascorbic acid can be added to some meats (bacons, hams, etc) interacting with food components modifying the colour to produce a more attractive product. Colour change has therefore been followed to observe any correlation that exists between colour and the other analytical methods used for following lipid oxidation in meat.

#### 6.3.1 Colour Parameters

The colour of an object can be described by the three parameters Hue (H), Chroma (C) and Lightness (L), all of which can be incorporated into a colour solid, Figure (6.10). The colour solid is useful as it allows a scientific characterization of colour to be determined. All colour changes can be followed within the colour solid. If the sample is analysed a point is defined within the cylinder. Therefore, after several analysis, trends in the colour parameters can be followed and plotted in the colour solid if required. The present colour studies were carried out using a ICS Micro-Match 2000 Diffuse Reflectance Spectrophotometer. Within the instrument white light is produced which is

scattered by a reflective surface and uniformly hits the sample. Detectors at 20 nm intervals between 380 and 720 nm measure the percentage of light reflected. Due to the inhomogeneity of the burgers both sides of the samples were analysed and two to five readings recorded on each until consistent results were obtained.

### Hue (H Value)

Hue is simply another name for what most people call colour. Hue is the term used to distinguish everyday colours such as red, blue, yellow, and mixtures of these primary components. The magnitude of the hue value (angle) when used in conjunction with Figure (6.10) indicates the hue present. For example, a lemons hue may be 85 (yellow-green) while the hue of a carrot may be 32 (red-yellow or orange). DH is the difference in hue values between standard and batch samples.

### Chroma (C Value)

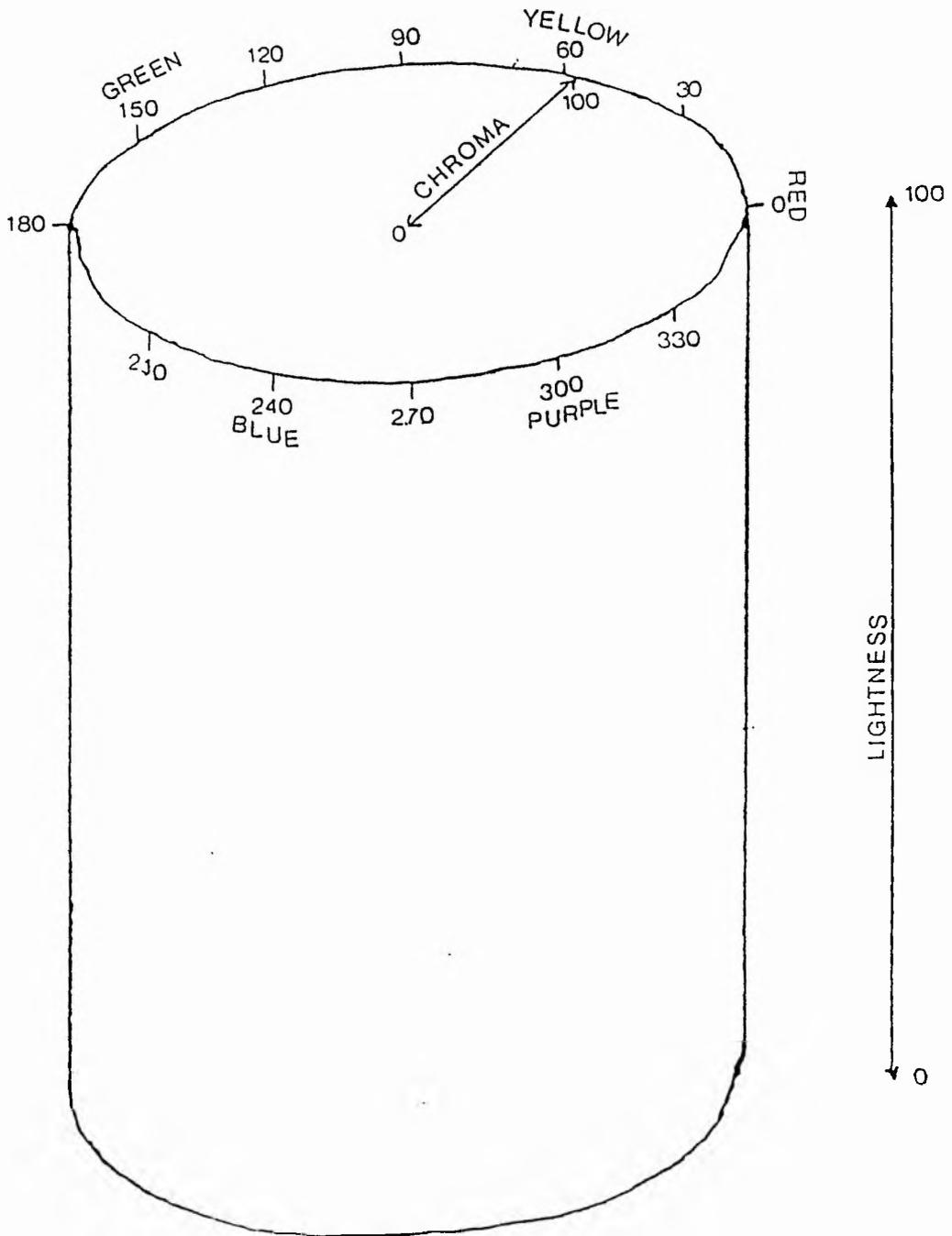
Chroma describes the saturation or intensity of a colour (hue). A scale from 0 to 100 is used where low values indicate pastel colours and high values indicate vivid or intense colours. For example, a red stick of chalk will have a low chroma value whereas red paint will have a high value. From Figure (6.10) low chroma values lie near the centre of

the circle whereas high chroma values lie towards the circumference.

### Lightness

This term describes the lightness of a sample. it is measured on an achromatic scale from 0 to 100 (ie black to white) where a low value indicates a dark sample whilst a high value shows a light sample. For example, a lemons lightness value will be quite high whilst a banana's lightness value will be quite low. DL is the difference in lightness values between standard and batch samples.

FIGURE 6.10



### 6.3.2 Results and Discussion

The results from the colour analysis are tabulated in Appendices (6.9 - 6.11) and present graphically in Figures (6.11 - 6.22).

The contribution made by lipids and other food components is difficult to establish as the observed colour is mainly due to the highly coloured myoglobins.<sup>(179)</sup> Reduced myoglobin (Mb) oxymyoglobin ( $MbO_2$ ) and metmyoglobin ( $Mb^+$ ) are the main components responsible for colour in meats. Reduced myoglobin is responsible for the purplish colour of freshly cut meat and meat held under anaerobic conditions, eg vacuum packing. On exposure to air myoglobin combines readily with oxygen forming bright red oxymyoglobin which gives meat its typical attractive colour. Brown metmyoglobin formed from the oxidation of the ferrous derivative ( $MbO_2$ ) is chiefly responsible for the discoloration of meat and occurs at low oxygen pressures, for example, below the surface of the meat. The conversion of oxmyoglobin to metmyoglobin has also been reported to be a result of lipid oxidation.<sup>(79)</sup>

6.3.2.1 The Stability of Uncooked Pork Burgers containing 1% salt at Different Temperatures

The results are presented in Figures (6.11 - 6.13) and Appendix (6.9). It can be seen from Figure (6.11) that there were considerable differences in the hue values of the burger samples when first analysed. Generally, the lower the temperature at which the burgers were stored the lower was the hue value. The difference in hue between samples continued on increased storage with a slight increase in the value. The exact nature of all compounds responsible for an increase in hue are not known. However an increase in metmyoglobin (brown) would be expected to increase hue. As discussed earlier, an increase in hue may result from lipid oxidation, ie, the conversion of oxymyoglobin to metmyoglobin.<sup>(165)</sup> However this conversion may occur to some extent by freezing the burgers causing a lower solubility of oxygen at the surface due to ice formation.

It was difficult to establish distinct links between chroma and increased storage temperature of the burgers, Figure (6.12). Burgers stored at -8°C (sample B) after 9 weeks had shown a considerable increase in chroma. The chroma of burgers stored at -13°C (sample C)

exhibited a slight increase then decrease followed by an increase at 38 weeks. Samples stored at  $-20^{\circ}\text{C}$  (sample D) generally had a lower chroma than those stored at  $-8$  and  $-13^{\circ}\text{C}$ . However, chroma values of samples stored at  $-30^{\circ}\text{C}$  were higher than those stored at  $-20^{\circ}\text{C}$ . Also, chroma values of burgers stored in the absence of salt (sample F) were higher than those stored with salt (sample D) at the same temperature.

Results from the lightness (L) parameter were very erratic. Although burgers stored at  $-4^{\circ}\text{C}$  (sample A) initially had the lowest lightness it was not possible to predict future changes as the burgers were not analysed further. Samples B and C stored at  $-8$  and  $-13^{\circ}\text{C}$  respectively had roughly equivalent L values at the end of the trial although sample C initially had a considerably higher value than sample B. In general the L values increased with increased storage temperature between  $-8$  and  $-20^{\circ}\text{C}$  (samples B - D) then decreased to a minimum at  $-30^{\circ}\text{C}$  (sample E) which would make it difficult to establish the degree of oxidation from this value alone.

6.3.2.2 The Stability of Cooked Pork Burgers  
Containing Different Amounts of Salt at -20°C

There appeared to be very little change in the hue values of all burger samples with increased storage time (Figure 6.14). However, the hue values from all the different burger treatments were initially high indicating the brownness which developed on cooking. Burgers containing salt generally had higher hue's than those stored without salt (sample G). This is in agreement with Miller et al<sup>(178)</sup> who found that salt caused an increase in brownness of cooked beefsteaks.

The chroma values of burgers decreased with increased storage time, (Figure 6.15). Burgers stored with increasing levels of salt had generally lower chroma values throughout storage. However, some results were erratic and could not easily be explained.

It was difficult to interpret the lightness values as they also appeared erratic. Burgers stored in the absence of salt (sample G) had a higher L value that in burgers containing 1% salt (sample H) but burgers containing 3 w/w% salt (sample G) started with the highest L value of all the samples and at the end of the trial had the lowest L value.

6.3.2.3 The Stability of Uncooked Pork Burgers  
Containing Different Amounts of Salt

There were substantial differences in hue at the beginning of the trial and with increased storage. Burgers stored with increasing concentrations of salt also had higher hue values throughout storage, (Figure 6.17). As discussed earlier lipid oxidation can promote the production of the brown metmyoglobin.<sup>(164)</sup> However, there remains some doubt as to the contribution of metmyoglobin to an increase in hue. Lamkey et al<sup>(179)</sup> found that salt decreased the metmyoglobin content in beef steaks. However, this study was carried out on beef with a maximum of 0.5% salt and stored only for four weeks. Burgers that contained 1% potassium chloride (sample P) in place of 1% sodium chloride (salt, sample L) had lower hue values. This is in agreement with other researchers who found that potassium chloride imparted more redness to meat than sodium chloride. Burgers stored with 2, 3 and 4% salt, samples (M, N and O) all had approximately equivalent hues throughout storage.

The chroma values of the burger samples varied considerably when first analysed. Generally, the higher the level of salt in the burgers the lower was the

chroma value. On increased storage time the chroma values of all samples appeared to fall. There did appear to be a greater decrease in the chroma of burgers stored with low levels or no salt. Burgers stored with 1 w/w% sodium chloride (sample L) consistently had a lower chroma than burgers stored with 1 w/w% potassium chloride (sample P).

With increased storage time there was a slight increase in lightness in all samples. Sample N, 3% salt had the highest L value followed by samples M and O which contained 2 and 4% salt respectively. Burgers containing 1% sodium chloride and 1% potassium chloride had similar L values.

#### 6.3.2.4 The Stability of Uncooked Pork Burgers Containing Sodium Nitrite and Different Amounts of Salt at -20°C

Samples stored with increasing levels of salt generally had lower hue values at the beginning of the storage trial. (Figure 6.20) This was in contrast with the previous experiment (6.2.5.3) in which the burgers did not contain nitrite. It is generally believed that nitrite can promote the conversion of oxymyoglobin (red) to metmyoglobin<sup>(179)</sup> (brown). As discussed in the

previous storage trial salt may also promote the production of metmyoglobin. Therefore, salt and nitrite may have had an additive effect on the production of metmyoglobin. However nitrite and nitric oxide have both been implied in the conversion of metmyoglobin to nitrosylmoglobin (pink).<sup>(179)</sup> Therefore as metmyoglobin was formed it was rapidly converted to the pink nitrosylmoglobin which is characteristic of cured meat.

At the beginning of the storage trial all the samples had a similar chroma but as the storage trial progressed significant changes occurred. At the end of the trial burgers containing 2 and 4 w/w% salt (samples S and U) had the lowest chroma and burgers with 1 w/w% salt sodium chloride, (sample R) and potassium chloride (sample V) had the highest chroma values. Burgers stored without salt had an intermediate chroma.

There were considerable differences in lightness between samples throughout the storage trial. Burgers with no salt (sample Q) had the highest L value throughout the storage trial. Burgers containing 3 w/w% salt (sample T) had the lowest L value from 3 weeks until the end of the trial. Samples R, S, U and V all had similar L values which were intermediate from samples Q and T.

#### 6.3.2.5 Conclusion

As can be seen from the results there did seem to be interesting changes in the colour parameters on increased storage time. Burgers stored at higher temperatures and the same level of salt had higher hue values. This may signify the formation of metmyoglobin which can occur as a result of lipid peroxidation. Differences also occurred in the chroma and lightness parameters although it was difficult to make any definite conclusions from the data obtained.

The hue of cooked burgers remained high throughout storage at around 80. This high hue was probably partly due to the formation of brown Maillard reaction products which are believed to be formed from the interaction of various food components when heated.<sup>(3)</sup> However there were more conclusive changes in the chroma where on increased salt content a decreased in chroma occurred.

In the experiment investigating the effect of salt content on the colour of raw pork burgers a hue of not more than 70 was obtained in burgers stored without salt (sample k) throughout the storage trial. As the salt content increased so did the value of hue to around 85 for burgers with 2 to 4% salt, which may suggest that

salt increased the formation of metmyoglobin, ie, the burgers become browner. However no definite conclusion can be made about the metmyoglobin content of the meat as other chemical modification may cause an increase in hue.

From the final storage trial which investigated the effect of salt concentration and nitrite on meat colour it was found that burgers containing nitrite but no added salt (sample Q) had a high hue of 75 to 80. With increased levels of salt the hue decreased to around 60 to 65, ie, the burgers became more red which suggests the formation of nitrosylmyoglobin is favoured at high salt concentrations. The reason may be that in this experiment high levels of salt also increase the production of metmyoglobin as discussed earlier and therefore promotes the subsequent reaction of nitric oxide with metmyoglobin producing the pink nitrosylmyoglobin. Discussion in section (6.6) will try and correlate these changes with the results from the other methods of analysis.

FIGURE 6.11

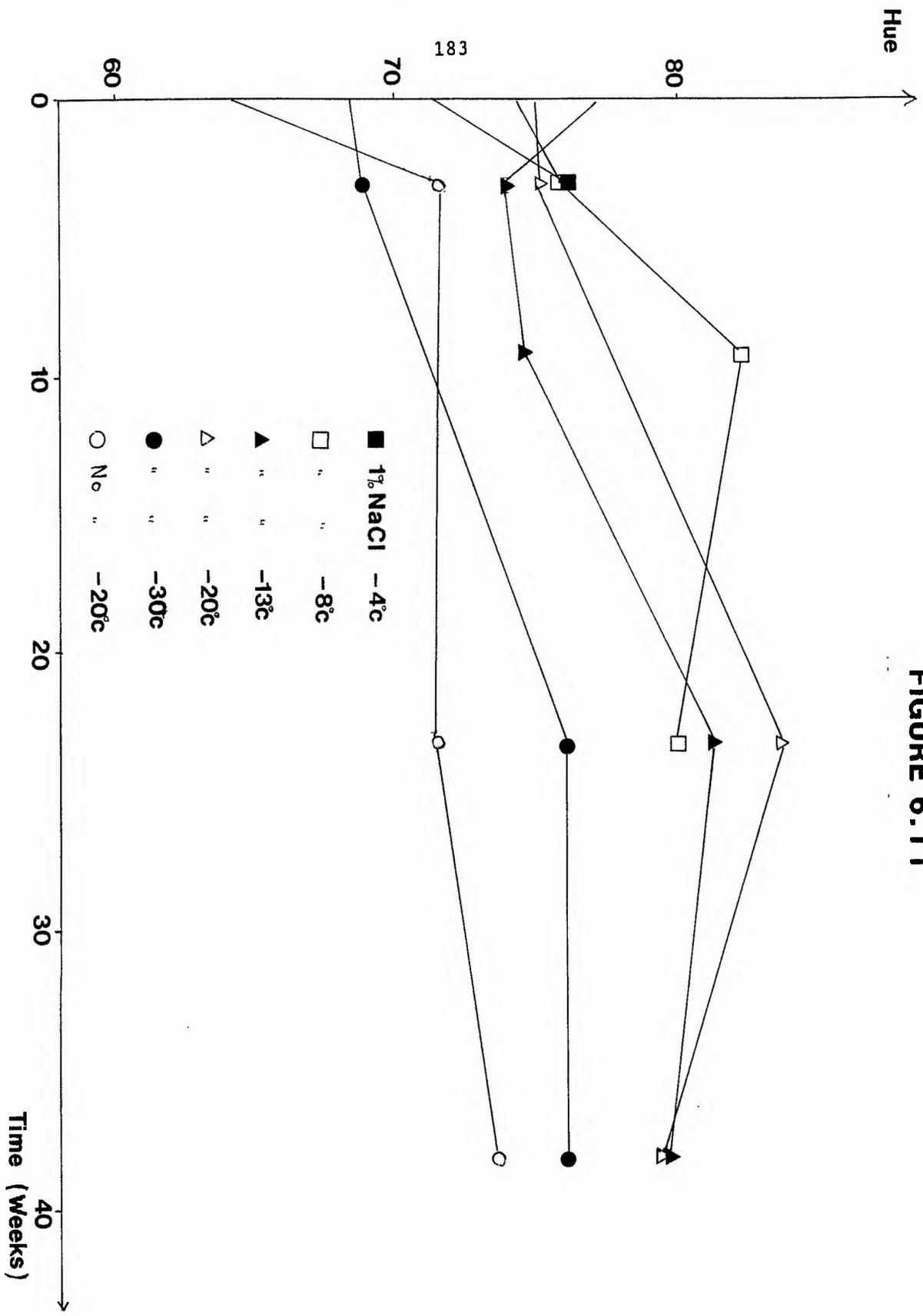
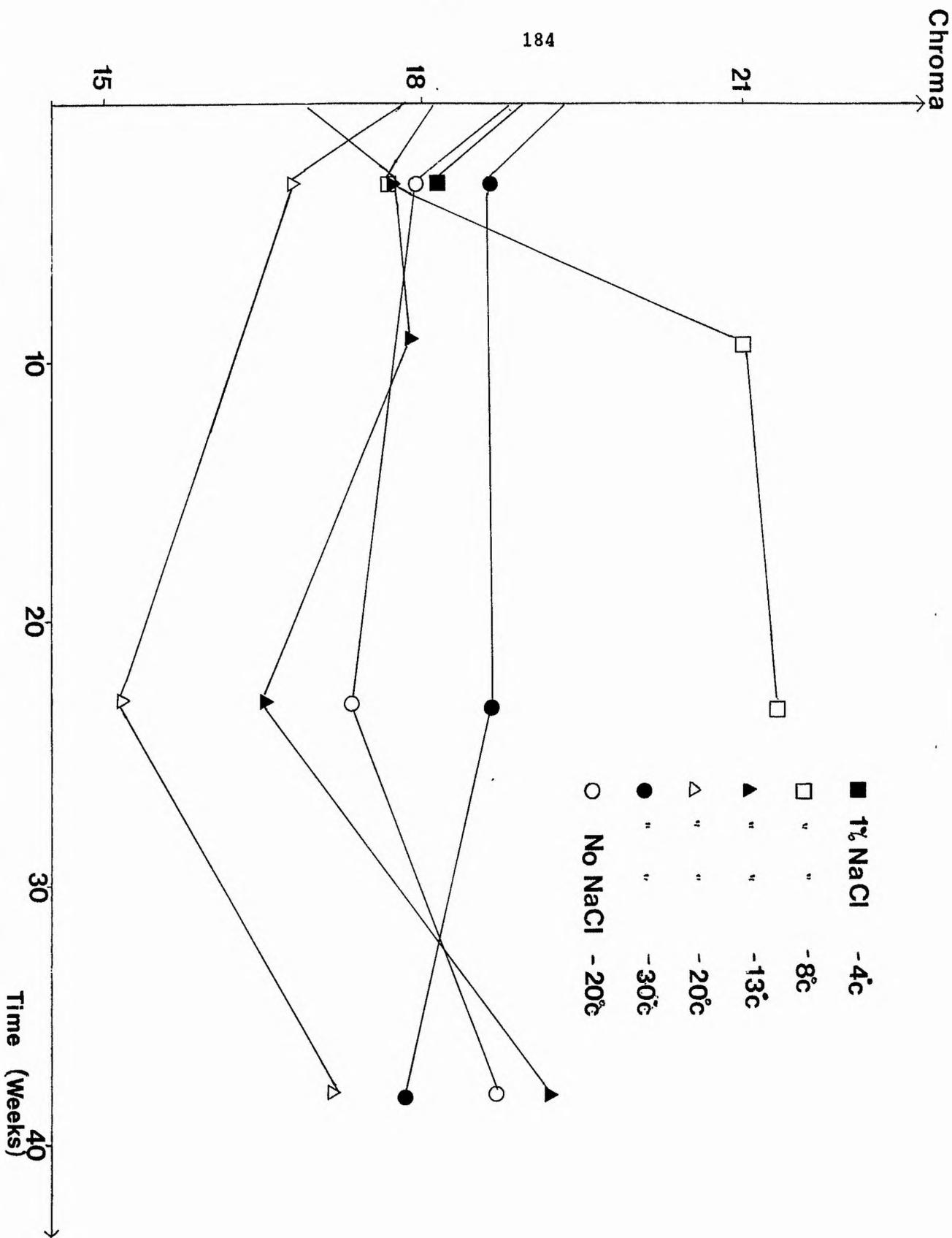
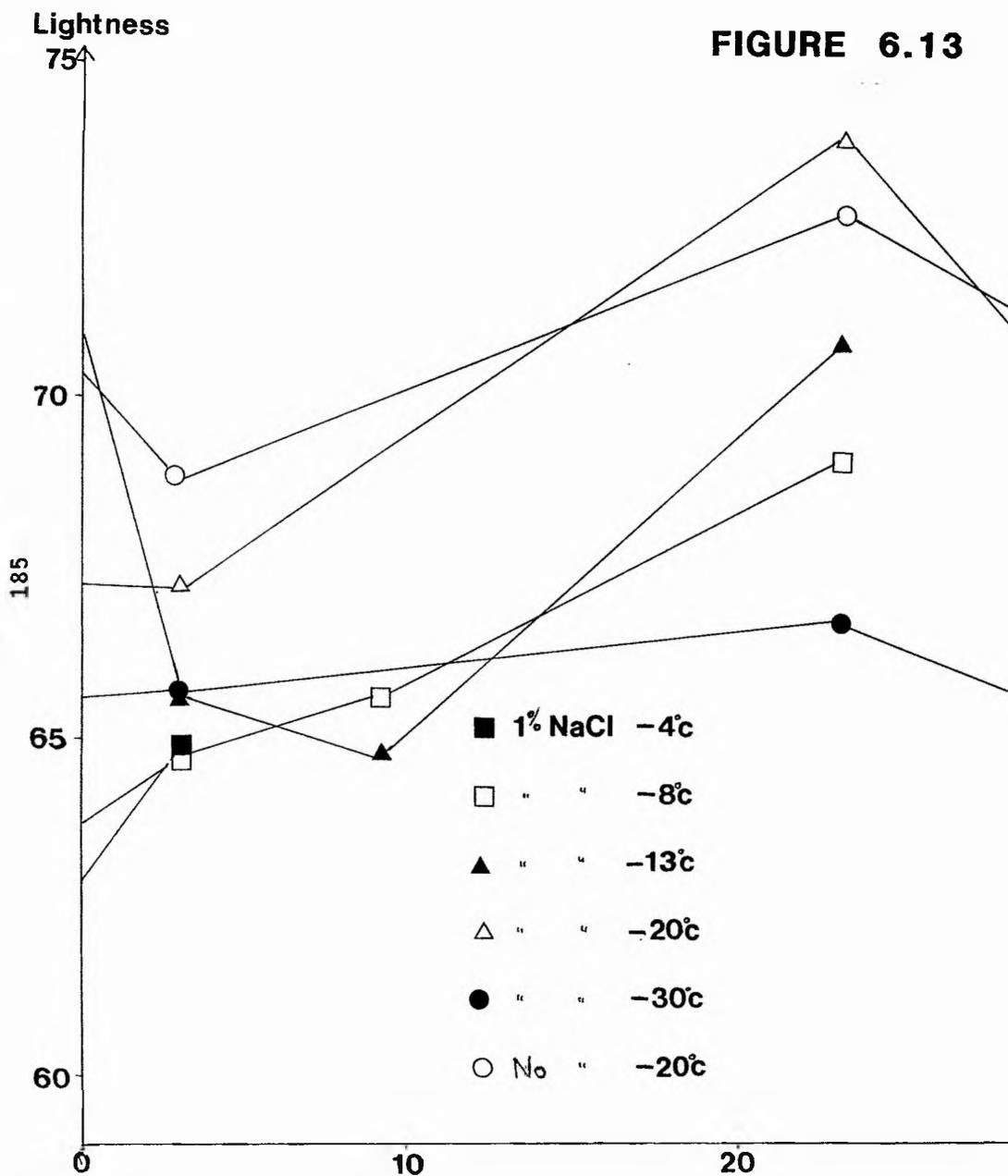
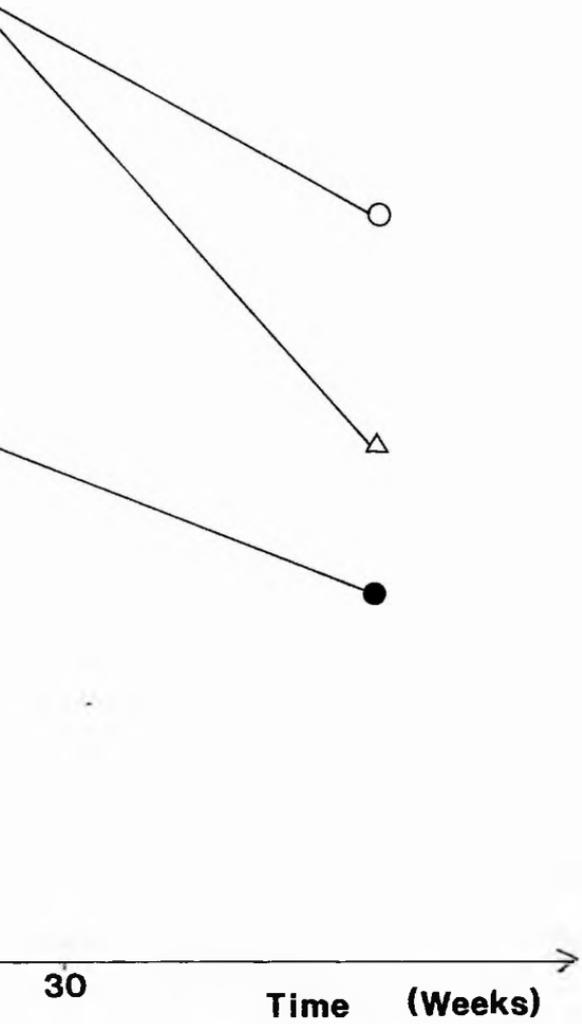


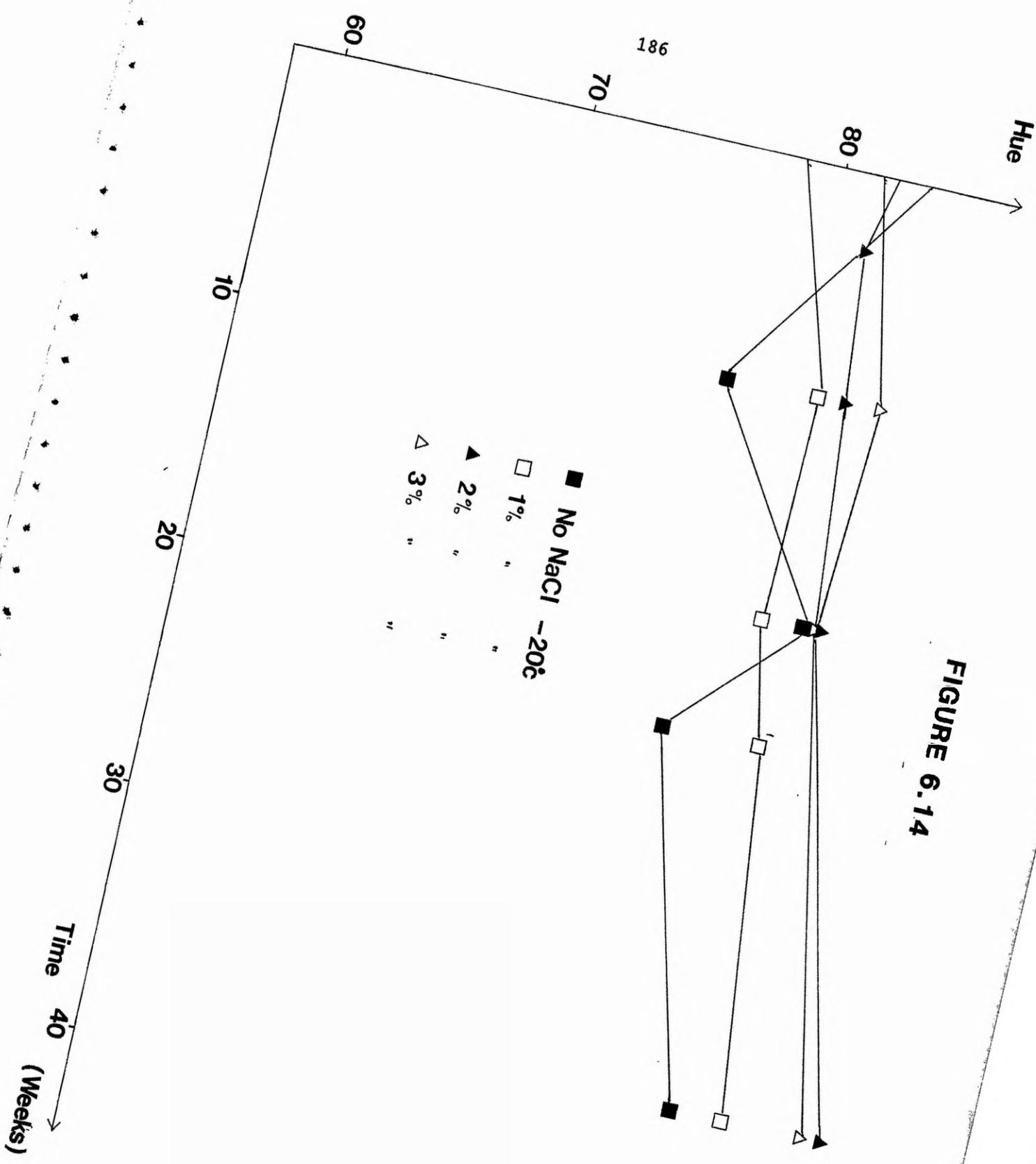
FIGURE 6.12



**FIGURE 6.13**







**FIGURE 6.14**

186

Hue

80

70

60

10

20

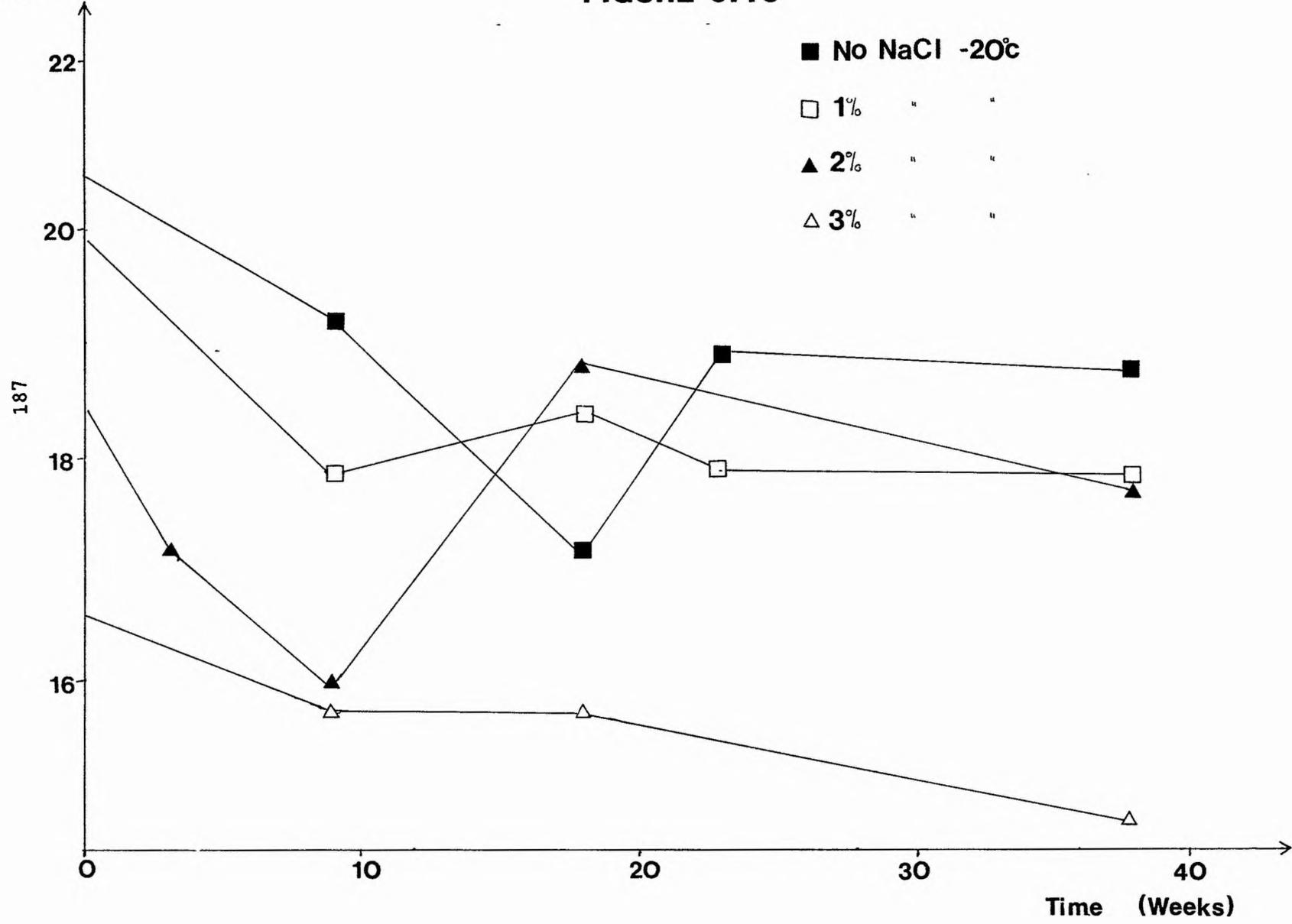
30

Time 40

(Weeks)

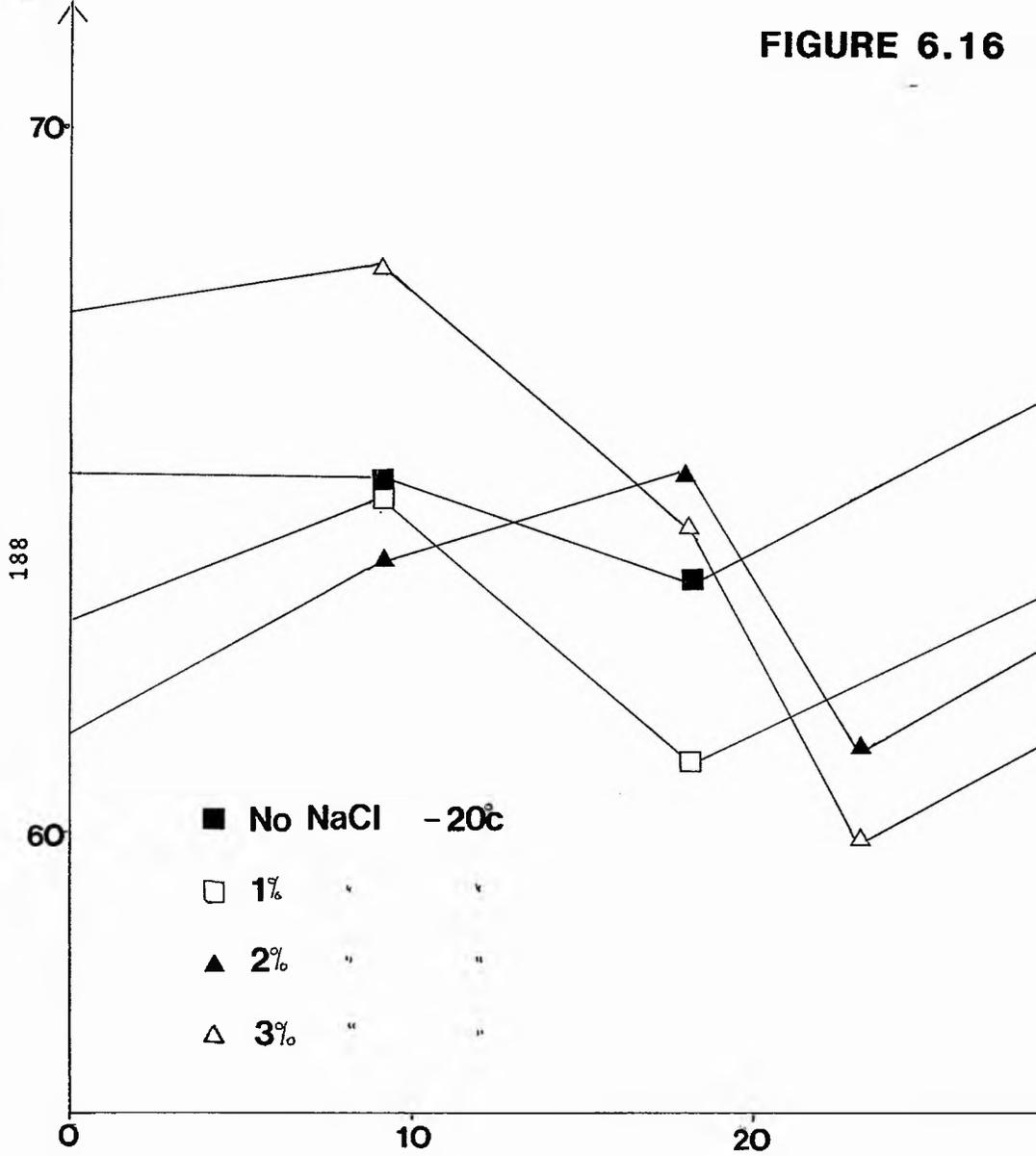
Chroma

FIGURE 6.15



**FIGURE 6.16**

**Lightness**



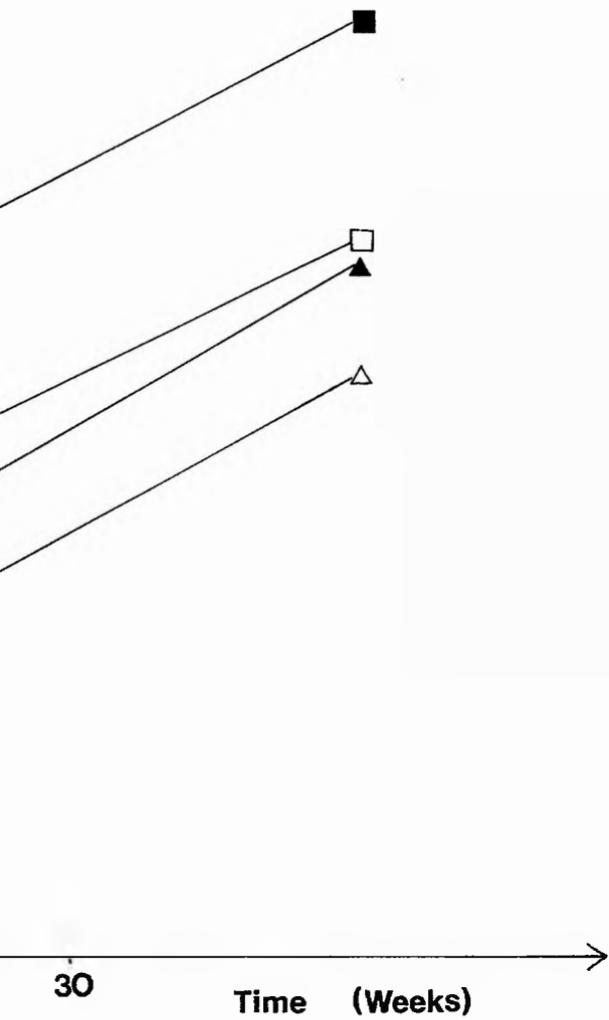
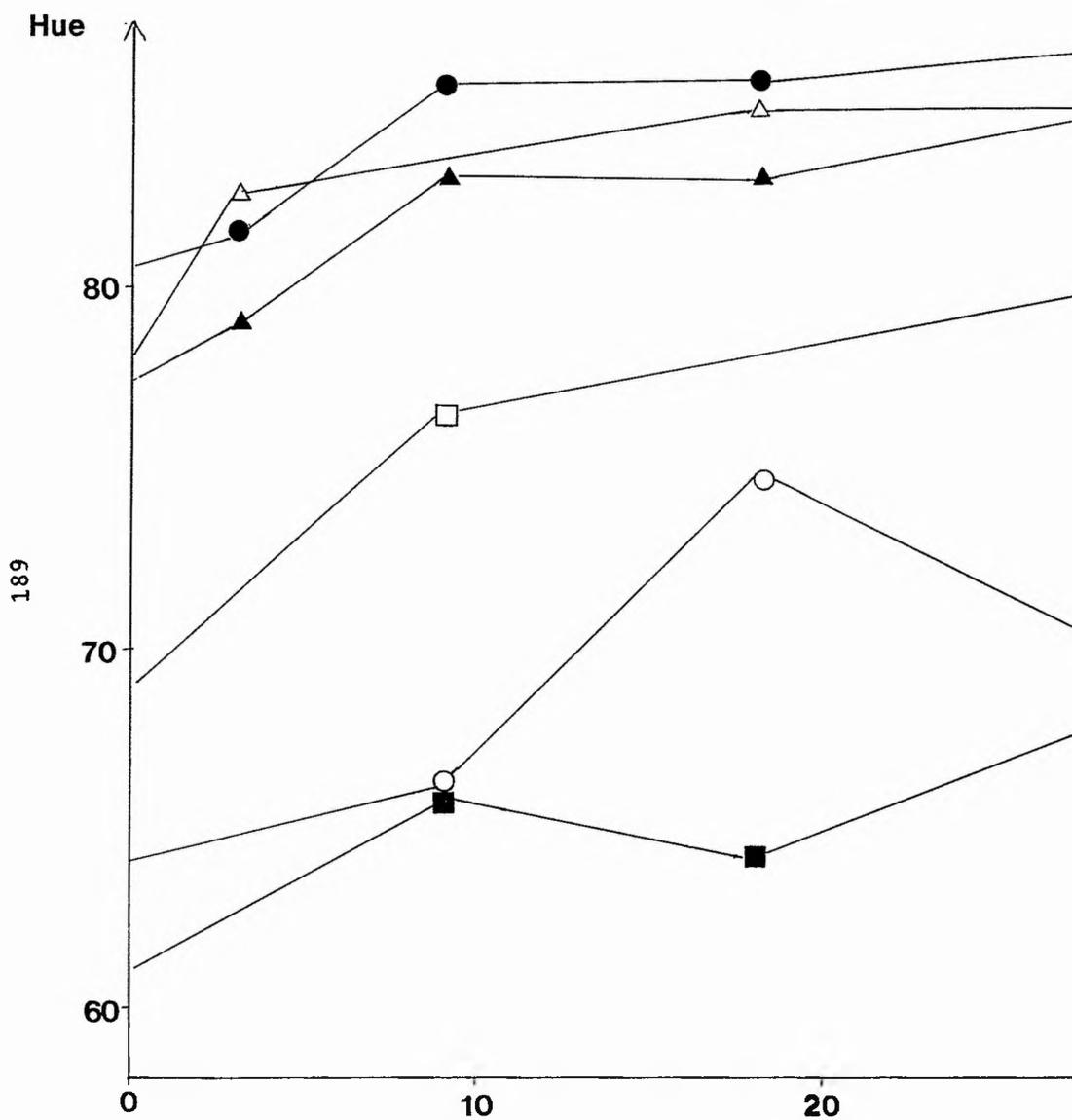
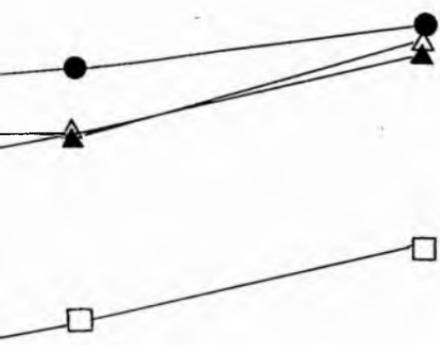


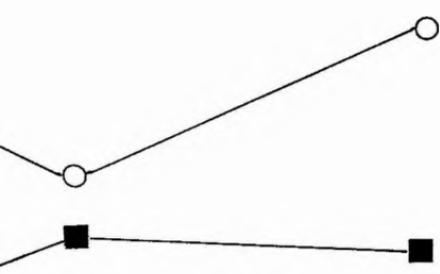
FIGURE 6.17





■ No NaCl -20°C

□ 1% " "



▲ 2% " "

△ 3% " "

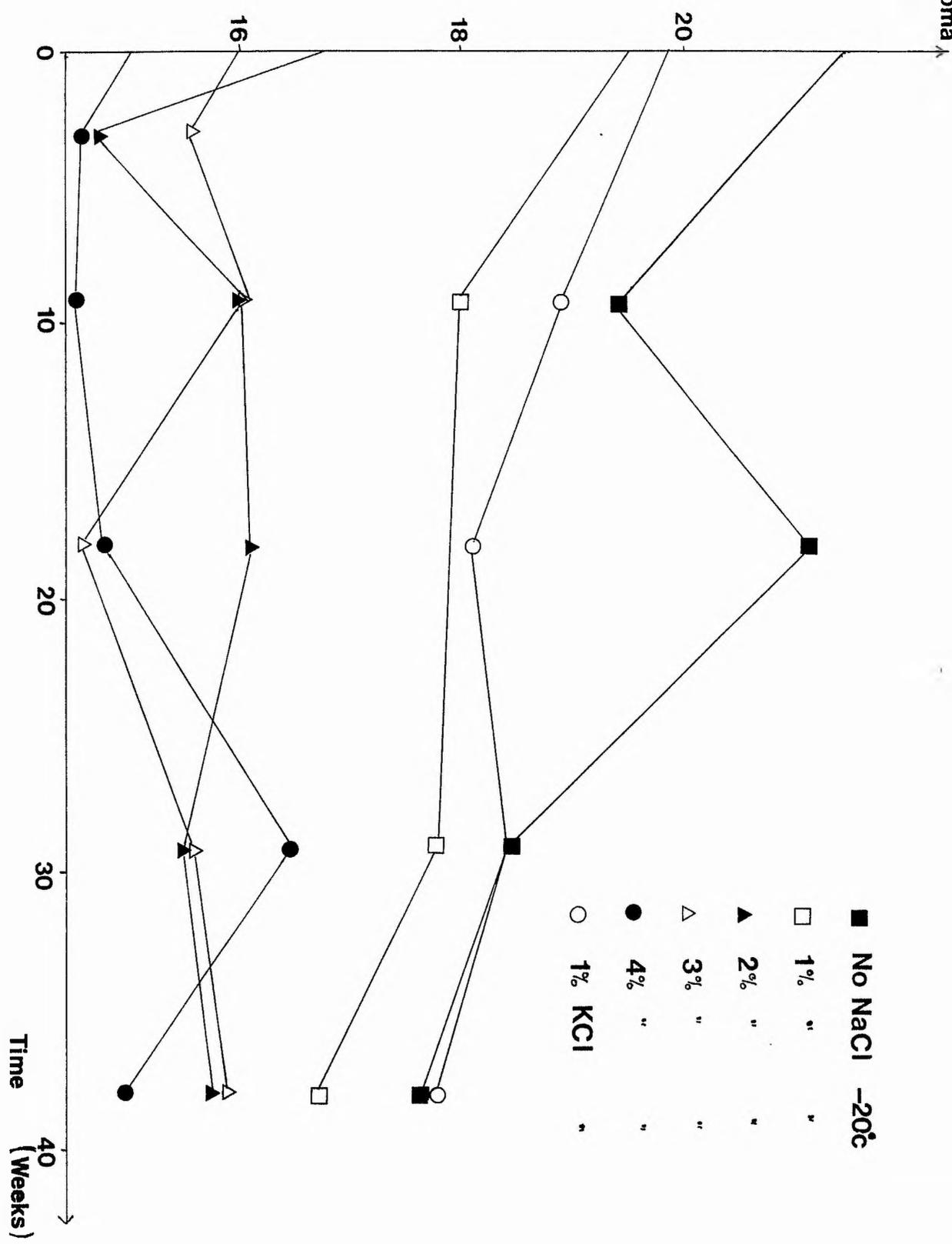
● 4% " "

○ 1% KCl " "

30

Time (Weeks)

FIGURE 6.18



■ No NaCl -20°C

□ 1% " "

▲ 2% " "

△ 3% " "

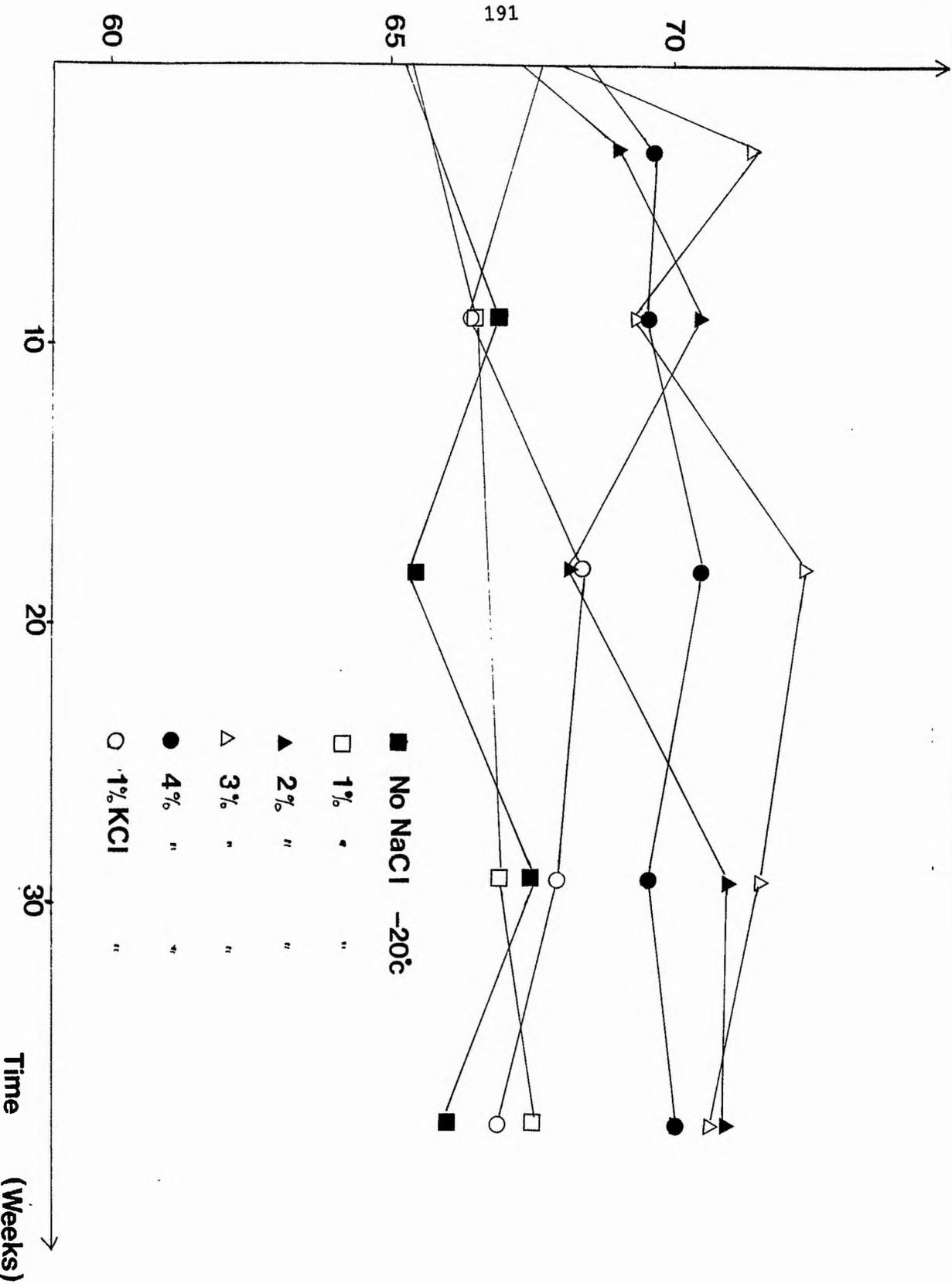
● 4% " "

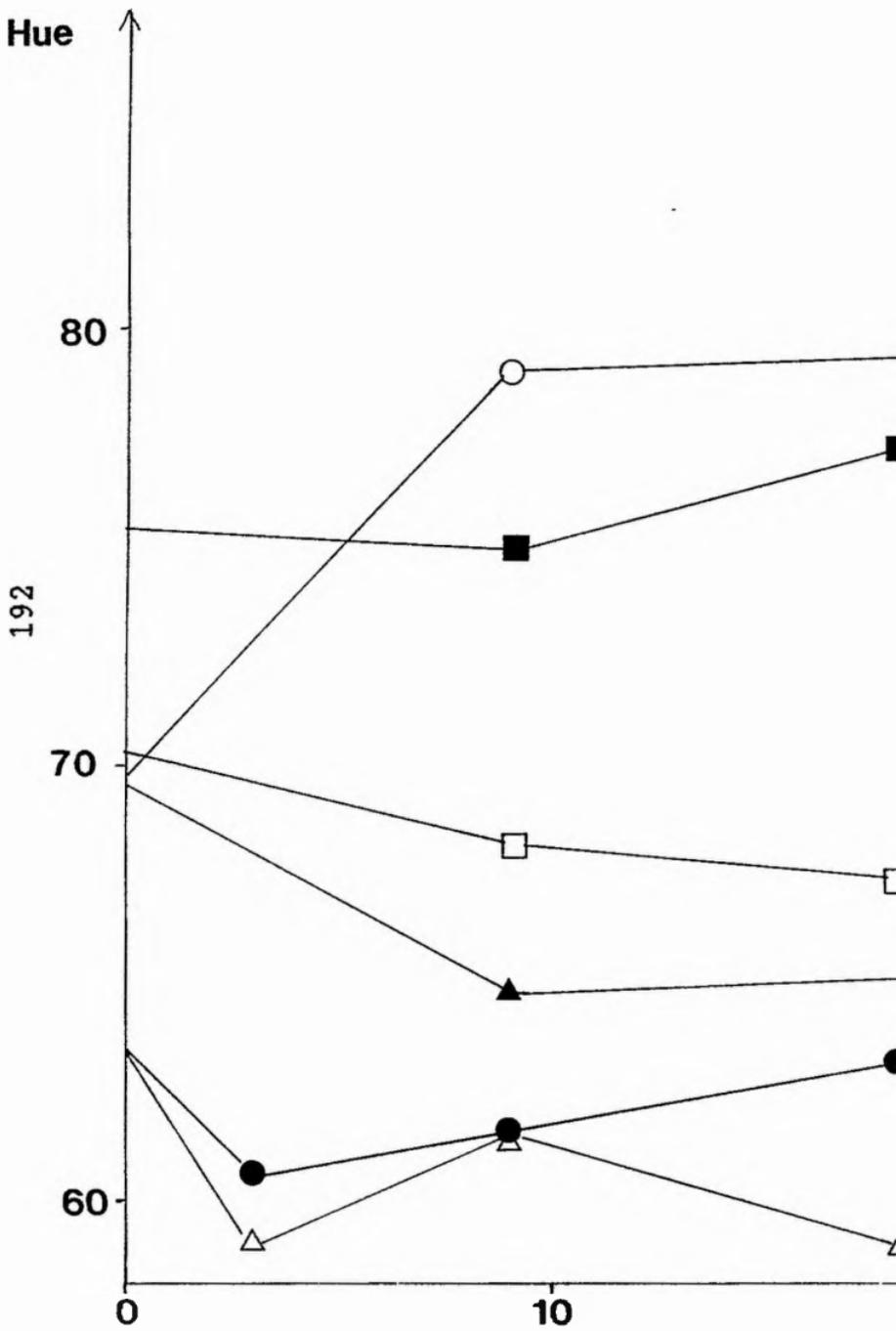
○ 1% KCl "

Time (Weeks)

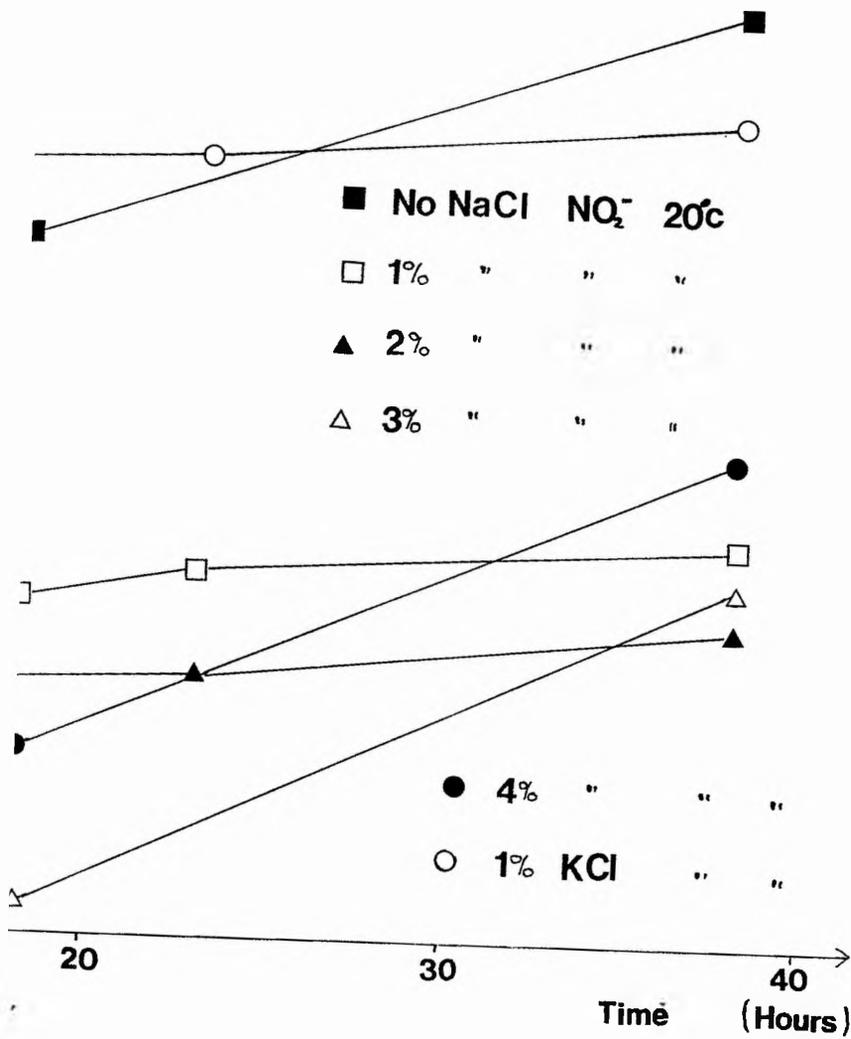
Lightness

FIGURE 6.19





**FIGURE 6.20**



Chroma

22

20

193

18

16

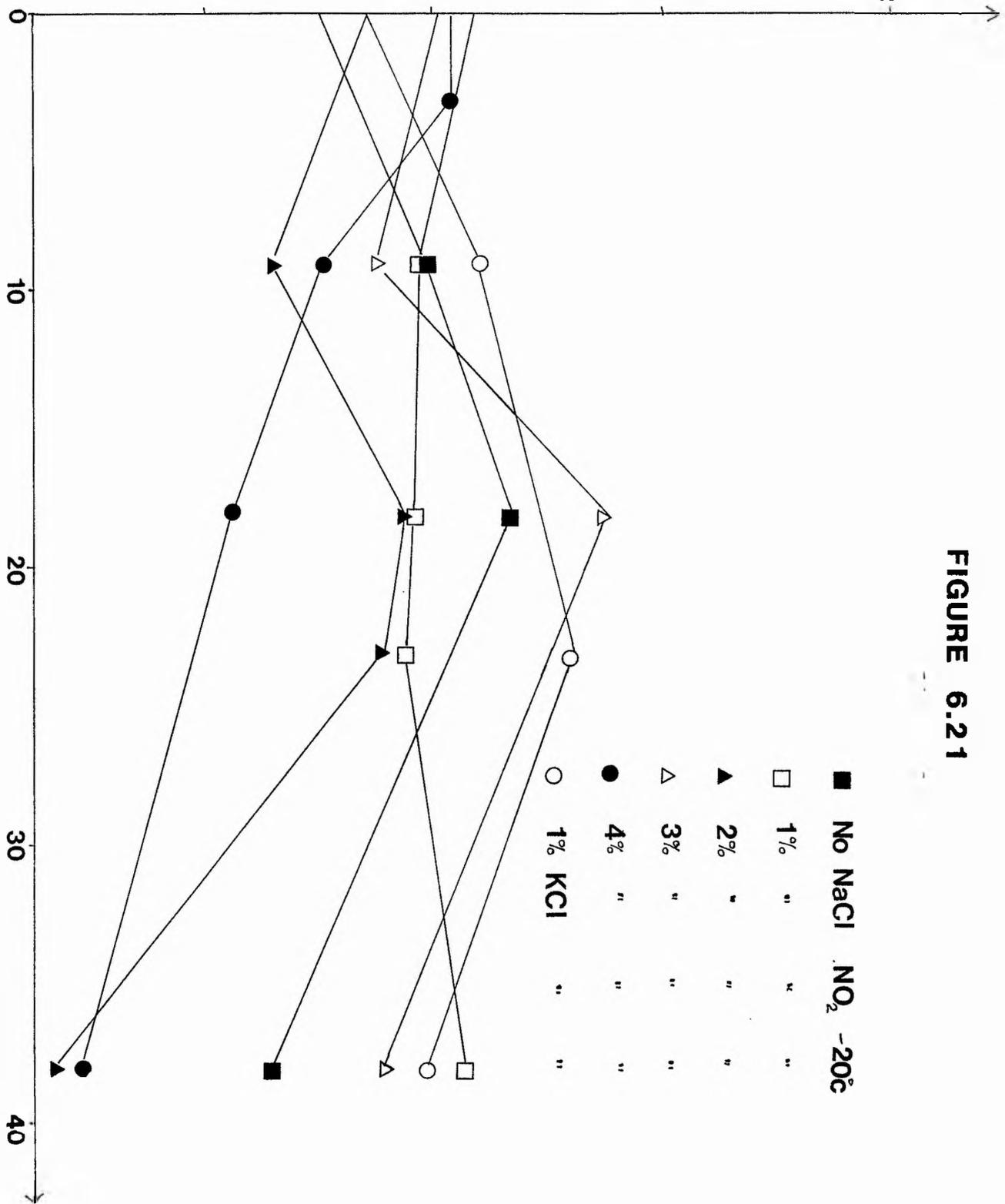


FIGURE 6.21

■ No NaCl NO<sub>2</sub> -20°C

□ 1% " " "

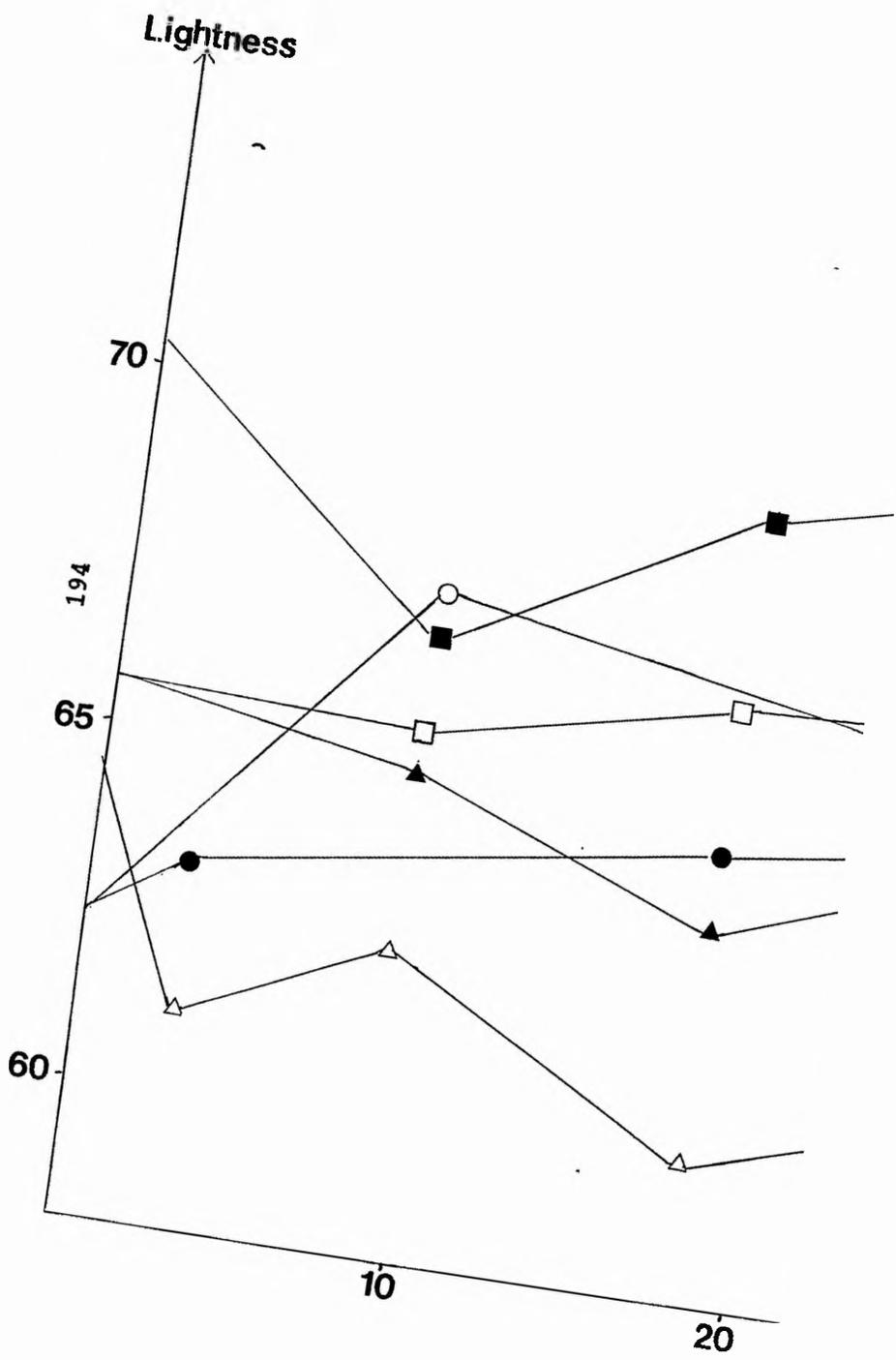
▲ 2% " " "

△ 3% " " "

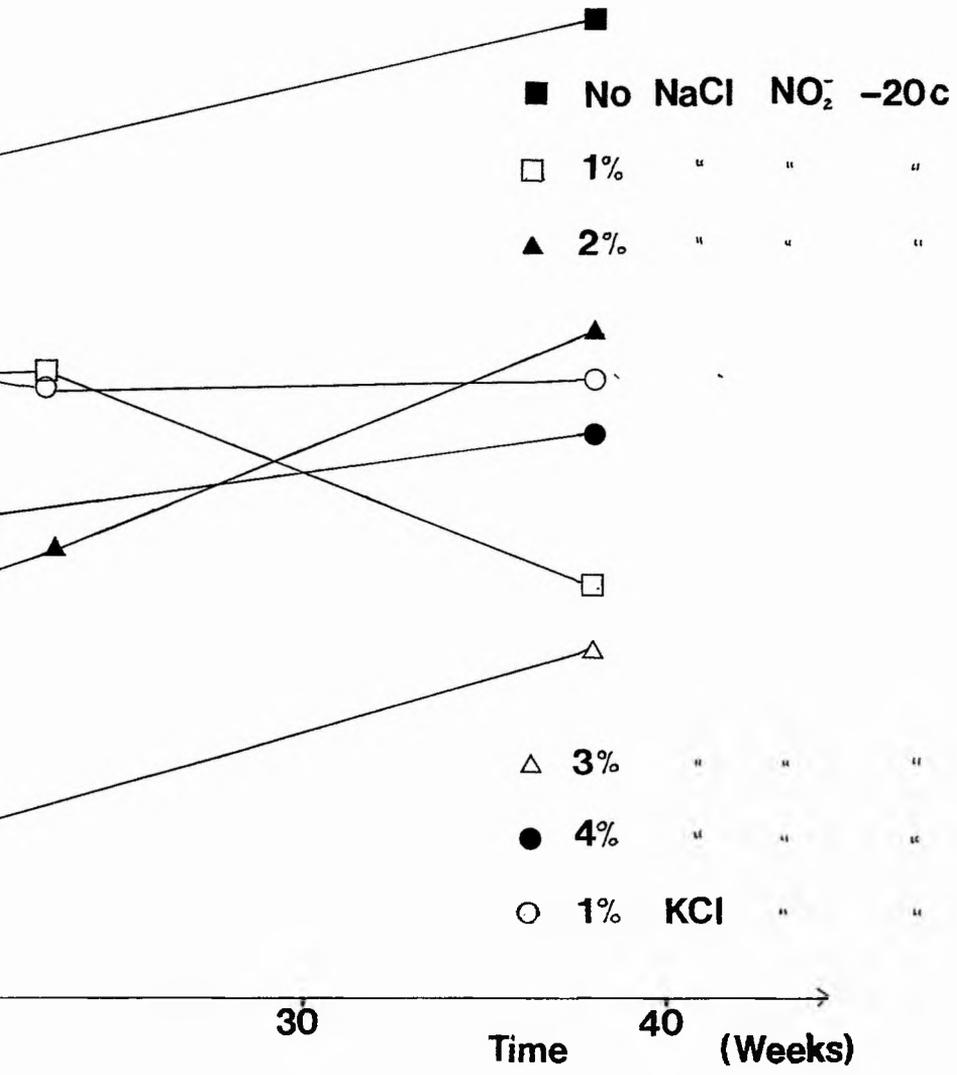
● 4% " " "

○ 1% KCl " "

Time (Weeks)



**FIGURE 6.22**



## 6.4 HPLC OF PORK PURGER NEUTRAL LIPIDS

### 6.4.1 Introduction

High Performance Liquid Chromatography (HPLC) of lipids has chiefly been used to analyze fatty acids, esters and their oxidation products.<sup>(180,181)</sup> This study set out to analyze pork burger neutral lipid oxidation products by HPLC and to see if there existed any agreement between this and the other methods of analysis.

The analysis was carried out on samples G to J (experiment 6.1.3) and K to P (experiment 6.1.4) in two sets (February 87 t = 4 month and June 87 t = 8 month). The HPLC system is described in Table (6.2). Two detection systems were used (mass and UV) because some minor oxidation products have relatively little percentage mass compared to the total sample but absorb strongly in the UV. Unfortunately UV absorption could only be followed at one particular wavelength and with the vast range of oxidation products now known to be produced during lipid peroxidation it was probable that we were observing the absorption of more than one species. The solvent system used absorbed at 234 nm and therefore conjugated diene formation could not be followed. To help identify pork burger neutral lipids and their oxidation products lipid standards, an oxidised oil and an

epoxidised triglyceride were all analysed under identical conditions as the pork burger neutral lipids.

TABLE 6.2

HPLC System

<u>Column</u>	-	silica - 15 cm x 0.46 mm, 5u ChromagaspHERE
<u>Pumps x 2</u>	-	Biothech Instruments Limited HPLC pump 64
<u>Detectors</u>		
mass	-	Applied Chromatography Systems Limited mass detector. Attenuator - 128 or 8
uv	-	Pye Unicam PU 4025 uv detector operated at 260 nm. Attenuation 0.16 or 0.64.
<u>Recorder</u>	-	PM 8252 dual-pen recorder chart speed 300 mm/hr

Solvent system and gradient

Solvent A	-	Hexane/dichloromethane (2:1 v/v)
Solvent B	-	Dichloromethane/Hexane/Acetonitrile (250:160:80 v/v/v)

Gradient

<u>Solvent Mix</u>	<u>Time</u>	<u>%A</u>	<u>%B</u>
1	0.0	100	0
2	0.5	97	3
3	10.0	70	30
4	23.0	50	50
5	24.0	0	100
6	29.0	0	100
7	29.5	100	0

TABLE 6.3

<u>Component</u>	<u>Retention Distance (mm)</u>	<u>Absorption (260 nm-uv)</u>
triglyceride	25 - 30	strong
epoxide of triglyceride (F1)	35 - 37	weak
epoxide of triglyceride (F2)	2 peaks 51 and 58	weak
cholesterol	52 - 55	weak
cholesterol esters	9	strong
diglyceride	50 - 52	weak
free fatty acids	45 - 47	weak

TABLE 6.4

Component	Retention Distance (mm)	Absorbtion (260 nm)
	three sets of absorptions	
linseed oil	(37, 39 and 42)	v. strong
safflower oil	(50 to 54 four peaks)	strong
coconut oil (oil mixture PV-65)	(63 and 66)	weak
rancid pork burger	(39 and 41)	strong
porkburger samples	(39 and 41) (+ v low conc of other products)	strong

In Figures 6.20 to 6.29 output from both mass detector and uv detector were recorded. The mass detector has its baseline at the bottom of the figure and the output from the uv detector has its baseline at the top.

#### 6.4.2 Epoxidation

Epoxidation was carried out by the method of Gunstone and Schuler.<sup>(182)</sup> Pork triglyceride (800 mg) was dissolved in chloroform (10 ml) and added to a solution of m-chloroperbenzoic acid (320 mg) in chloroform (10 ml). The solution was stirred for 5 hours at room temperature then washed successively with aqueous sodium bicarbonate (5%) and brine. Each aqueous wash was re-extracted with ether and the product recovered from the combined organic layers. Separation of the product by TLC (petrol-ether - formic acid, 80:20:1) gave three fractions that separated from the

baseline. The retention distances and chromatograms for fractions 1 and 2 are reported in Table (6.3) and Figure (6.20) respectively.

#### 6.4.3 Neutral Lipid Extraction and Isolation from Pork Burgers

Frozen burgers were removed from storage and divided into four using a fixed blade scalpel then immediately placed in methanol (40 ml) and allowed to thaw for 30 minutes. After thawing the meat was homogenised (2 minutes) using an Ultra Turrax homogenizer then 150 ml of chloroform-methanol (2:1) added and the mixture rehomogenized. The samples were allowed to stand for 1 hour with occasional mixing before the homogenate was passed through filter paper. The filter paper and residue were washed twice with chloroform (10 ml) then the combined filtrates were transferred to a clean separating funnel containing 1% sodium chloride (40 ml). The funnels were gently shaken and the two layers allowed to settle before the lower chloroform layer was collected. The chloroform was removed by blowing nitrogen over the surface and heating gently (30°C). Total lipid (200 mg) was dissolved in chloroform (0.5 ml) and added to a Sep-pak silica cartridge. Neutral lipid was eluted from the column using chloroform (15 ml) and the chloroform removed by blowing nitrogen over the solution. Neutral lipid was

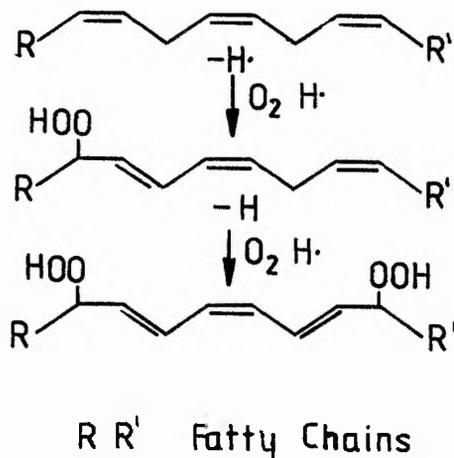
dissolved in Hexane/dichlormethane (2:1 v/v) at a concentration of 50 mg/ml prior to analysis.

#### 6.4.4 Results and Discussion

Due to limited time no quantitative information concerning the oxidation products was obtained. This was mainly due to the lack of standards and the uncertainty of the chromophore(s) being detected. Tables (6.3 - 6.4) present the retention distances and a qualitative assessment of the strength of the absorbing signal. The chromatograms are presented in Figures (6.21 - 6.29).

As can be seen from Figure (6.21) triglycerides were by far the largest neutral lipids. They also absorbed in the uv although the exact nature of the absorption was unresolved. One possibility could have been the carbonyl  $\pi$  to  $\pi^*$  of the triglyceride. However when the triglycerides were epoxidised the strength of signal fell. Another possibility for the observed chromophore could have been conjugated trienes which absorb in the range 250 to 280 nm. Although such chromophores may exist in highly oxidised material through the formation of dihydroperoxides, Figure (6.30), it is questionable as to whether they were present in 'fresh' pork triglyceride.

FIGURE 6.30



The formation of this chromophore requires the presence of an unsaturated acyl chain with three or more double bonds which were present in pork triglycerides but only at small concentrations.

The epoxide fractions F1 and F2 obtained by TLC were run separately then combined and analysed together, Figure (6.20). Their elution profile would have been dependent on both the number of epoxide groups per triglyceride molecule and on the nature of a particular isomer. For example, in a triglyceride containing two saturated fatty acyl chains and oleate epoxide (S, S, and O), SOS would elute from a silica column before SSO since SSO would have more contact with the

silica. However, the epoxide F1 appeared as one component while F2 was composed of two. Figures (6.22 - 6.27) represent the chromatograms of samples (K - P) for February 1987. The sensitivity of the uv detector was the same for all samples (0.64) but the attenuation of the mass detector was changed to observe the corresponding minor oxidation products (sample M). All samples showed a twin absorption which increased significantly between February 1987 and June 1987 (the uv detector was reduced in sensitivity x4 between February and June). It was interesting to note that burgers stored with 4% salt (Figure 6.27) exhibited a doublet which was less intense than burgers stores with 2 and 3% salt, (Figures 6.25 and 6.26) respectively. If the intensity of the doublet was a measure of the extent of oxidation then burgers stored with 4% salt oxidised slower than burgers with 2 and 3% salt. The twin absorption in all samples was of similar retention distance to that of epoxidised triglyceride F1. However as mentioned previously epoxides did not strongly absorb at 260 nm and since the concentration of the pork burger oxidation products was far less than the concentration of the synthesised epoxidised triglyceride then the increase in intensity of the doublet was unlikely to have been due to epoxides. The formation of carbonyls has been shown to occur from the termination of peroxy radicals<sup>(27)</sup> and via the decomposition of hydroperoxides (via B scission) which can produce dienals. The formation of both cannot be

discounted. An oxidised oil with a peroxide value (PV) of 65 was analysed and gave a similar twin absorption to that of the burgers, Figure (6.28). Since hydroperoxides would be expected to be the major products from initial oxidation it may be possible that the doublet represents hydroperoxides, although this again raises questions regarding the absorbing chromophore. The splitting which occurred may have been due to inside/outside isomers as discussed earlier. The traces recorded for samples G to J, experiment two were very similar to samples K to P with the increase in uv absorbance of the doublet being  $G < H < I < J$ . A rancid porkburger, Figure (6.29) was analysed and exhibited a similar absorbing doublet. However the concentration of the first component of the doublet was substantially greater than the second but the significance of the result is not known.

These results suggest that the strength of absorption of the doublet is dependent on both the level of salt in the samples. The results in connection with the other methods of analysis are discussed in section (6.6). HPLC may prove to be a highly useful technique for following lipid oxidation in meats and other food stuffs although much work is still required to be carried out. The use of a HPLC - MS system would prove invaluable in identifying intact minor oxidation products.

FIGURE 6.20

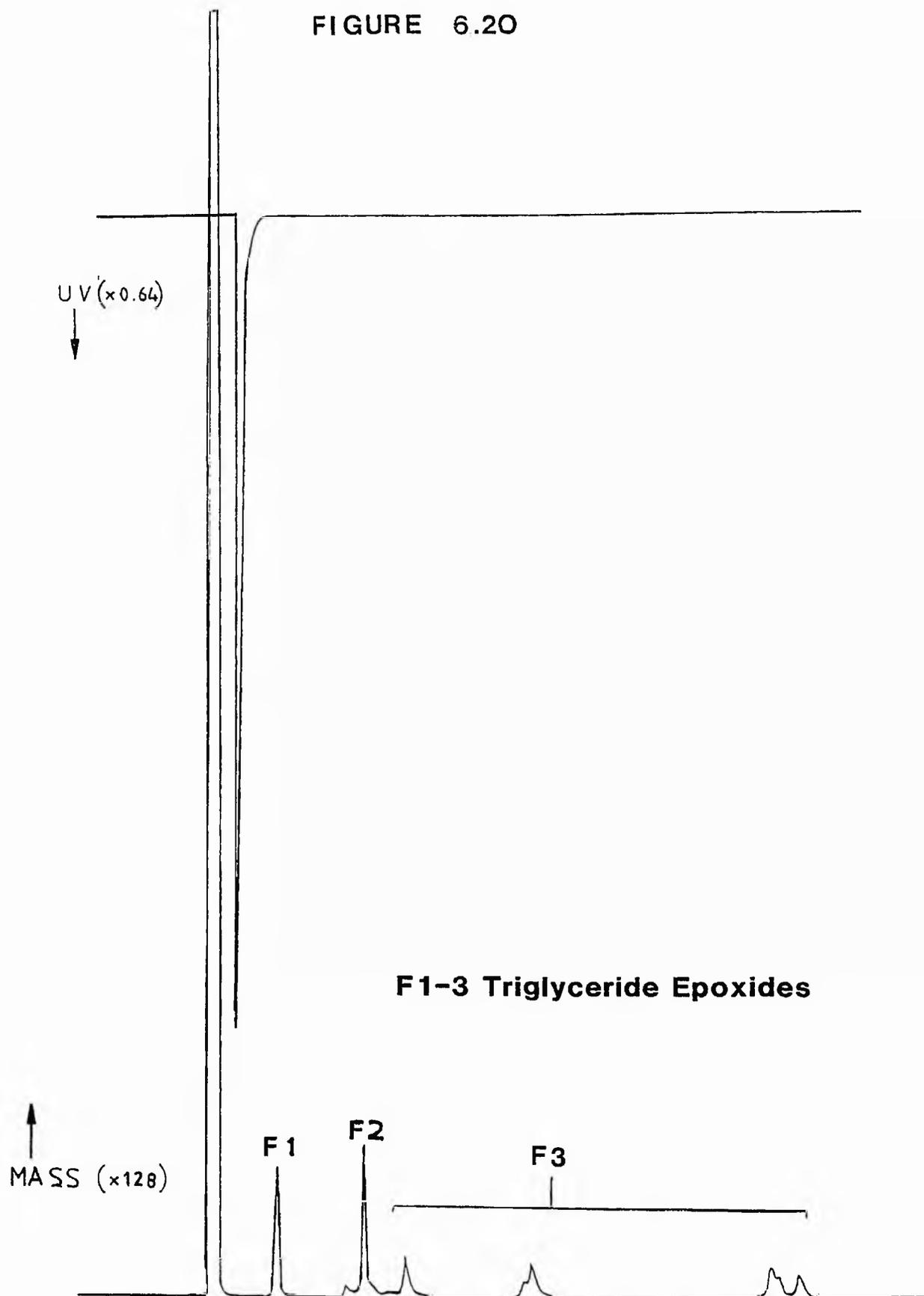
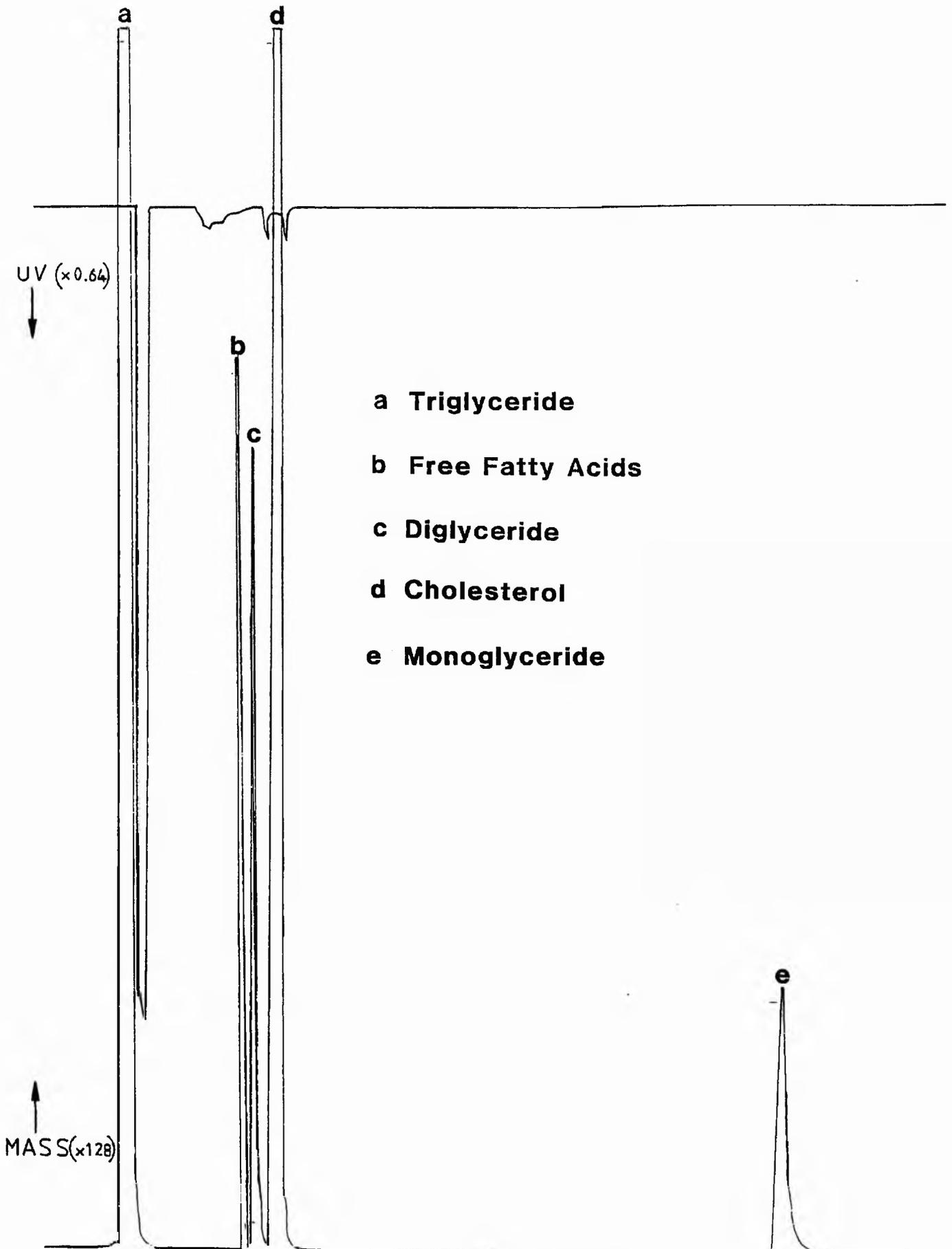


FIGURE 6.21



**FIGURE 6.22**

**No NaCl -20°C**

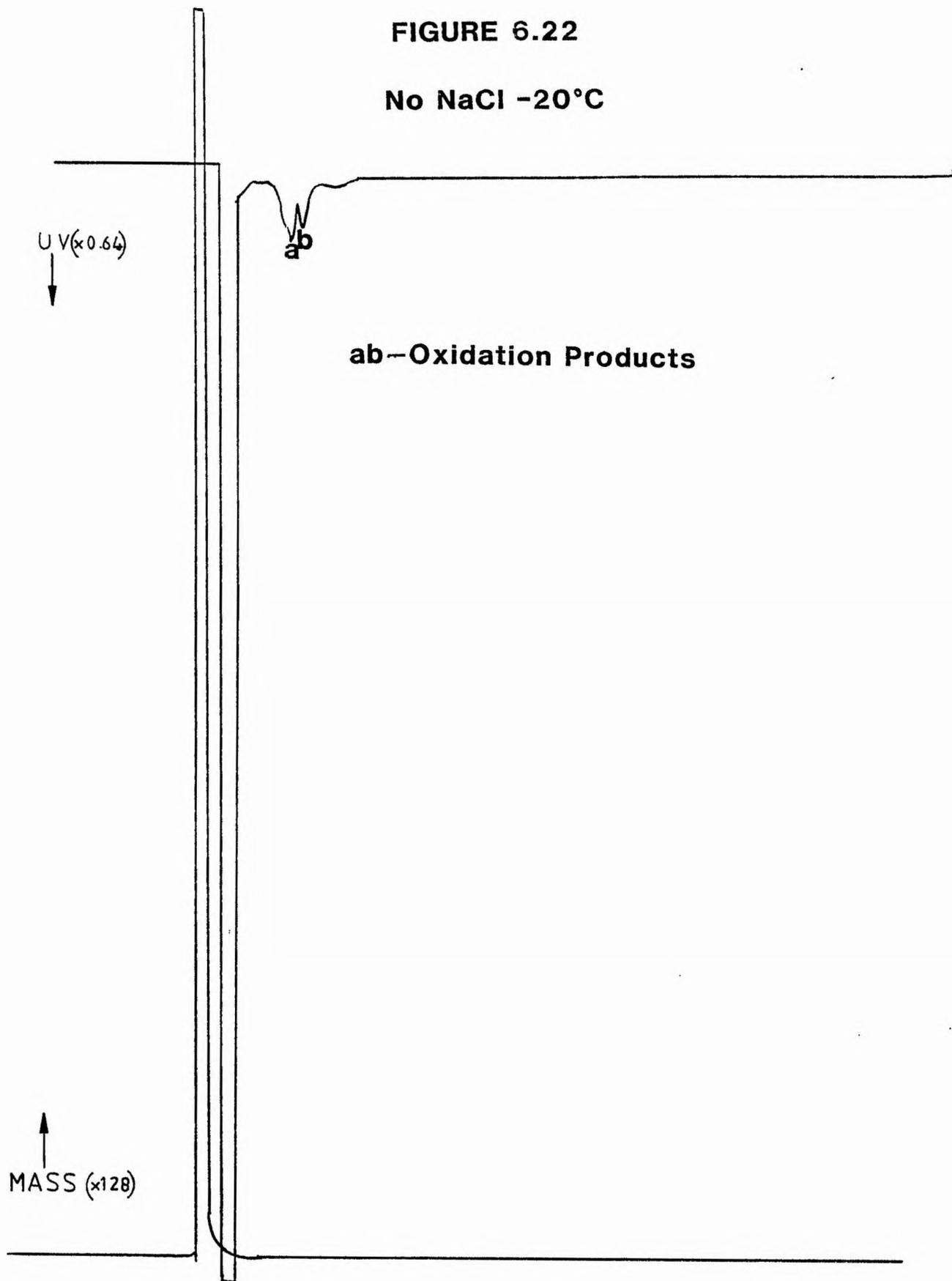


FIGURE 6.23

1% NaCl -20°C

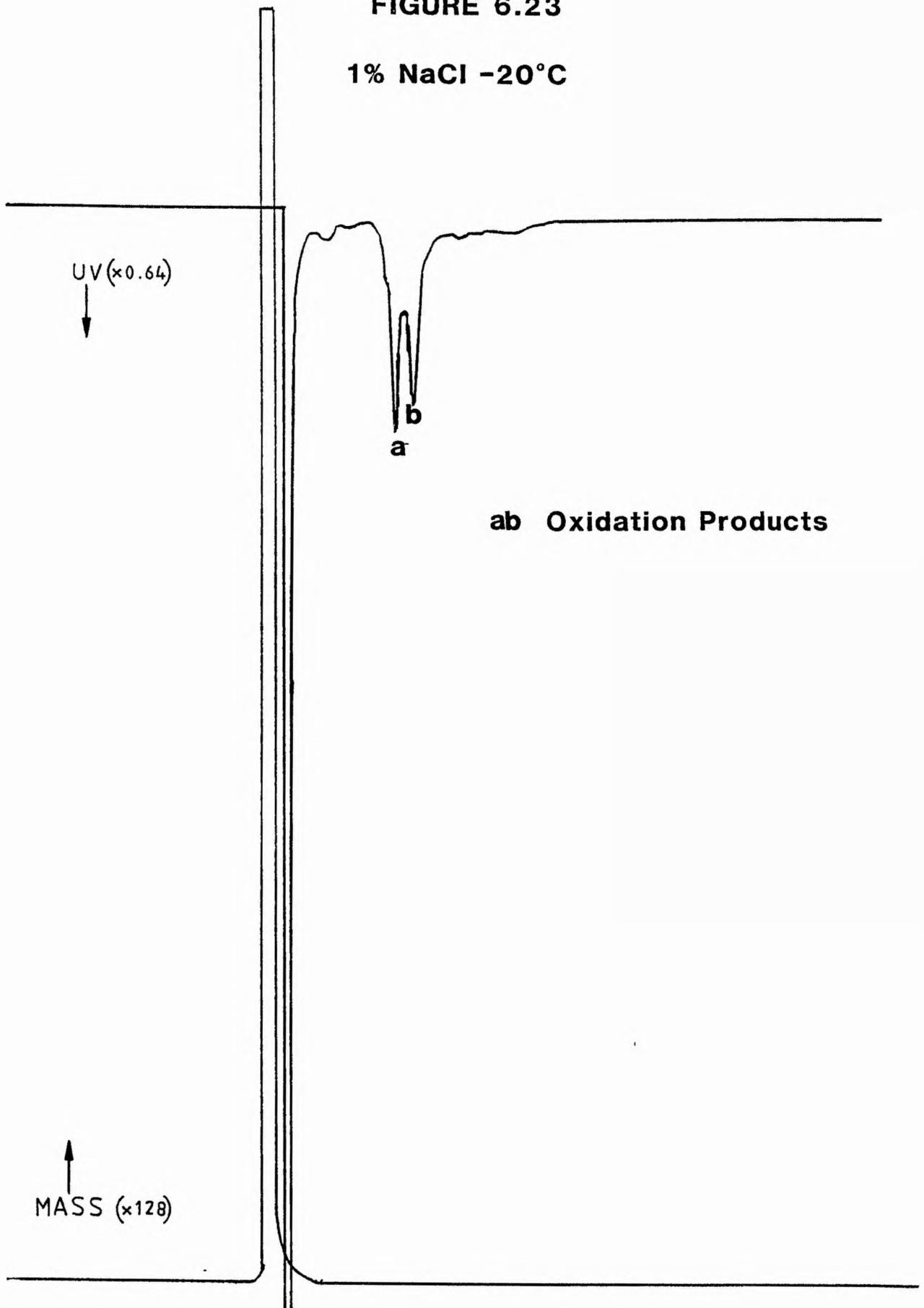
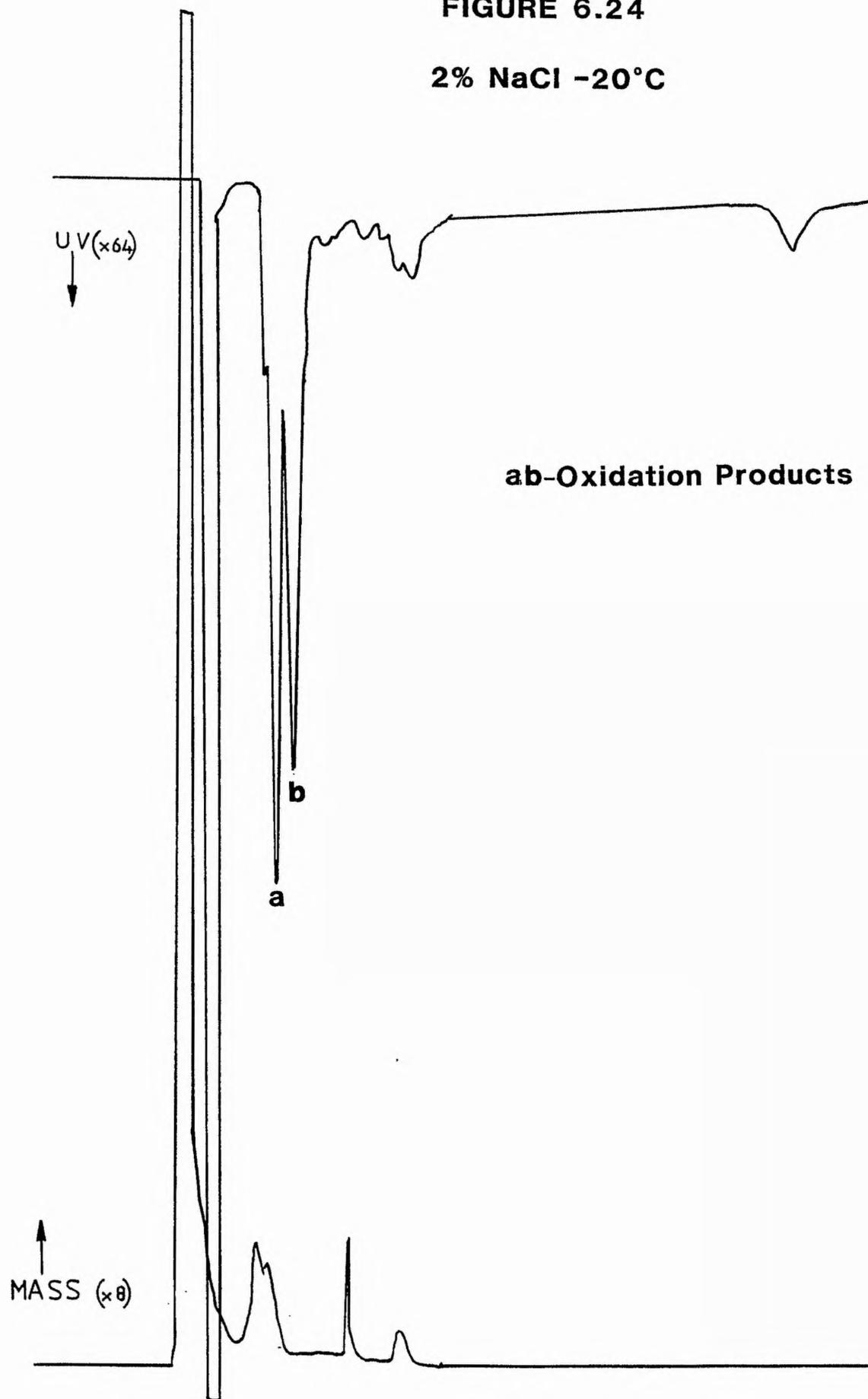


FIGURE 6.24

2% NaCl -20°C



**FIGURE 6.25**  
**3% NaCl -20°C**

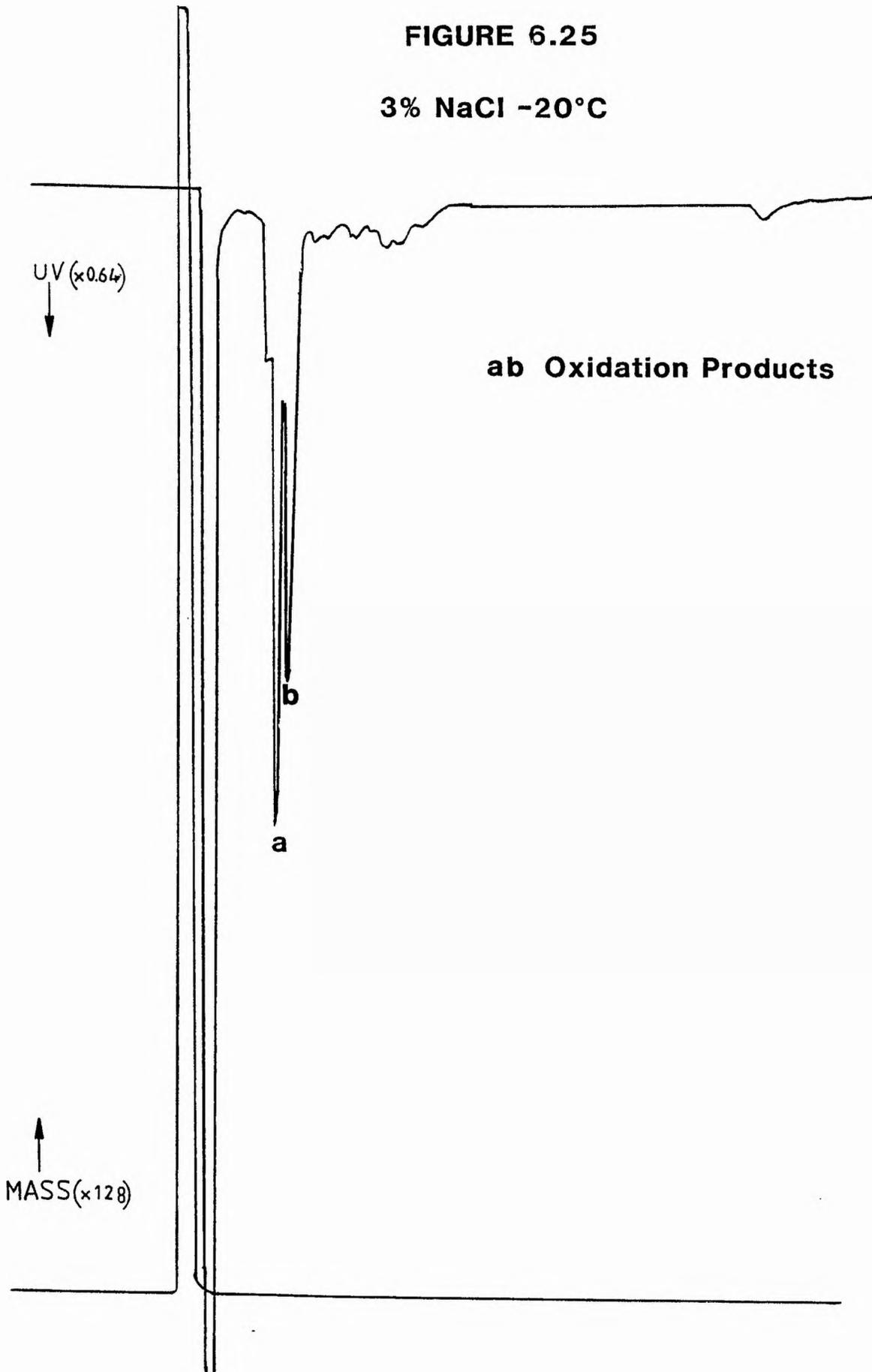


FIGURE 6.26

4% NaCl -20°C

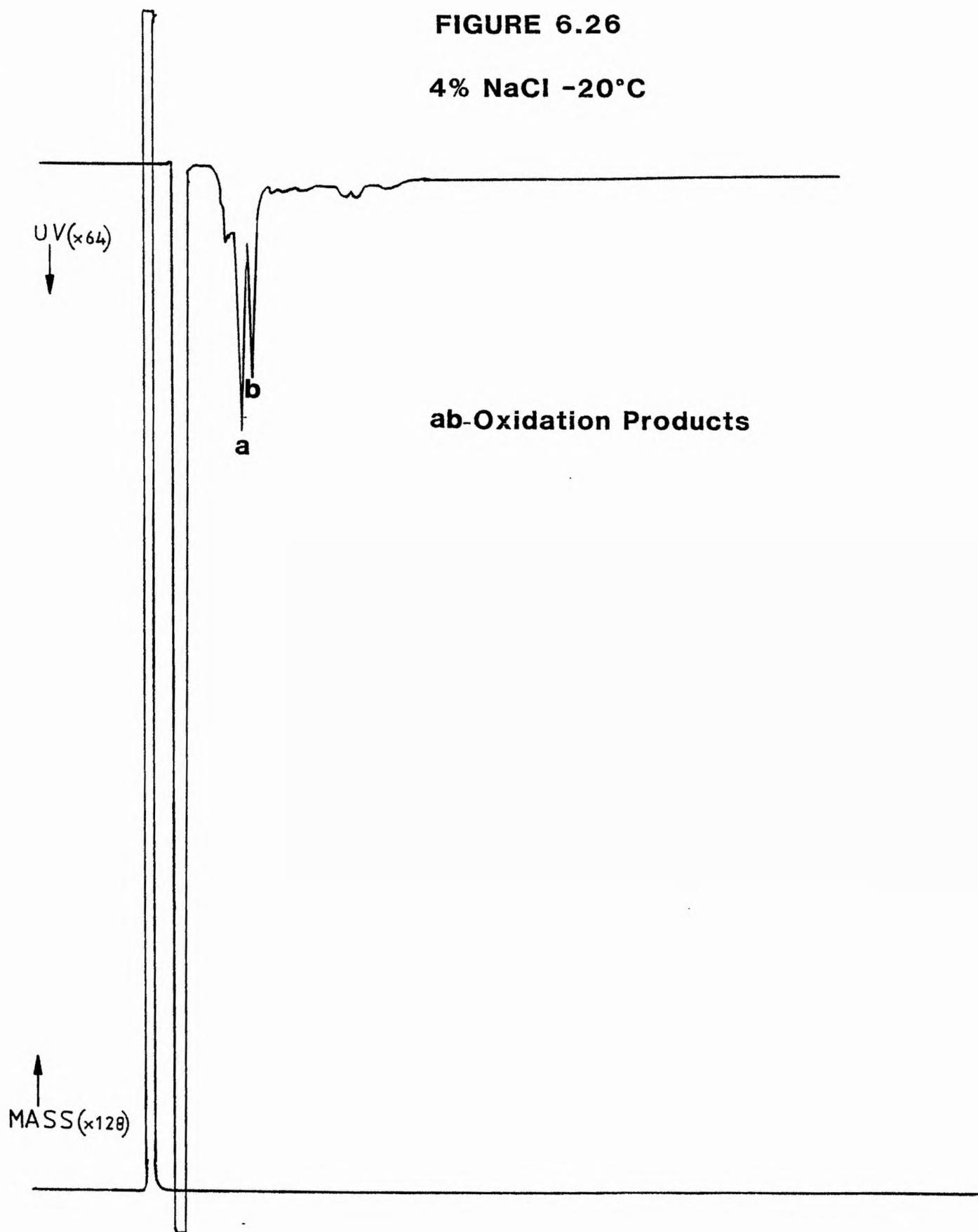
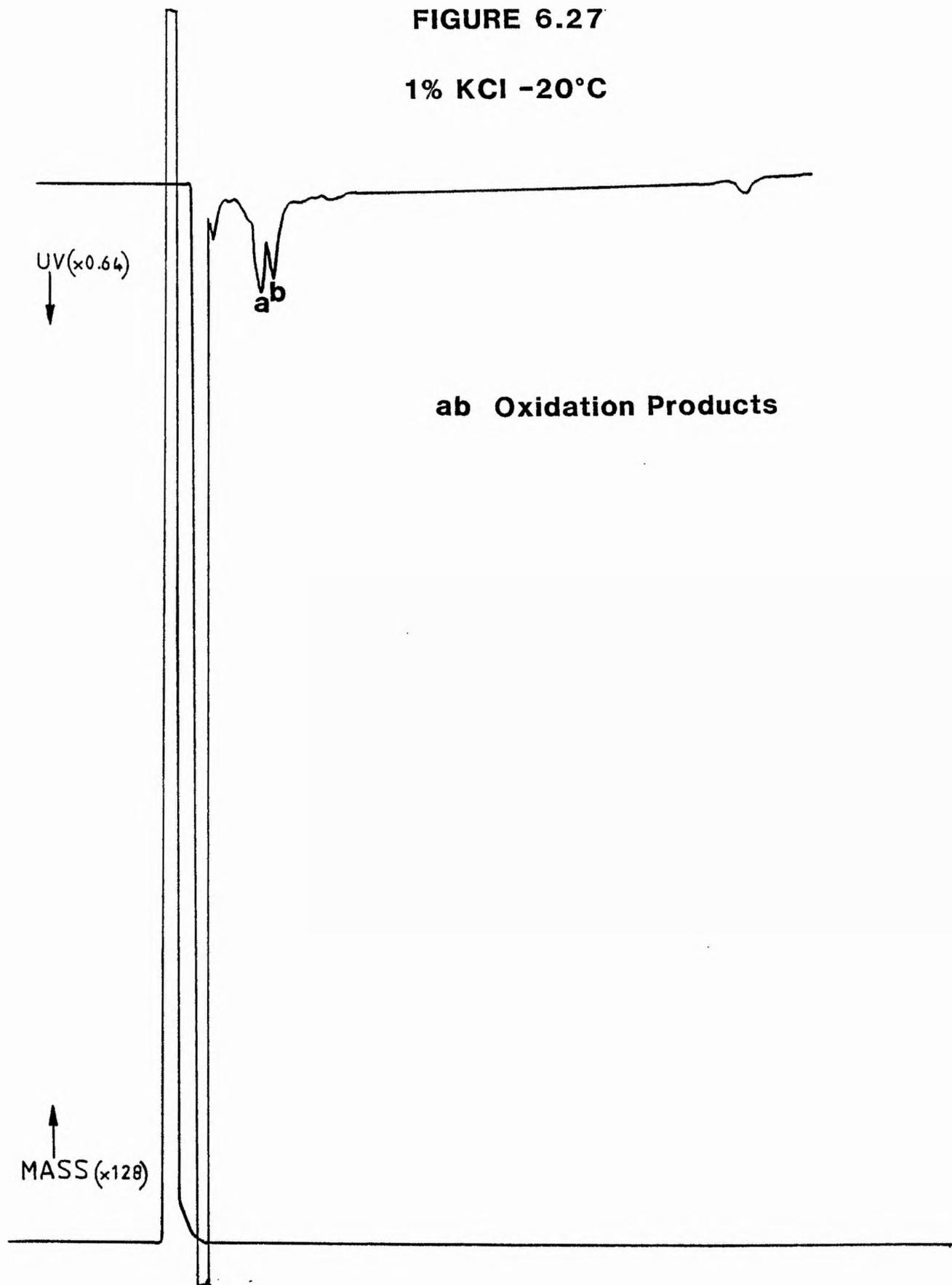
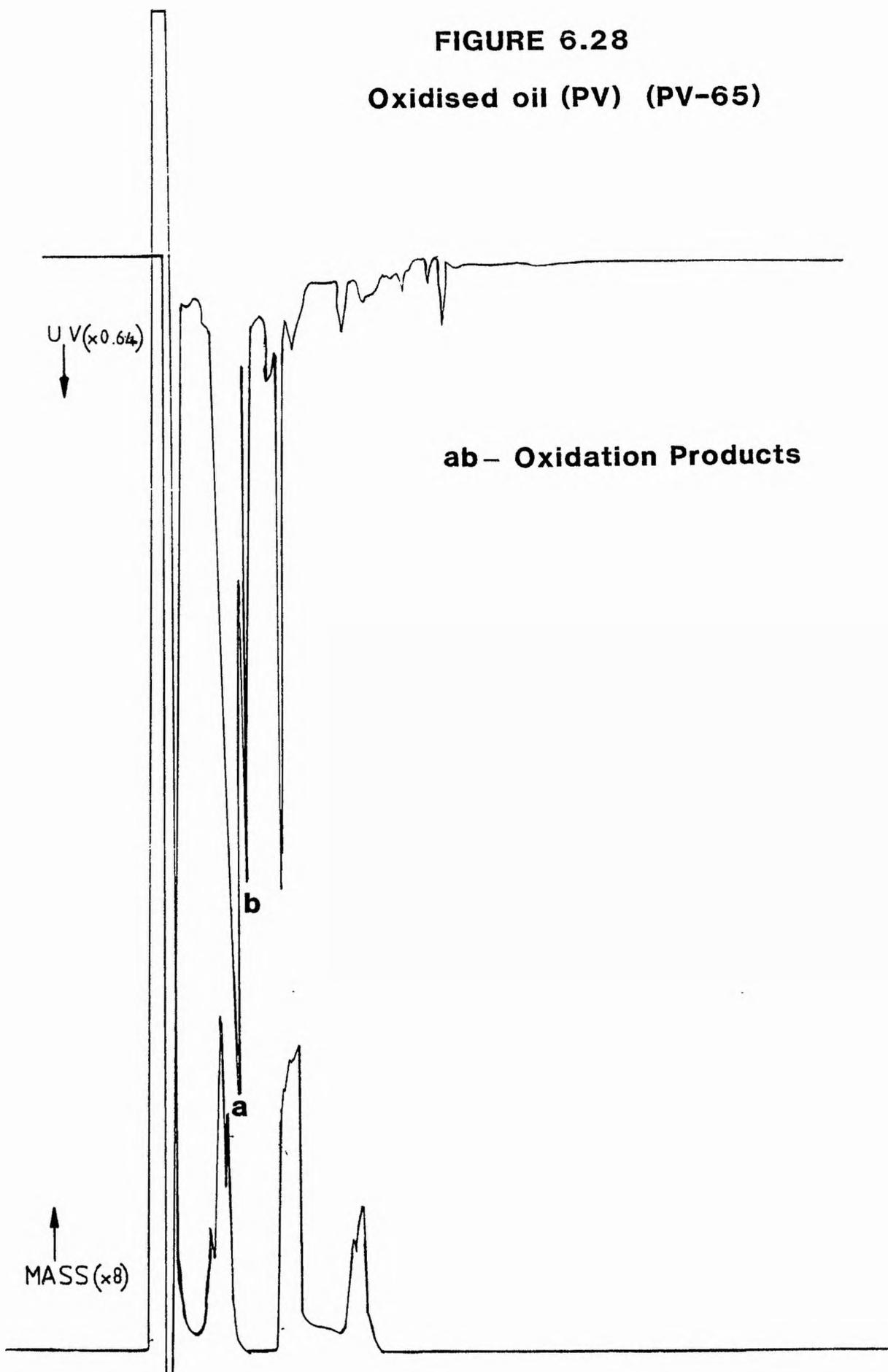


FIGURE 6.27

1% KCl -20°C

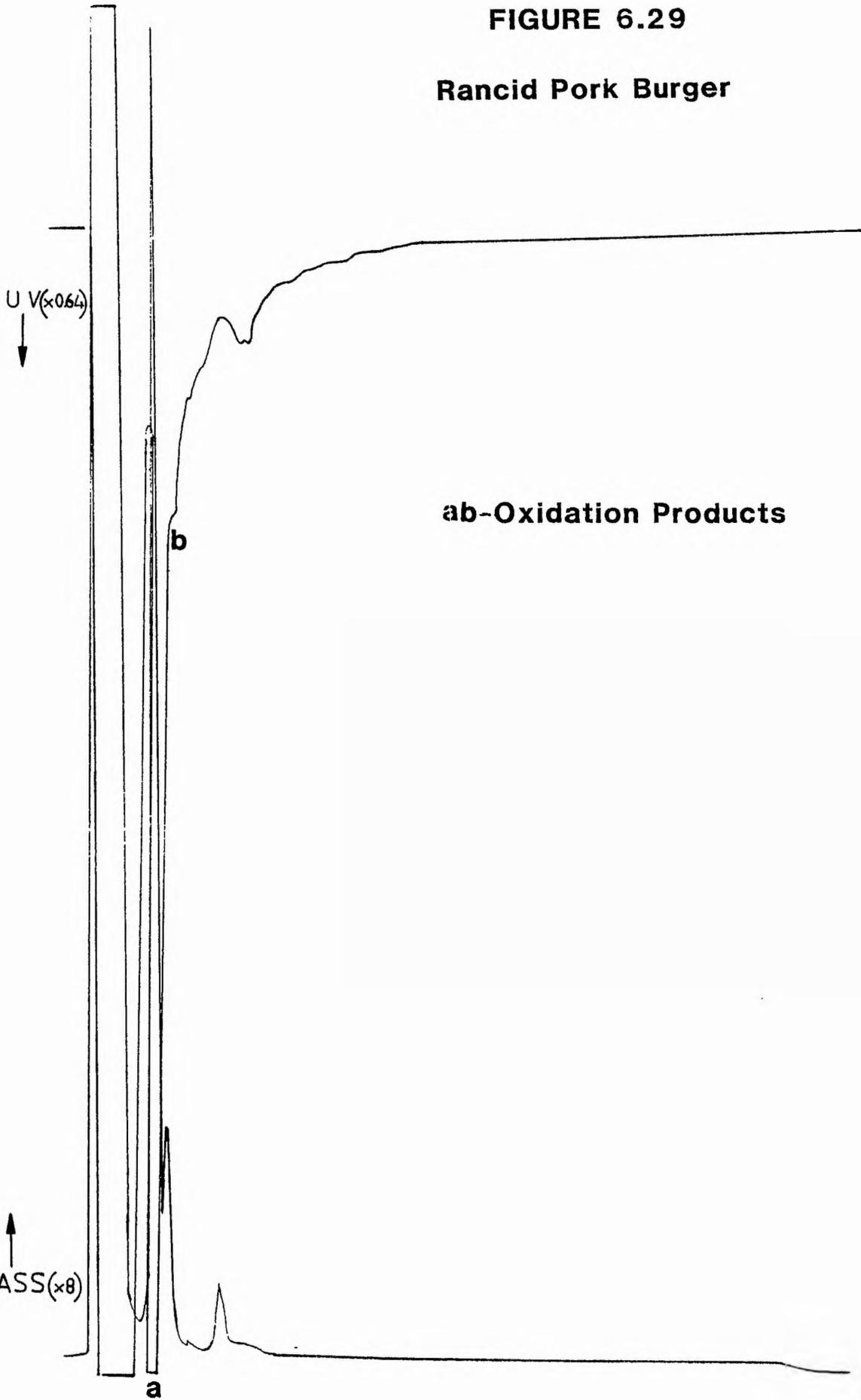


**FIGURE 6.28**  
**Oxidised oil (PV) (PV-65)**



**FIGURE 6.29**

**Rancid Pork Burger**



### 6.5 ORGANOLEPTIC ASSESSMENT

Organoleptic assessment was carried out at Colworth House to study burger quality and deterioration as the storage trial progressed. It was hoped that some agreement would be achieved between sensory evaluation and results from the other methods of analysis.

At each take-off the burgers were grilled to a centre temperature of 75°C for approximately 8 minutes. They were kept warm in a hostess tray until served to a trained panel in tasting booths under red lights. There were 12 trained panelists in a session each tasting 4 out of 6 samples which resulted in 8 replications of each sample at a session. At each take-off date there were 4 sessions, ie, each sample was tasted 24 times. Each panelist was asked to grade the burgers from 1 to 10 in each of the aroma and flavour parameters, Figure (6.31). The results were analysed statistically and the means and standard deviations obtained. The results are presented graphically in Figures (6.32-6.39) and tabulated in Appendices (6.12-6.15).



## 6.5.1 Results and Discussion

### 6.5.1.1 The Stability of Uncooked Pork Burgers Containing 1% salt at Different Temperatures

The tasting panel results are tabulated in Appendix (6.13) and a selection presented graphically in Figures (6.32-6.39). Samples A, B, and C were not tasted at all take-off dates as they became rancid. At the first two take-off dates burgers stored at  $-4^{\circ}\text{C}$  (sample A) generally had the highest aroma scores. They also scored highest in Overall Strength, Overall Other, Rancid, Stale/Musty and Other flavours. These increases in flavour scores corresponded with a decrease in the Overall Quality flavour score which represents the overall acceptability of the burger to the tasting panel. There were very little differences in the Overall Strength and Pork aroma's of the burgers stored between  $-8$  and  $-30^{\circ}\text{C}$  (samples B - F). However, throughout storage the Other Aroma scores were generally greater the higher the temperature of storage. Overall Strength, Smokey, Fatty, Sweet, Overall Other and Other flavour scores remained relatively constant throughout the storage period. At the beginning of the trial very little difference was seen in pork and bacon flavour but as storage time increased it appeared that pork flavour

was best maintained at lower storage temperatures. A slight increase in bacon flavour occurred at higher storage temperatures. On increased storage time there was an increase in Rancid and Stale/Musty flavours which was greater at higher storage temperatures. For example, burgers stored at  $-20^{\circ}\text{C}$  (sample D) had a Rancid flavour score of 1.379 at the start of the trial and reached 3.207 by the end. Burgers stored at  $-30^{\circ}\text{C}$  (sample E) started with a Rancid flavour score of 1.208 and finished with a score of 2.317 at the end of the trial. An increase in Rancid flavour score was usually accompanied by a decrease in the Overall Quality flavour score. Burgers stored in the absence of salt (samples F) scored lower than burgers containing 1% salt (sample D) in Rancid and Stale/Musty flavours with increased storage time. Sample F also scored higher than sample D in the Overall Quality Flavour score.

These results suggest that lower storage temperatures help prevent the deterioration of pork which results in the development of off-odours and off-flavours. In addition, burgers stored at  $-20^{\circ}\text{C}$  with no added salt appeared to retain their quality over burgers stored with salt.

6.5.1.2 The Stability of Cooked Pork Burgers  
Containing Different Amounts of Salt at -20°C

The results are tabulated in Appendix (6.14) and some presented graphically in Figures (6.32-6.39). There was very little change in Overall Strength aroma scores both at the start of the trial and on increased storage time in all samples. However in burgers with high levels of salt (2 and 3%, samples I and J) there was a decrease in Pork aroma and an increase in Other aroma scores on increased storage time. There was also practically no change in Overall Strength, Smokey, and Fatty flavours throughout the trial. Panelists could easily pick out burgers with different salt contents as measured by the salt flavour scores. There was an increase in Bacon, Overall Other, Rancid, Stale/Musty and Other flavours both as the salt content of the burgers increased and as the storage trial progressed. The above changes were accompanied by a general decrease in Overall Quality flavour scores which were greater in burgers stored with higher levels of salt. It was interesting to note that in burgers stored with no salt, (sample G) a slight increase in the Overall Quality flavour score. These results suggest that cooked pork burgers are best stored in the absence of salt.

6.5.1.3 The Stability of Uncooked Pork Burgers  
Containing Different Amounts of Salt at -20°C

The results are presented in Appendix (6.15) and Figures (6.3 -6. .). There was very little change in the Overall Strength aroma and Smokey, Fatty and Sweet flavours with increased storage time and between burgers with different salt concentrations. There was a slight increase in Overall Strength and Bacon flavours both with increased salt content and storage time. Panelists could easily differentiate between burgers stored with different concentrations of salt as indicated by the Salt flavour scores. Overall Other, Rancid, Stale/Musty and Other flavours increased with increased storage time and salt content. These changes corresponded with a general decrease in Overall Quality flavour scores with increased storage time and salt content. However, burgers stored with no added salt (sample K) slightly increased in the Overall Quality flavour score on increased storage time. Burgers stored with 1% potassium chloride in place of 1% sodium chloride scored higher in Overall Other, Rancid, Stale/Musty, Other and lower in Overall Quality flavour scores which suggests that potassium chloride is not a desirable replacement for sodium chloride in raw pork burgers stored frozen at -20°C. These results agree with Hadden et al<sup>(174)</sup> who

showed that salt caused an increase in rancid odour scores of comminuted pork when held under frozen storage.

6.5.1.4 The Stability of Uncooked Pork Burgers Containing Sodium Nitrite and Different Amounts of Salt at -20°C

The results are tabulated in Appendix (6.16) and a selection presented graphically in Figures (6.32-6.39). There was practically no change in Overall Strength aroma throughout the trial. Pork aroma scores tended to decrease with increased storage time. However, a larger decrease appeared to occur in burgers stored without salt (sample Q) than in burgers stored with salt (samples R - V). Other aroma scores generally increased on increased storage time which was greatest in samples with low levels or no added salt. There was very little change in Overall Strength, Pork, Smokey, Fatty, Sweet and Overall Other flavours on increased storage time. However at any particular take off date pork flavour generally scored higher in burgers containing low levels of salt and Smokey flavour higher at higher levels of salt. It was also interesting to observe that Bacon flavour scores were higher in burgers containing 2, 3 and 4% salt (samples S, T and U) both at the start and

throughout storage. Burgers that contained increasing concentrations of salt had higher Rancid, Stale/Musty and Other flavour scores on increased storage which was accompanied by a general decrease in the Overall Quality flavour score. These results show that raw cured pork burgers are best stored at  $-20^{\circ}\text{C}$  in the absence of salt. In addition, potassium chloride reduced overall quality in pork burgers.

#### 6.5.1.5 Comparison of Storage Trials

The direct comparison of aroma and flavour scores between individual storage trials must be carried out with caution since the meat used in individual experiments was obtained and mixed on separate days. As can be seen from the lipid FAME analysis, both phospholipids and total lipid FAME profiles were different in each of the four storage trials. Differences in total fat content of burgers in the different storage trials would also have been important in the development of off-odours and flavours detected on increased storage time. However that apart, comparisons can be made since burgers in all four experiments were tasted at the same tasting sessions.

The Overall Strength aroma scores in the four experiments were all of similar value at the start of

the trial but decreased slightly on increased storage time. The Pork aroma and flavour scores were lower in burgers containing nitrite than in the other three storage trials where burgers were stored in the absence of nitrite. Other aroma scores were comparable at the beginning of storage and increased in all experiments on increased storage. The Overall Strength flavour scores were relatively stable in the experiment investigating the effect of storage temperature on burger stability but were more varied in the other three storage trials where high salt levels of burgers resulted in increased scores. Smokey flavour scores in all four storage trials were approximately 1 to 2 and did not vary much on increased storage. Bacon flavour scores were considerably greater in cured burgers at the beginning of storage and increased in all four experiments as the trial progressed. Bacon flavour scores were also higher in burgers containing more salt. Fatty flavour scores were slightly lower for cooked burgers than for uncooked burgers which was probably due to fat loss through cooking. In all experiments panelists were easily able to detect changes in salt content and found little change in salt flavour scores on increased storage time. Burgers stored with potassium chloride had comparable scores to those stored with sodium chloride. Sweet flavour scores were approximately 1 to 2 in all four

trials both at the start and throughout storage. Initially, Overall flavour scores were similar in all four trials. However, with increased storage, scores increased in all experiments except where burgers were cured (nitrite present). Initially Rancid, Stale/Musty and Other flavour scores were all comparable and low, (1 - 2), in all four storage trials. However there was a general increase in their scores with increased temperature, salt content and storage time. Additionally, there seemed to be a larger increase in the above parameters in raw burgers stored at  $-20^{\circ}\text{C}$  with high levels of salt. Burgers stored at  $-4$ ,  $-8$  and  $-13^{\circ}\text{C}$  (samples A, B and C) were not analysed at all take off dates as they had become rancid and would have presumably also scored higher in these parameters if they had been tasted. Overall Quality flavour scores generally decreased both on increased salt content and increased storage time in all four experiments. However a larger decrease occurred in uncured burgers with high levels of salt. In contrast, there was an increase in the Overall Quality flavour score of cooked burgers containing no added salt on increases storage.

From the discussion above it appeared that the most obvious changes to occur with increased storage time were an increase in Overall Other, Rancid, Stale/Musty

and Other flavours and a decrease in Overall Quality flavour scores which gave clear indications of the deterioration in the burgers. From this it is apparent that raw pork burgers are best stored at lower temperatures in the absence of salt. It is also clear that cooked burgers containing no salt stored better than cooked burgers with added salt which deteriorated faster with increased salt content. Burgers that were cured (added nitrite) also had greater storage stability in the absence of salt. However, as discussed earlier, these conclusions ignore the fact that burgers prepared for different trials had different total lipid and phospholipid FAME profiles which is obviously important to the development of oxidative rancidity. This is discussed in more detail in Section 6.6.

**FIGURE 6.32**

*Pork Flavour Storage Trial.*

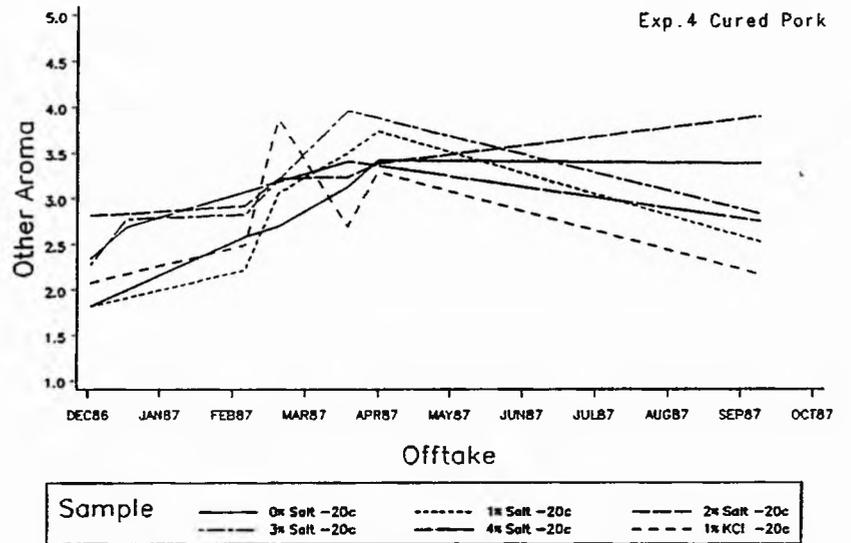
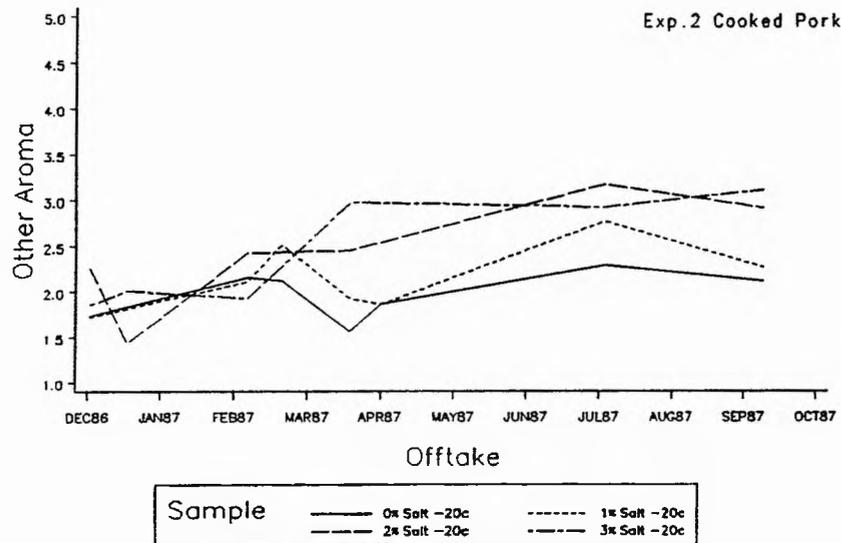
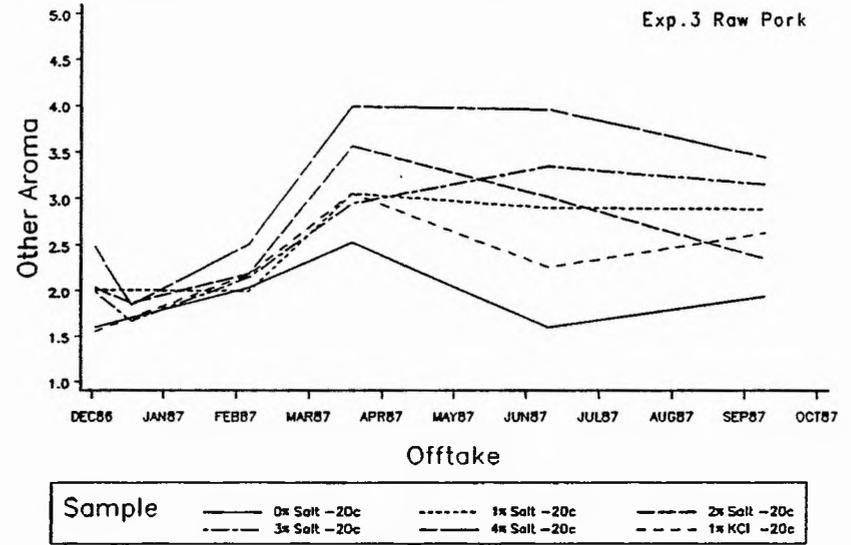
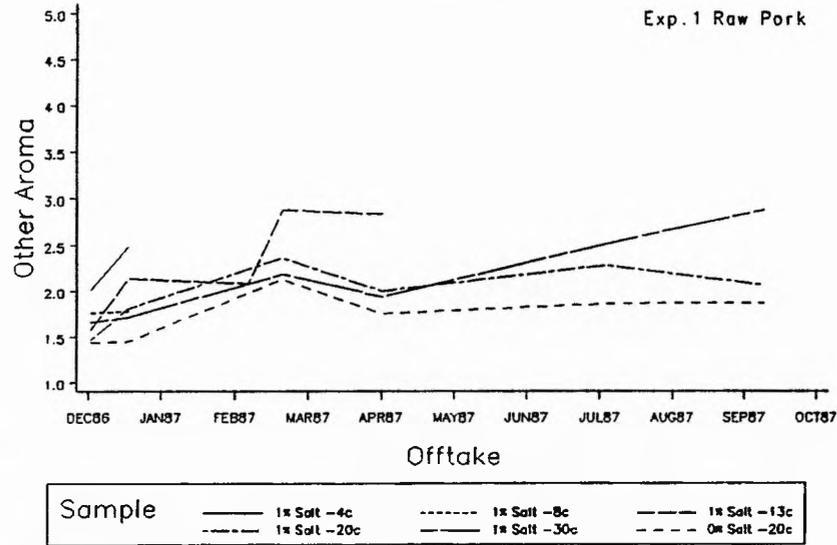
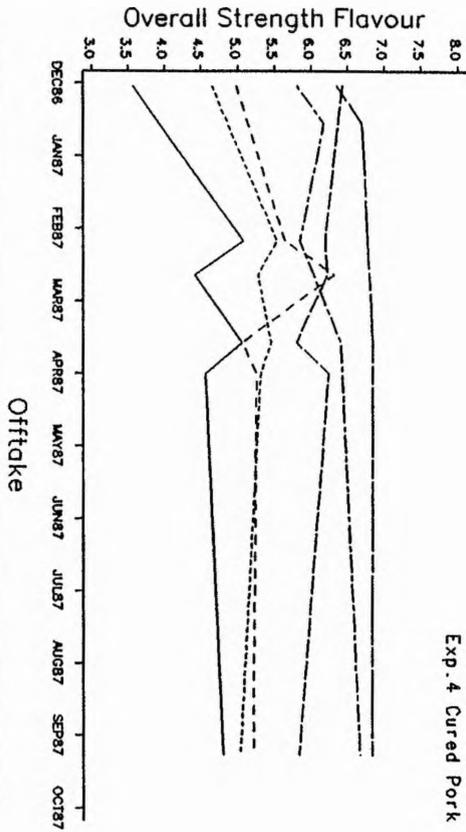
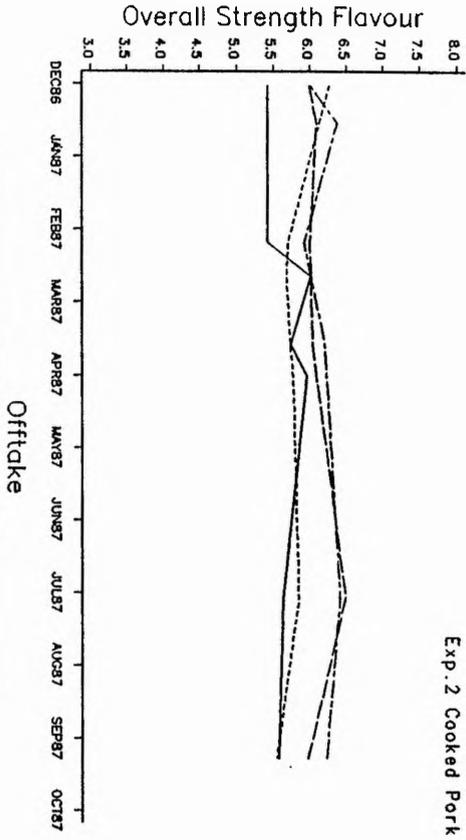
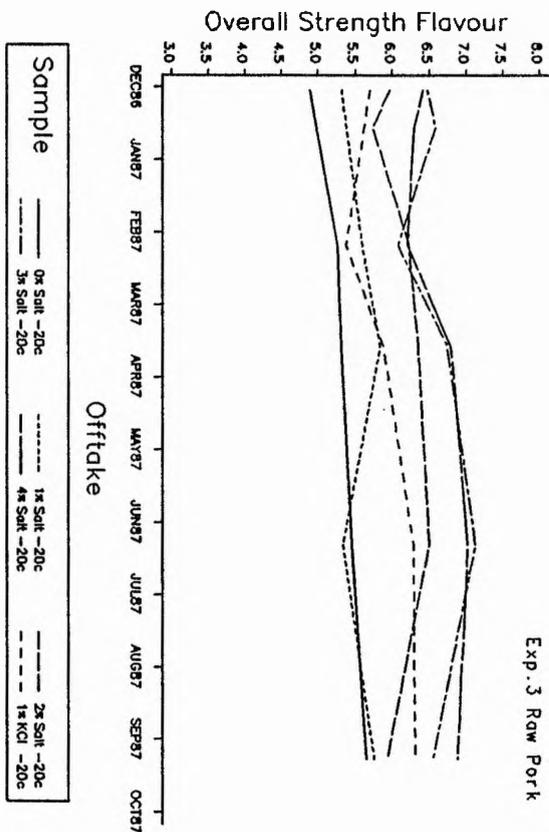
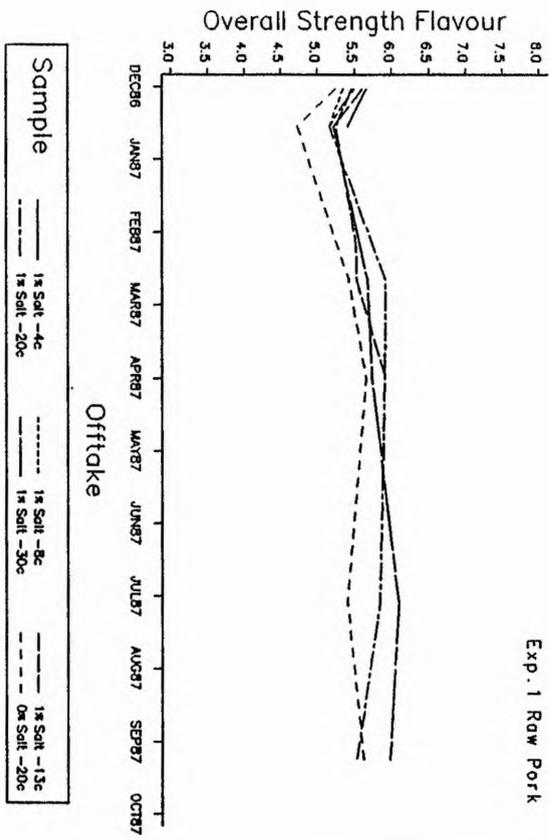


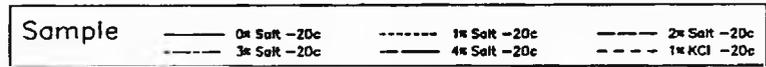
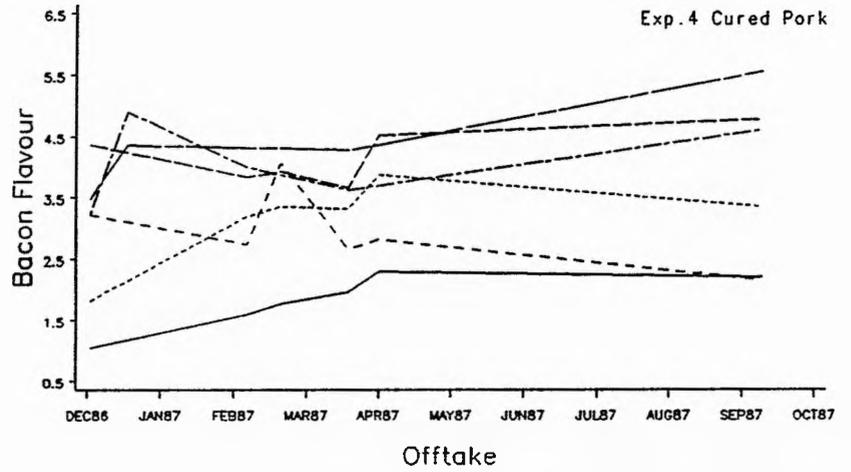
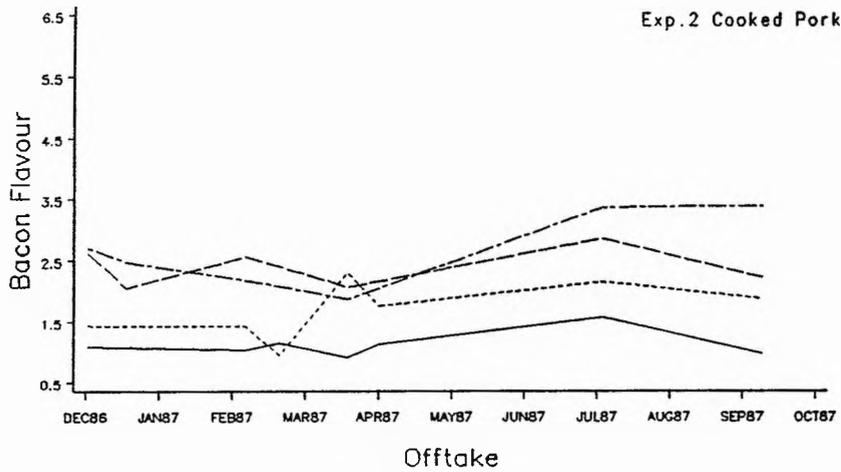
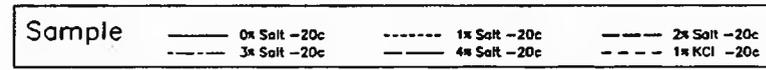
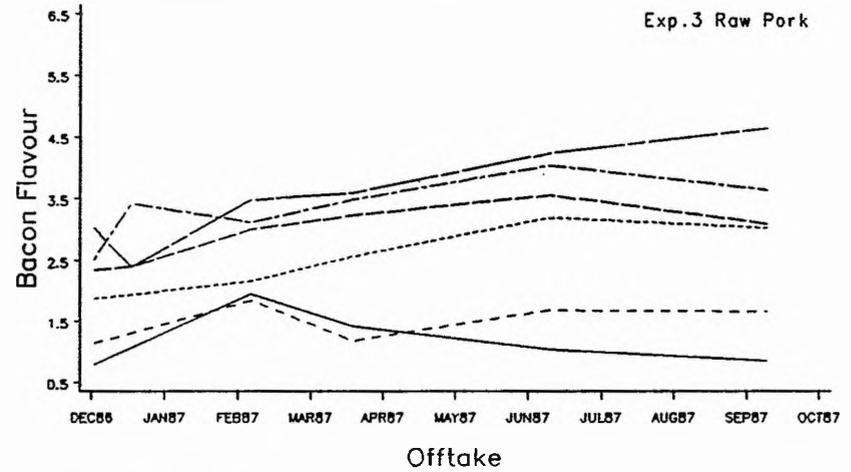
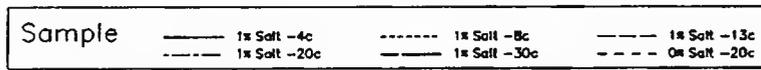
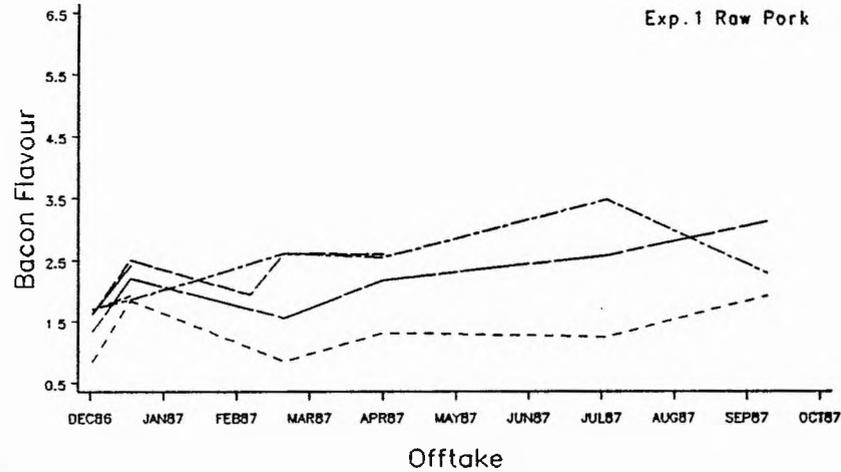
FIGURE 6.33

# Pork Flavour Storage Trial.



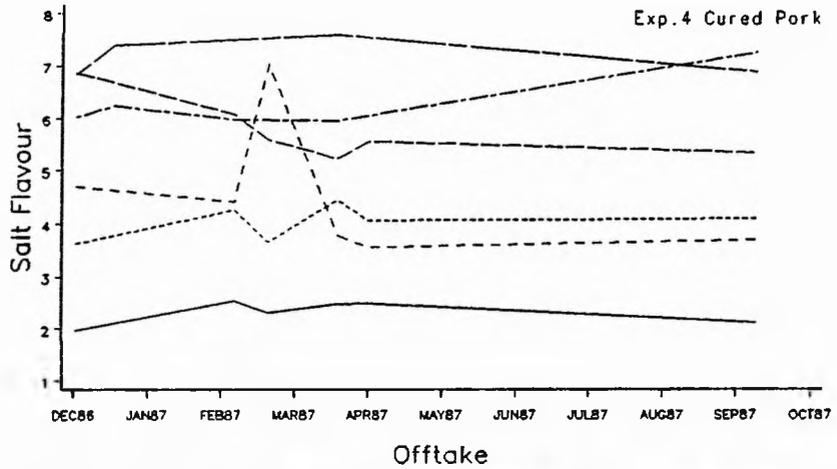
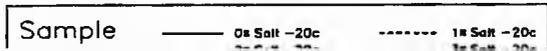
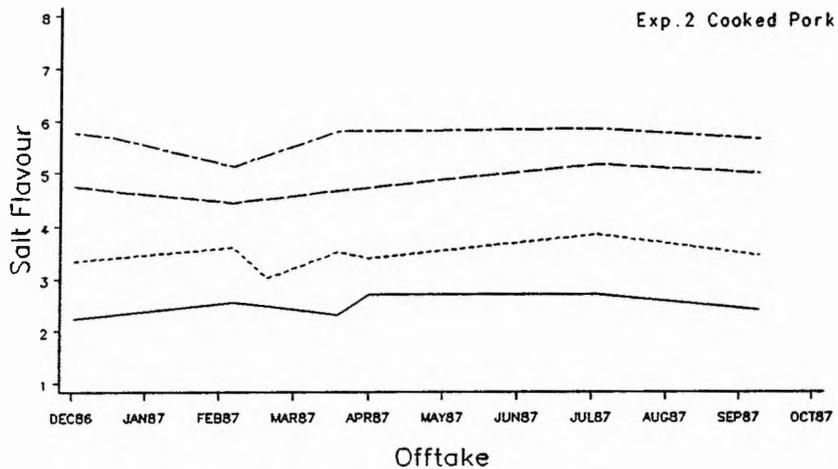
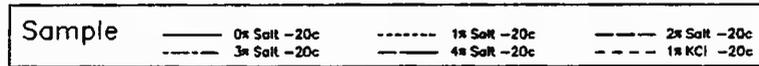
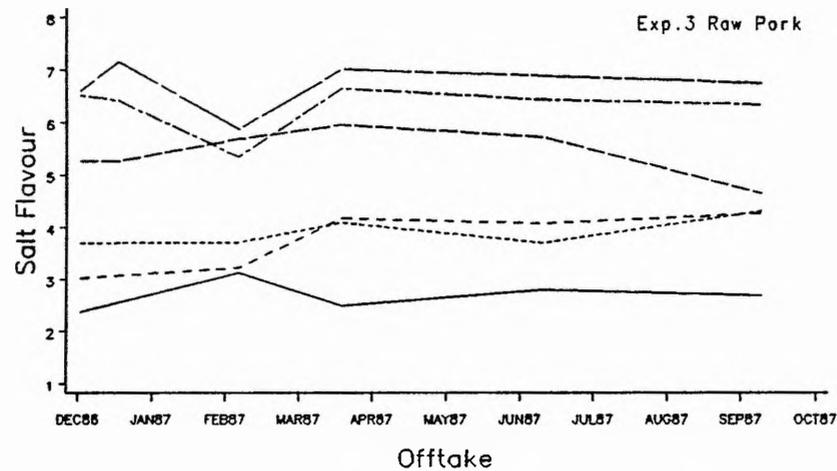
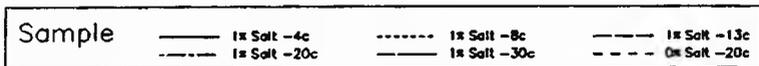
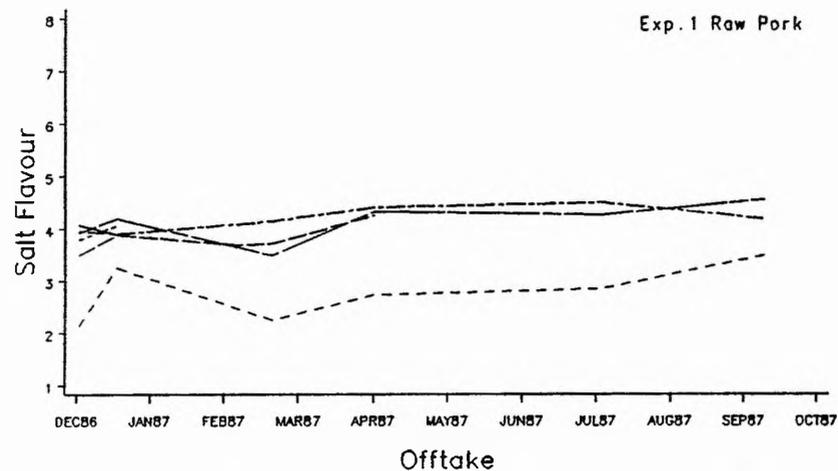
**FIGURE 6.34**

*Pork Flavour Storage Trial.*



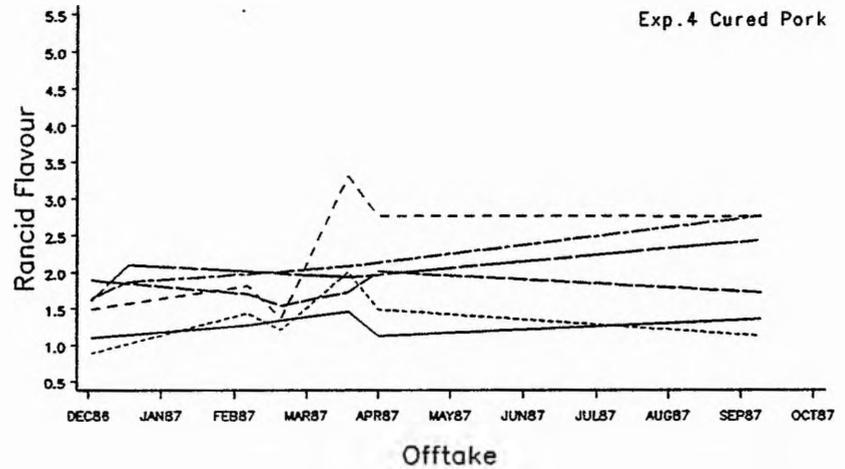
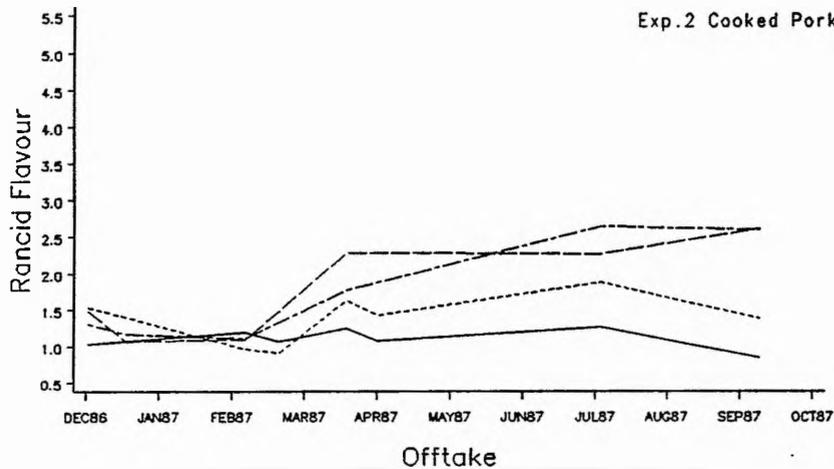
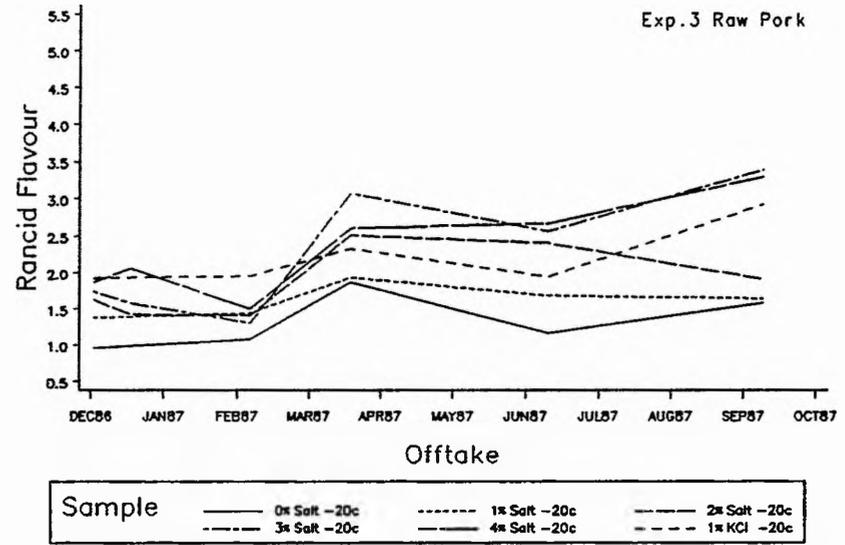
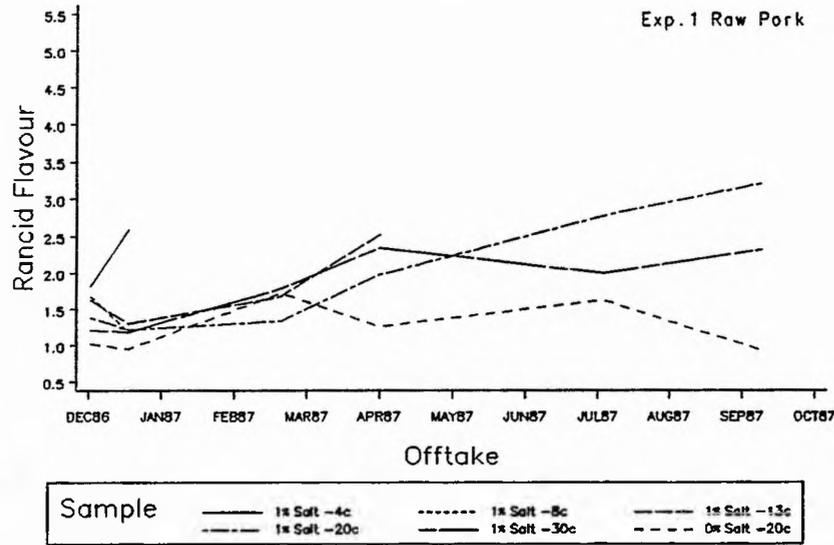
**FIGURE 6.35**

*Pork Flavour Storage Trial.*



**FIGURE 6.36**

*Pork Flavour Storage Trial.*



**FIGURE 6.37**

*Pork Flavour Storage Trial.*

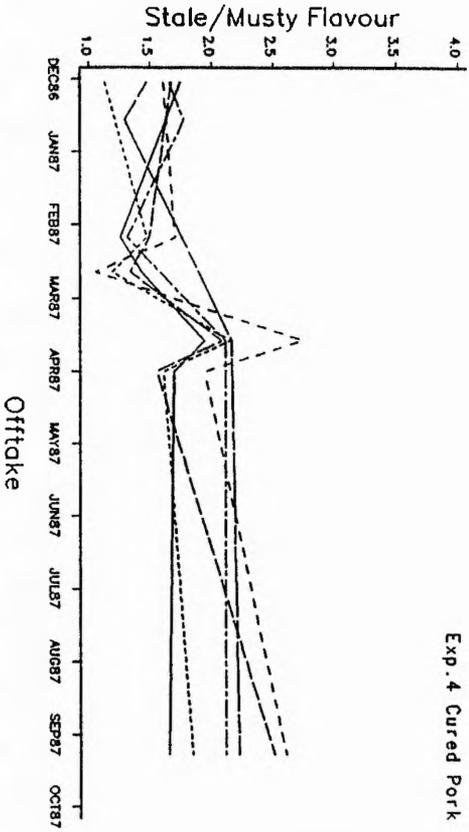
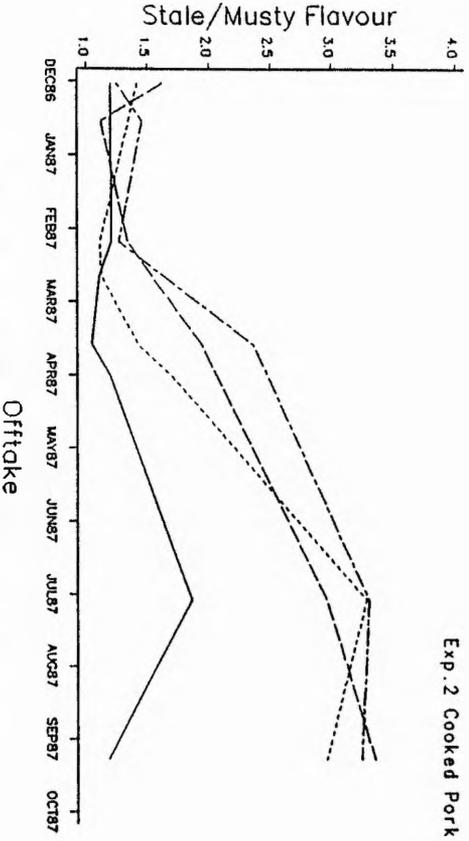
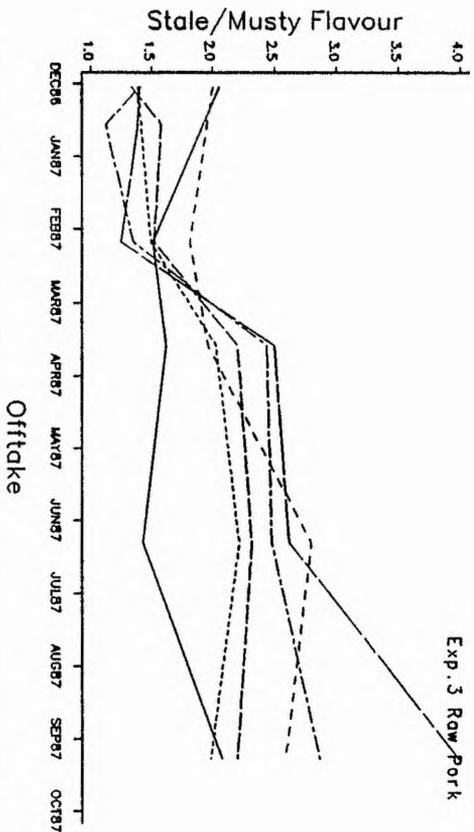
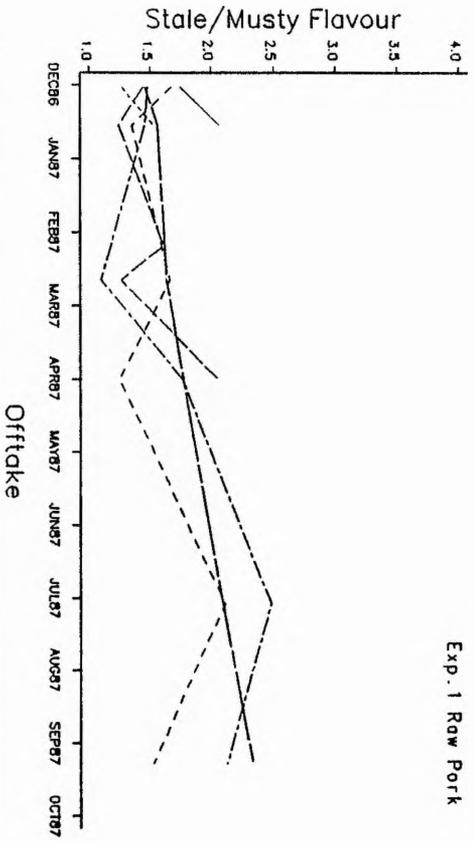


FIGURE 6.39

# Pork Flavour Storage Trial.

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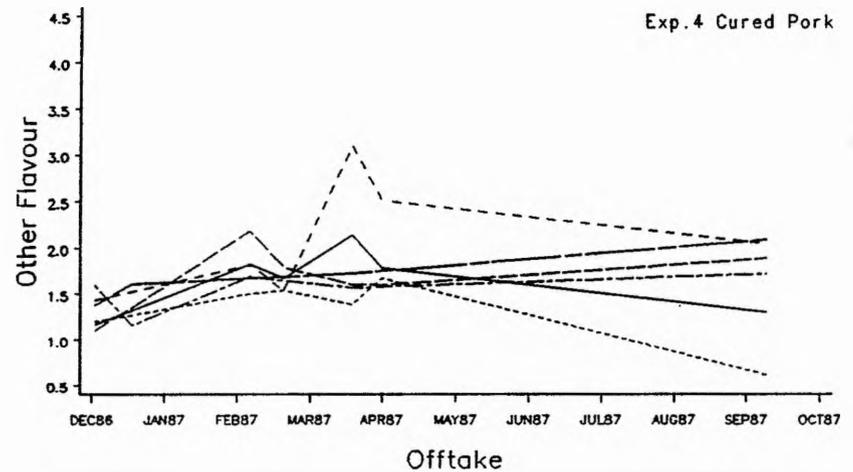
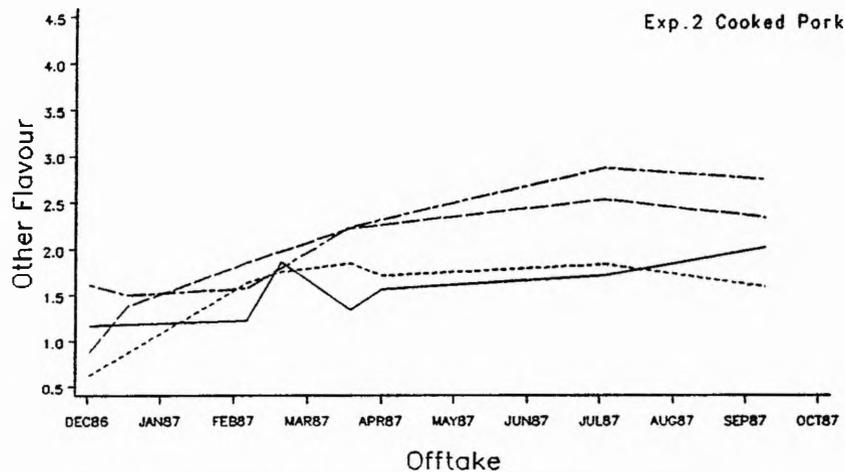
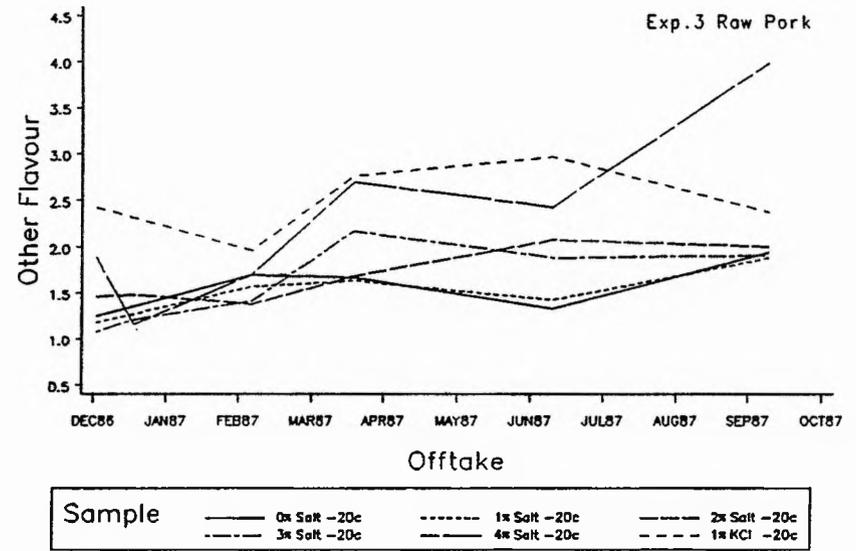
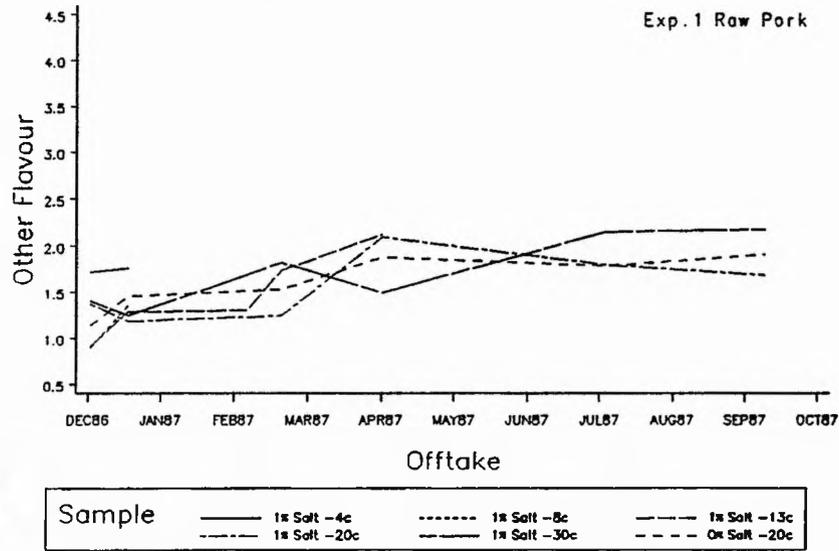
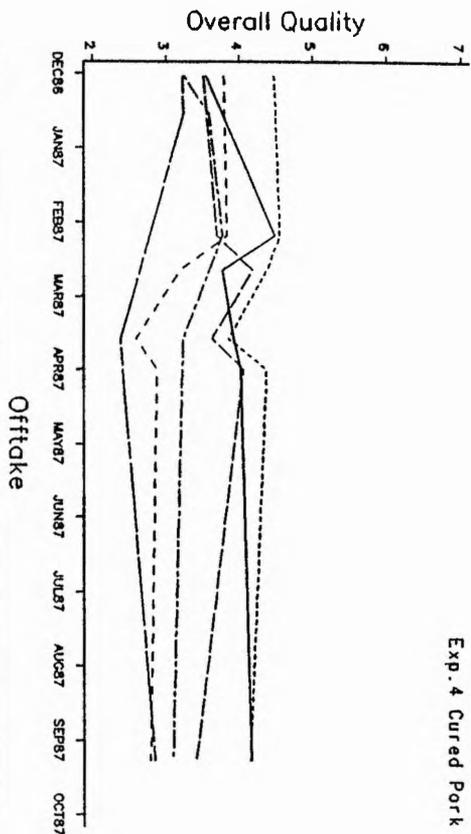
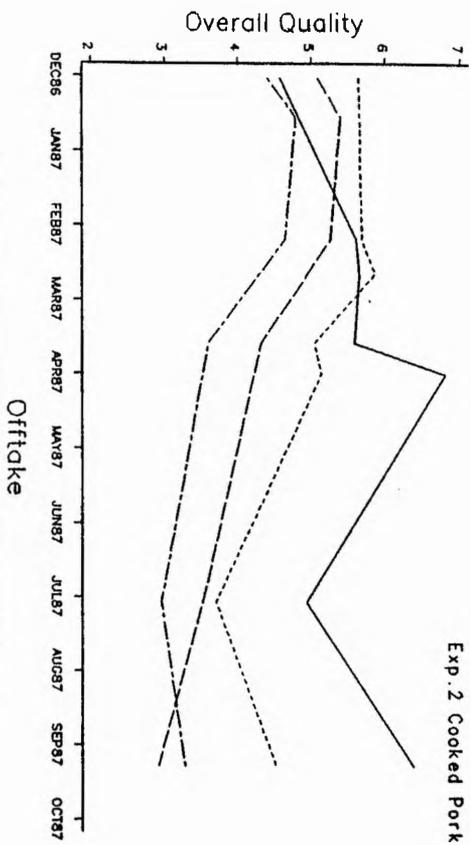
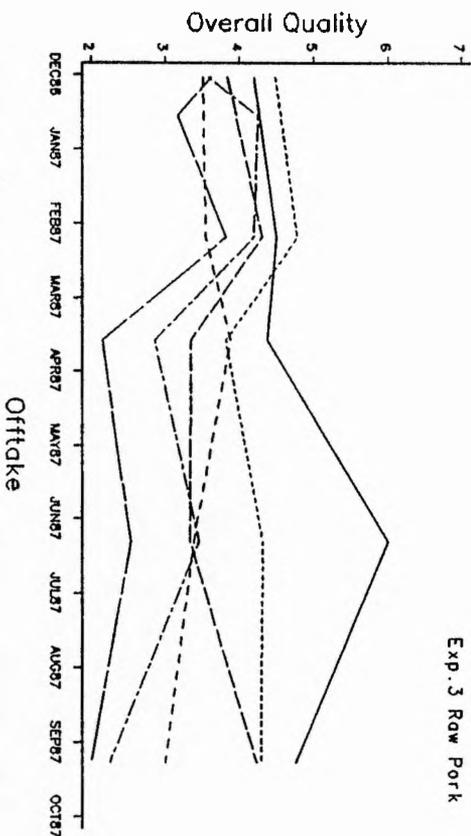
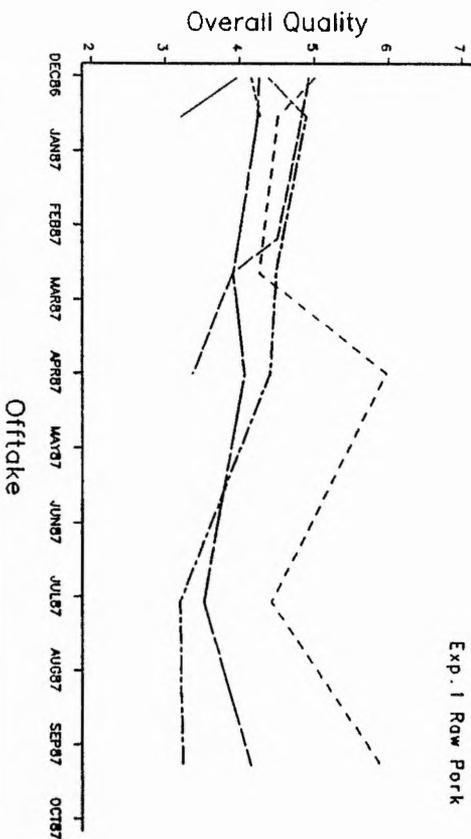


FIGURE 6.39

# Pork Flavour Storage Trial.



## 6.6 COMPARISON OF METHODS FOR FOLLOWING THE OXIDATIVE DETERIORATION OF PORK

An important objective of the four storage trials was to determine comparative changes between the various methods of analysis. These are considered below.

### 6.6.1 The Stability of Uncooked Pork Burgers Containing 1% salt at Different Temperatures

An increase in the oxidation indices (OI) of the total lipid and phospholipid FAME's occurred with increased storage time and temperature. This corresponded with changes in the colour parameters and tasting panel scores.

Throughout storage the OI of both total lipid and phospholipid FAME's provided a measure of the extent of lipid oxidation. However due to the possibility of hydrolysis and protein lipid interactions it would be unsatisfactory to rely solely on this analysis. The increase in oxidation (FAME analysis) was accompanied by an increase in hue. An increase in hue has been partly attributed to the presence of metmyoglobin which can occur at low oxygen pressures and as a result of lipid oxidation. The greater the hue value the higher was the storage temperature which may indicate that lipid oxidation occurred at higher temperatures. This would

be in agreement with the FAME analysis and organoleptic assessment. There were no conclusive changes in chroma and lightness which made these colour parameters unsuitable for following oxidation. When the burgers were first tasted, burgers stored at  $-4^{\circ}\text{C}$  scored highest in Overall Other, Rancid, Stale/Musty and Other flavours and lowest in the Overall Quality flavour score which provides a clear indication of how sensitive organoleptic studies were in detecting oxidative deterioration in meat. Organoleptic studies showed that the deterioration of pork occurred more rapidly at higher storage temperatures and that salt reduced the quality of the meat at  $-20^{\circ}\text{C}$ .

Recommendations from this storage trial are to store pork at lower temperatures and preferentially in the absence of salt. Organoleptic assessment, lipid FAME analysis and changes in hue were acceptable methods for following the deterioration of uncooked pork held at different sub-zero temperatures.

6.6.2 The Stability of Cooked Pork Burgers Containing  
Different Amounts of Salt at -20°C

In contrast to what was found in the investigation into the effect of storage temperature on burger stability, this study revealed greater differences in the OI of the phospholipid FAME's. There was also a general increase in the OI of the total lipid FAME's with increased storage time and salt content. However in sample G it was interesting to note that although there was a general increase in the OI of the total lipid FAME's there was little change in the OI of the phospholipid FAME's.

Initially, all samples had high hue values which increased slightly on increased storage time and salt content. An exception to this were burgers stored without salt (sample G), which had the highest hue at the beginning of the trial and the lowest at the end. This observation would agree with the previous trial in that increasing hue values represented increased oxidation. The fact that the hue of sample G decreased on increased storage may show that sample G was less prone to oxidation which was in agreement with the FAME analysis. At the beginning of storage chroma values were lower in burgers containing increasing levels of salt and decreased with increased storage. Therefore a decrease in

chroma accompanies lipid oxidation in cooked pork. The lightness (L) values generally increased on increased storage time. However, due to the erratic changes that occurred it would be unsatisfactory to use L values as an indicator of oxidation. It is unlikely that the differences in the colour parameters at the beginning of the trial were entirely due to lipid peroxidation. There were only small changes in the colour parameters on increased storage time but more dramatic changes in the FAME analysis and organoleptic scores. This probably means that salt on its own plays an important part in the initially observed colour. Therefore initial colour values could not easily be used as a measure of the extent of lipid peroxidation. At the beginning of storage, the tasting panel found a small increase in Rancid and Stale/Musty flavours with increased salt content. However, not until the end of the trial were more dramatic differences perceived and at which stage a significant loss of the phospholipid PUFA's had occurred. As discussed previously the mechanism of salts action is unclear. The observation that low concentrations of copper II were more catalytic than high concentrations in the rate of peroxidation of phospholipid liposomes may have been an important factor. The higher production of metmyoglobin with increased salt content may have showed that proteins were also catalytic in peroxidation. The decrease in lipid PUFA's as measured by the FAME analysis does not discriminate between the various mechanisms of peroxidation.

Therefore the contribution of enzymic reaction to peroxidation is difficult to establish as is their dependance on salt concentration. In addition to the above factors the ability of salt to extract protein from membranes may also have contributed to the differences in rate. Cooking can also cause dramatic changes to the structure of meat. In addition to the production of Browning Maillard reaction products cooking is believed to denature proteins which can cause an increase in their catalytic activity.

The cooking process itself would have been expected to promote lipid oxidation. When the pork was then frozen higher levels of lipid hydroperoxides may have been present which may have accelerated peroxidation.

In summarising the oxidative deterioration of cooked pork burgers in this storage trial may best be followed by an increase in the oxidation index of both total lipid and phospholipid FAME's. This was accompanied by a slight increase in the hue. No obvious changes occurred in the lightness colour parameter with increases oxidation, therefore, it would be unsuitable for following oxidation. Organoleptic assessment was very sensitive to lipid peroxidation and identified the faster deterioration of pork with higher levels of salt.

Recommendations from this storage trial are to store cooked pork in the absence of salt. This should increase the quality, shelf-life and nutritional value of the product.

6.6.3 The Stability of Uncooked Pork Burgers Containing Different Amounts of Salt at -20°C

The oxidation index of the total lipids changed very little with increases storage time. There was however, a more significant change in the phospholipid FAME's between samples with increased storage time. The phospholipid analysis showed that burgers which contained intermediate levels of salt (2 and 3%) oxidised faster than those stored with 4% salt. This was not detected in the total lipid analysis.

There were considerable differences in the hue of samples at the beginning of the trial. Samples stored with increasing levels of salt had higher hue values. Burgers stored with 2, 3 and 4% salt (samples M, N and O) had very similar hues. All hue values increased with increased storage time. The large difference in hue at the beginning of storage was probably due to the effect of salt content and not lipid peroxidation. However, the increase in hue in all samples with increased storage may have been influenced by lipid oxidation. Burgers stored with 1% potassium chloride (sample

P) in place of sodium chloride (sample L) had a lower hue throughout storage. At the beginning of the trial chroma values were lower in burgers containing high levels of salt. however there was a more dramatic decrease in chroma in burgers with low levels or no added salt as compared with burgers stored with high levels of salt. Since oxidation was greatest in burgers with high levels of salt (FAME analysis) the decrease in chroma should not have resulted from lipid oxidation. the observation that there was relatively no change in the chroma of burgers stored with high levels of salt may show that the underlying trend in chroma is upwards on increased oxidation. It seems likely that the observed chroma values are dependant on more than lipid peroxidation and would therefore not be suitable for following peroxidation. Lightness values were generally higher in burgers with high salt content. Although erratic, they appeared to increase slightly with increased storage. In general, the initial values of the colour parameters do not give a satisfactory indication of the extent of lipid peroxidation.

The analysis of pork burger neutral lipids by HPLC demonstrated that this technique could follow minor changes in meat neutral lipids with increased storage. Unfortunately the exact nature of the absorbing chromophore could not be identified and therefore no quantitative information was

obtained. However, from a qualitative evaluation it appeared that there was a general increase in absorption of a doublet both with increased storage time and salt content. It was interesting to note that burgers stored with 4% salt (sample O) exhibited a lower absorption in this doublet than samples stored with 2 and 3% salt. If this represented a lower level of oxidation then it agreed with the phospholipid FAME analysis. Rancid and Stale/Musty flavour scores were generally higher in burgers stored with increasing concentrations of salt at the beginning of the trial and with increased storage. However, although a lower level of oxidation occurred in samples stored with 4% salt (sample O) than those stored with 2 and 3% salt (FAME analysis) sample O was perceived organoleptically as more rancid. It may be that at high levels, salt itself was contributing to the perception of rancid flavour. The addition of 1% potassium chloride (sample P) in place of 1% sodium chloride (sample L) appeared to enhance both Rancid and Stale/Musty flavours throughout storage. However, this did not agree with the FAME analysis in which sample P was more stable to oxidation than sample L. This clearly shows that FAME analysis on its own cannot be used solely as a measure of the extent of oxidative deterioration of meat. Potassium chloride may have contributed directly to the perceived flavour in addition to the contributions made by oxidising lipid.

As discussed for cooked pork the mechanism of salts catalytic action may be complex. However, in this trial the pork was not cooked and factors such as protein denaturing through cooking and oxidation brought about by heating in air should not apply. It is not known why the 4% salt level caused an apparent reduction in the rate of peroxidation (FAME analysis) relative to burgers contains 2 and 3% salt. One possible explanation is based on the concentration of trace amounts of copper (II). Dilution of copper (II) by addition up to 3% salt may have diluted copper (II) to such a level when it was more catalytic but on further dilution (4% salt) its catalytic activity was rapidly reduced. However, this explanation is questionable since the copper (II) concentration was not determined and the mobility of metal ions in meat is not known. Another possible explanation is that at a salt content of 4% excessive protein was removed from membranes which lowered the susceptibility of phospholipids to protein induced peroxidation.

Recommendations from this storage trial are to store uncooked pork in the absence of salt. Replacement of 1% sodium chloride with 1% potassium chloride was detrimental to the perceived quality of the meat and is therefore undesirable.

6.6.4 The Stability of Uncooked Pork Burgers Containing Sodium Nitrite and Different Amounts of Salt at -20°C

Changes in the OI of both total lipids and phospholipid FAME's were erratic. However, it did appear that burgers stored with high levels of salt (samples T and U) were less susceptible to peroxidation than burgers stored without salt. The formation of nitrosylmyoglobin in cured pork indicated by a lower hue was more pronounced at higher salt concentrations. This may have masked underlying changes brought about by lipid oxidation. There was not obvious trend in the chroma and lightness values with increased salt content. Therefore colour change in cured pork may not be a satisfactory method for following oxidation. Results from the organoleptic studies showed that pork produced off-flavours at high levels of salt which correlated with a decrease in the Overall Quality flavour score. However this did not agree with the FAME analysis. Reaction of nitrite with food constituents other than oxidising lipids may have been important to the perceived flavour of the meat. The possibility that nitrosylmyoglobin formation is favoured at high salt levels may suggest that this compound or subsequent reactions of it are important to the perceived flavour. Burgers stored with potassium chloride in place of sodium

chloride exhibited a greater stability to peroxidation as indicated by the lower OI in the phospholipid FAME analysis. However, the tasting results again showed that potassium chloride caused an increase in Rancid and Stale/Musty flavours as compared to sodium chloride. It therefore appears that both FAME and colour analysis were of little value in predicting the deterioration of meat as detected by organoleptic assessment.

Recommendations from this trial are to store cured pork in the absence of salt.

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## APPENDICES

## APPENDIX 2.1

Sample 1

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	12.22		14.90		
18:0	9.97		12.58		
18:1	10.04	2.21	12.79	2.09	2.15
18:2	20.70	1.07	26.24	1.02	1.04
18:3	1.03	21.53	1.28	20.84	21.18
20:4	9.44	2.35	12.16	2.19	2.27
20:5	2.75	8.06	3.57	7.47	7.76
22:5	2.14	10.36	2.79	9.56	9.96
22:6	2.14	10.36	2.82	9.46	9.91

Sample 2

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	13.91		13.62		
18:0	12.29		12.20		
18:1	11.69	2.24	11.60	2.23	2.23
18:2	27.03	0.97	26.76	0.96	0.96
18:3	1.57	16.69	1.53	16.88	16.78
20:4	13.91	1.88	14.22	1.82	1.85
20:5	4.36	6.01	4.41	5.86	5.94
22:5	3.17	8.26	3.12	8.28	8.27
22:6	3.41	7.68	3.56	7.25	7.46

Sample 3

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	12.45		12.41		
18:0	12.54		12.35		
18:1	12.02	2.08	11.98	2.07	2.08
18:2	27.37	0.91	27.50	0.90	0.90
18:3	1.49	16.77	1.48	16.73	16.75
20:4	14.50	1.72	14.66	1.69	1.70
20:5	4.57	5.47	4.57	5.42	5.44
22:5	3.44	7.26	3.44	7.20	7.23
22:6	3.64	6.86	3.65	6.78	6.82

Sample 4

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	14.31		14.38		
18:0	10.54		10.75		
18:1	11.63	2.14	11.86	2.12	2.13
18:2	25.72	0.97	26.15	0.96	0.96
18:3	1.57	15.83	1.47	17.10	16.46
20:4	13.99	1.78	13.59	1.85	1.82
20:5	4.79	5.18	4.65	5.40	5.29
22:5	3.18	7.81	3.22	7.80	7.80
22:6	3.62	6.86	3.68	6.83	6.80

Sample 5

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	14.31		14.38		
18:0	10.54		10.75		
18:1	11.63	2.14	11.86	2.12	2.13
18:2	25.72	0.97	26.15	0.96	0.96
18:3	1.57	15.83	1.47	17.10	16.46
20:4	13.99	1.78	13.59	1.85	1.82
20:5	4.79	5.18	4.65	5.40	5.29
22:5	3.18	7.81	3.22	7.80	7.80
22:6	3.82	6.86	3.68	6.83	6.84

## APPENDIX 2.2

Sample 1

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	29.42		29.48		
18:0	10.57		10.64		
18:1	20.01	2.00	20.03	2.01	2.00
18:2	20.11	1.99	20.14	1.99	1.99
20:4	1.74	22.98	1.63	24.61	23.80
20:5	0.99	40.39	1.00	40.12	40.26
22:5	0.80	49.99	0.95	42.23	46.11
22:6	0.41	97.54	0.29	138.34	117.94

Sample 2

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	29.05		29.89		
18:0	10.68		10.13		
18:1	20.21	1.97	20.18	1.98	1.98
18:2	20.00	1.99	20.16	1.98	1.98
20:4	1.60	24.83	1.75	22.87	23.85
20:5	1.04	38.20	1.09	36.72	37.46
22:5	0.73	54.42	0.75	53.36	53.89
22:6	0.45	88.23	0.43	93.07	90.65

Sample 3

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	29.90		30.46		
18:0	10.21		10.43		
18:1	20.15	1.99	20.24	2.02	2.00
18:2	20.23	1.98	20.31	1.44	1.71
20:4	1.55	25.88	1.63	25.02	25.45
20:5	1.09	36.80	0.91	44.82	40.81
22:5	0.82	48.91	0.63	64.75	56.83
22:6	0.26	154.27	0.52	78.44	116.36

Sample 4

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	31.01		31.04		
18:0	10.48		10.56		
18:1	20.87	1.99	20.83	2.00	2.00
18:2	20.94	1.98	20.95	1.99	1.98
20:4	1.80	23.05	1.81	22.98	23.02
20:5	1.07	38.78	1.01	41.19	39.02
22:5	0.73	56.84	0.75	55.47	56.16
22:6	0.65	63.83	0.62	67.10	65.46

Sample 5

<u>FAME</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>x%</u>	<u>16:0+18:0/x</u>	<u>av</u>
16:0	30.24		31.00		
18:0	10.11		10.13		
18:1	20.03	2.01	20.11	2.05	2.03
18:2	20.49	1.97	20.56	2.00	1.98
20:4	1.74	23.19	1.73	23.77	23.48
20:5	0.83	48.61	0.89	46.21	47.41
22:5	0.89	45.34	0.74	55.58	50.46
22:6	0.51	79.12	0.48	85.67	82.40

## APPENDIX 2.3

FAME	% composition					
	time (hrs)					
	0			2.5		
16:0	19.88	19.91	19.82	19.47	20.38	20.48
17:0	3.98	3.98	4.25	4.18	4.32	4.33
18:0	2.85	2.86	3.08	2.99	3.06	3.06
18:1	14.04	14.05	14.27	14.32	14.70	14.67
18:2	29.72	29.59	29.71	29.87	30.49	30.42
20:4	6.61	6.61	6.78	6.82	6.55	6.56
20:5	5.41	5.17	5.21	5.27	5.20	5.25
22:5	2.18	2.16	2.15	2.18	2.32	2.36
22:6	2.62	2.60	2.68	2.70	2.67	2.72

FAME	5.5		9.5		22	
	16:0	21.37	21.39	17.83	16.80	21.65
17:0	5.34	5.35	3.46	3.76	5.03	5.00
18:0	4.84	4.82	3.24	3.24	4.25	4.24
18:1	16.72	16.71	12.25	12.24	16.13	16.16
18:2	27.45	27.49	21.94	21.92	27.15	27.31
20:4	6.06	6.07	4.12	4.31	4.92	4.98
20:5	3.98	3.99	3.09	3.12	3.26	3.28
22:5	2.22	2.22	4.62	4.62	2.31	2.37
22:6	2.50	2.51	0.84	0.88	1.69	1.74

<b>FAME</b>	<b>31</b>		<b>46</b>		<b>71</b>	
16:0	22.80	22.45	22.48	22.69	25.76	24.55
17:0	5.84	5.68	6.31	6.12	6.12	6.79
18:0	5.34	5.27	6.27	6.26	6.17	6.35
18:1	17.68	17.61	18.20	18.21	19.44	19.41
18:2	26.38	26.42	21.86	21.82	24.30	23.82
20:4	4.40	4.49	3.04	3.04	2.77	2.82
20:5	2.71	2.79	1.63	1.63	1.38	1.41
22:5	2.09	2.19	1.76	1.76	3.94	3.98
22:6	1.08	1.14	0.81	0.81	0.35	0.35

## APPENDIX 2.4

16:0 + 18:0 as Internal Standard

16:0 + 18:0/x%						
time (hrs)						
FAME(x)	0	2.5	5.5	9.5	22	31
18:1	1.60	1.60	1.57	1.68	1.60	1.58
18:2	0.76	0.77	0.95	0.96	0.94	1.06
20:4	3.39	3.59	4.32	4.48	5.21	6.28
20:5	4.38	4.48	6.58	6.62	7.90	10.16
22:5	10.48	9.97	11.81		11.04	13.06
22:6	8.58	8.65	10.46		15.07	25.36
FAME(x)	46	71				
18:1	1.60	1.62				
18:2	1.33	1.30				
20:4	9.55	11.24				
20:5	17.81	22.52				
22:5	16.54	7.93				
22:6	35.84	89.76				

## APPENDIX 2.5

17:0 as Internal Standard

17:0/x%						
time (hrs)						
FAME (x)	0	2.5	5.5	9.5	22	31
16:0	0.21	0.21	0.25	0.20	0.23	0.26
18:0	1.40	1.42	1.10	1.12	1.18	1.08
18:1	0.39	0.30	0.32	0.30	0.31	0.32
18:2	0.14	0.14	0.20	0.16	0.18	0.22
20:4	0.61	0.66	0.88	0.86	0.01	1.36
20:5	0.79	0.82	1.34	1.16	1.53	2.08
22:5	1.90	1.85	2.40		2.14	2.69
22:6	1.55	1.60	1.14		2.94	4.18
FAME (x)	46	71				
16:0	0.28	0.26				
18:0	1.02	1.02				
18:1	0.34	0.34				
18:2	0.28	0.26				
20:4	2.04	2.32				
20:5	2.82	4.63				
22:5	3.54	1.63				
22:6	7.68	18.47				

## APPENDIX 2.6

18:0 as Internal Standard

		<u>18:0/x%</u>							
		<u>time (hrs)</u>							
<u>FAME(x)</u>	<u>0</u>	<u>2.5</u>	<u>5.5</u>	<u>9.5</u>	<u>22</u>	<u>37</u>	<u>46</u>	<u>71</u>	
18:1	0.21	0.29	0.29	0.26	0.26	0.30	0.34	0.32	
18:2	0.10	0.14	0.18	0.15	0.16	0.20	0.29	0.26	
20:4	0.44	0.66	0.80	0.77	0.86	1.19	2.06	2.24	
20:5	0.56	0.83	1.21	1.04	1.30	1.93	3.84	4.49	
22:5	1.36	1.85	2.18	0.70	1.81	2.48	3.57	1.58	
22:6	1.11	1.60	1.93	3.77	2.48	4.74	7.73	17.89	

## APPENDIX 2.7

% Composition (corrected)

time (hrs)								
FAME	2.5		5.5		9.5		22	
16:0	19.10	19.19	18.14	18.16	20.02	18.87	17.49	17.40
18:0	2.87	2.87	4.11	4.09	3.64	3.64	3.43	3.43
18:1	13.77	13.75	14.20	14.19	13.76	13.74	13.03	13.06
18:2	28.57	28.50	23.30	23.33	24.64	24.62	21.94	22.07
20:4	6.14	6.15	5.14	5.15	4.63	4.84	3.98	4.02
20:5	4.87	4.92	3.38	3.39	3.47	3.50	2.63	2.65
22:5	2.17	2.21	1.88	1.88	5.19	5.19	1.87	1.91
22:6	2.50	2.55	2.12	2.13	0.99	0.99	1.36	1.41

FAME	31		46		71	
16:0	17.42	17.15	14.54	14.68	16.67	15.88
18:0	4.08	4.03	4.06	4.05	3.99	4.11
18:1	13.51	13.45	11.78	11.78	12.58	12.56
18:2	20.15	20.18	14.14	14.12	15.72	15.41
20:4	3.36	3.43	1.97	1.97	1.79	1.82
20:5	2.07	2.13	1.06	1.06	0.89	0.91
22:5	1.60	1.67	1.13	1.14	2.55	2.58
22:6	0.82	0.87	0.52	0.52	0.23	0.23

## APPENDIX 2.8

$$\ln \left\{ \frac{a}{a-x} \right\}$$

time (hrs)							
FAME	2.5	5.5	9.5	22	31	46	71
18:1	0.00	0.02*	-0.05	0.00	0.01	0.00	-0.01*
18:2	-0.01	-0.22*	-0.23	-0.21-	-0.33	-0.56	-0.54*
20:4	-0.06	-0.24*	-0.36	-0.43	-0.68	-1.04	-1.20*
20:5	-0.02	-0.41*	-0.41	-0.59	-0.84	-1.40	-1.64*
22:5	-0.05	-0.12		-0.05	-0.22	-0.46	0.28
22:6	-0.01	-0.20		-0.56	-1.08	-1.43	2.53

## APPENDIX 2.9

$$\ln \left\{ \frac{a}{a-x} \right\}$$

time (hrs)							
FAME	2.5	5.5	9.5	22	31	46	71
16:0	0.00	-0.17	0.05	-0.09	-0.24	-0.29	-0.21
18:0	-0.01	0.24	0.22	0.17	0.26	0.32	0.32
18:1	-0.03	-0.10*	-0.03	-0.07	-0.10	-0.16	-0.16*
18:2	0.00	-0.36*	-0.13	-0.12	-0.45	-0.69	-0.62*
20:4	-0.08	-0.37*	-0.34	-0.50	-0.80	-1.21	-1.34*
20:5	-0.04	-0.53*	-0.39	-0.66	-0.97	-1.27	-1.77*
22:5	-0.03	-0.23		-0.12	-0.35	-0.62	0.15
22:6	-0.03	-0.31		-0.64	-0.99	-1.40	-2.48

## APPENDIX 2.10

$$\ln \left\{ \frac{a}{a-x} \right\}$$

	time (hrs)						
acid	2.5	5.5	9.5	22	31	46	71
18:1	-0.32	-0.32*	-0.21	-0.21	-0.36	-0.48	-0.42*
18:2	-0.34	-0.59*	-0.40	-0.47	-0.69	-1.07	-0.95*
20:4	-0.40	-0.60*	-0.56	-0.67	-1.00	-1.54	-1.63*
20:5	-0.39	-0.77*	-0.62	-0.84	-1.24	-1.92	-2.08*
22:5	-0.31	-0.47	-0.66	-0.28	-0.60	-0.96	0.15
22:6	-0.36	-0.55	-1.22	-0.80	-1.43	-1.94	2.78

\* Not used in the determination of the gradient (Appendix 2.11)

## APPENDIX 2.11

OI - 16:0 + 18:0

<u>FAME</u>	<u>a</u>	<u>bx10<sup>-3</sup></u>	<u>c</u>
18:1	-0.02	0.00	0.44
18:2	-0.025	11.00	0.94
20:4	-0.051	21.00	0.98
20:5	-0.01	29.00	0.98

OI - 17:0

<u>FAME</u>	<u>a</u>	<u>bx10<sup>-3</sup></u>	<u>c</u>
18:1	-0.01	3.00	0.98
18:2	-0.07	16.00	0.95
20:4	-0.03	25.00	0.99
20:5	-0.02	30.00	0.99

OI - 18:0

<u>FAME</u>	<u>a</u>	<u>bx10<sup>-3</sup></u>	<u>c</u>
18:1	-0.24	3.00	0.71
18:2	-0.23	16.00	0.96
20:4	-0.27	25.00	0.97
20:5	-0.24	34.00	0.98

a = intercept on y axis

b = gradient

c = correlation coefficient

## APPENDIX 4.1

SI liposomes/water

16:0 + 18:0/x

FAME (X)	time (hrs)				
	0	2	6	21.5	72
18:1	2.05	2.08	2.16	2.18	2.16
18:2	0.74	0.94	0.96	0.94	1.62
20:4	2.38	3.28	3.76	3.92	87.43
20:5	13.41	15.06	17.71	19.31	
22:5	11.32	14.20	17.00	18.76	
22:6	13.48	18.58	21.30	24.98	
	(26.98)	(31.88)	(33.57)	(33.51)	(69.16)

S2 liposomes FeSO<sub>4</sub>/ascorbic acid

16:0 + 18:0/x

FAME (X)	time (hrs)				
	0	2	6	21.5	72
18:1	2.05	1.95	2.12	2.26	2.82
18:2	0.74	0.70	0.89	1.19	15.85
20:4	2.38	2.26	3.13	5.68	
20:5	13.41	8.77	9.90	32.80	
22:5	11.32	10.17	15.57	26.50	
22:6	13.48	12.45	19.24	44.92	
	(26.98)	(31.88)	(33.57)	(37.90)	(45.70)

## APPENDIX 4.2

SI liposomes/water

16:0 + 18:0/x

FAME (X)	time (hrs)				
	0	1.5	7	21.5	74.5
18:1	2.76	2.62	2.53	2.52	2.43
18:2	0.82	0.84	0.90	0.94	1.02
20:4	6.92	7.16	7.88	8.92	12.08
20:5	13.36	14.94	18.04	22.38	34.17
22:5	17.42	24.36	25.28	28.00	39.26
22:6	18.10	31.78	36.36	40.91	61.18
	(30.20)	(33.30)	(34.26)	(35.14)	(37.50)

liposomes NaNO<sub>2</sub>

16:0 + 18:0/x

FAME (X)	time (hrs)				
	0	3	8	24	74
18:1	2.76	2.40	2.44	2.46	2.38
18:2	0.82	0.84	0.92	0.90	0.93
20:4	6.92	7.68	7.75	8.02	9.74
20:5	13.36	15.80	19.71	18.87	27.08
22:5	17.42	21.14	23.94	23.16	30.93
22:6	18.10	25.14	34.98	29.23	47.15
	(30.20)	(33.19)	(34.58)	(34.06)	(35.34)

Determination of Relative Rate in Control SI

<u>FAME</u>	<u>A</u>	<u>B</u>	<u>relative rate</u>	<u>C</u>
18:2	-0.0586	2.26 x 10 <sup>-3</sup>	1	-0.929
20:4	-0.0678	6.733 x 10 <sup>-3</sup>	3.02	-0.987
20:5	-0.195	-0.010	4.24	-0.971

A = intercept

B = slope

C = correlation coefficient

## APPENDIX 4.3

SI liposomes/water

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	3	8	22.5	30.5	47.5
18:1	2.52	2.53	2.52	2.34	2.26	2.48
18:2	0.82	0.81	0.89	1.80	3.08	9.42
20:4	6.63	7.22	9.95	70.63	119.47	
20:5	28.70	25.06	32.38	63.03		
22:5	26.40	28.02	30.28			
22:6	43.40	47.04	79.84			
	(32.72)	(32.22)	(33.32)	(44.14)	(50.15)	(59.86)

S2 liposomes/Cu(II)/water

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)						
	0	2.5	5	8	22	30	46.25
18:1	2.52	2.50	2.48	2.42	2.78	2.84	3.20
18:2	0.82	0.98	1.08	1.37	5.40	8.94	14.56
20:4	6.63	10.93	16.34	36.26	356.18		
20:5	28.70	54.98	67.98				
22:5	26.40	27.49	53.01				
22:6	43.40	98.08	218.06				
	(32.72)	(36.29)	(38.16)	(41.88)	(66.60)	(62.80)	(60.57)

## S3 liposomes/Cu(II) sodium nitrite

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	3	8	22.5	30.5	47.5
18:1	2.52	2.44	2.38	2.64	2.75	2.88
18:2	0.82	0.92	1.27	4.27	7.45	9.29
20:4	6.63	10.48	28.24	572.2		
20:5	28.70	43.83				
22:5	26.40	31.72				
22:6	43.40	108.4				
	(32.72)	(35.16)	(38.99)	(57.52)	(60.02)	(63.99)

## S4 liposomes/Cu(II)/ascorbic acid

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)						
	0	2.5	5	8	22	30	46.25
18:1	2.52	2.42	2.52	2.64	2.72	3.07	3.24
18:2	0.82	0.98	1.02	1.32	6.02	4.11	15.74
20:4	6.63	10.38	13.99	29.34	1019.1		
20:5	28.70	46.80	68.42				
22:5	26.40	36.75	39.61				
22:6	43.40	81.89	163.61				
	(32.72)	(36.77)	(37.63)	(41.94)	(61.14)	(67.00)	(66.55)

## APPENDIX 4.4

S1 liposomes/water

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)						
	0	3	8	22	29	46	71
18:1	2.40	2.40	2.76	2.58	2.44	2.45	2.37
18:2	0.88	0.73	0.82	0.82	0.88	0.84	0.89
20:4	6.71	5.80	6.54	6.31	7.17	6.74	8.04
20:5	27.36	22.36	23.20	25.05	30.52	26.27	38.14
22:5	23.15	22.28	26.16	24.39	24.48	23.26	28.64
22:6	37.44	33.55	36.60	39.12	41.17	37.66	53.99
	(33.74)	(30.63)	(32.20)	(32.26)	(33.54)	(32.58)	(34.39)

S2 liposomes/NaCl

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	3	8	22	29	46.5
18:1	2.40	2.58	2.56	2.92	3.22	3.17
18:2	0.88	0.94	1.60	8.64	12.51	17.44
20:4	6.71	11.22	38.59	130.15		
20:5	23.15					
22:5	27.36					
22:6	37.44					
	(33.74)	(33.64)	(44.57)	(62.38)	(67.13)	(67.92)

## S3 liposomes Cu(II)

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)						
	0	3	8	22	29	46	71
18:1	2.40	2.71	2.95	2.69	2.63	2.48	2.70
18:2	0.88	0.83	0.92	0.99	0.86	0.87	1.05
20:4	6.71	6.68	7.25	8.13	6.69	6.87	11.12
20:5	27.36	24.12	27.40	36.94	25.05	28.48	51.61
22:5	23.36	27.25	28.54	27.59	26.17	24.81	47.12
22:6	37.44	43.08	45.88	49.10	43.38	41.62	103.12
	(33.74)	(31.26)	(34.39)	(35.84)	(32.97)	(33.48)	(37.80)

## S4 liposomes/Cu(II)/NaCl

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	3	8	22	29	46.5
18:1	2.40	2.54	2.64	2.90	3.09	3.29
18:2	0.88	0.90	1.70	8.84	12.94	13.50
20:4	6.71	11.15	44.82	783.36		
20:5	27.36	42.4				
22:5	23.36	35.87				
22:6	37.44					
	(33.74)	(34.37)	(45.51)	(62.72)	(66.20)	(66.56)

Determination of Relative Rate in Control SI

<u>FAME</u>	<u>A</u>	<u>B</u>	<u>relative rate</u>	<u>C</u>
18:2	0.336	-0.056	1.00	1
20:4	0.33	-0.110	1.96	1.96
20:5	0.280	-0.047	0.84	0.84

A = intercept

B = slope

C = correlation coefficient

## APPENDIX 4.5

SI liposomes/water

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	4	21.75	30	71	144
18:1	1.99	1.98	1.97	2.08	2.01	1.98
18:2	0.86	0.89	0.91	0.89	0.92	0.92
20:4	1.64	1.67	1.73	1.75	1.80	1.86
20:5	5.70	6.12	6.55	5.94	6.82	7.00
22:5	7.76	7.21	4.42	4.70	4.04	8.16
22:6	5.30	6.74	9.83	10.23	9.99	8.31
	(22.45)	(22.48)	(23.52)	(24.80)	(24.89)	(25.63)

S2 liposomes/Cu(II)/water

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	1.25	3.25	5.25	22.5	25
18:1	1.99	1.98	2.01	2.03	2.20	2.38
18:2	0.86	0.90	1.02	1.14	2.83	3.32
20:4	1.64	2.04	2.66	3.98	74.08	110.52
20:5	5.70	7.38	10.46	14.56		
22:5	7.76	8.04	12.26	18.26		
22:6	5.30	11.14	14.43	25.78		
	(22.45)	(27.42)	(29.66)	(31.99)	(36.82)	(45.24)

## S3 liposomes/Phosphate

OI = 16:0 + 18:0/x

FAME (X)	time (hrs)					
	0	4	21.75	30	51.5	144
18:1	1.99	2.00	2.02	2.01	2.01	1.98
18:2	0.86	0.83	0.88	0.90	0.87	0.84
20:4	1.64	1.60	1.68	1.74	1.69	1.66
20:5	5.70	5.02	5.82	6.26	5.92	5.55
22:5	7.76	7.30	6.39	6.92	5.50	4.72
22:6	5.30	6.72	8.05	7.22	8.39	7.92
	(22.45)	(21.35)	(22.17)	(22.39)	(22.91)	(23.01)

S4 liposomes/Cu(II)/phosphate

16:0 + 18:0/x

	0	1.25	3.25	5.5	9.25	22.5	25	53
18:1	1.99	1.94	2.02	2.02	2.00	2.04	2.06	2.10
18:2	0.86	0.94	1.04	1.16	1.15	1.34	1.36	1.48
20:4	1.64	2.13	2.82	4.06	4.05	6.84	6.78	8.24
20:5	5.70	7.90	11.39	17.32	16.48	26.56	28.74	34.58
22:5	7.76	9.86	13.12	17.93	14.95	21.46	11.44	16.49
22:6	5.30	10.36	15.35	26.39	29.90	53.10	75.62	100.24
	(22.45)	(22.86)	(23.99)	(24.03)	(25.11)	(26.92)	(29.11)	(31.41)

## S2 liposomes/Cu(II)

$$\ln \left\{ \frac{a}{a-x} \right\}$$

FAME (X)	time (hrs)				
	1.25	3.25	5.25	22.5	25
20:4	-0.218	-0.483	-0.886	-3.81	-4.21

## S4 liposomes/Cu(II)/Phosphate

$$\ln \left\{ \frac{a}{a-x} \right\}$$

FAME (X)	time (hrs)						
	1.25	3.25	5.25	9.25	22.5	25	53
20:4	-0.261	-0.542	-0.906	-0.904	-1.430	-1.43	-1.61

## APPENDIX 4.6

S1 liposomes/hand shaken

OI = 16:0 + 18:0/x

	FAME (X)		time (hrs)				
	0	3	7	22.5	30.06	49.5	75.5
18:1	1.54	1.55	1.66	1.66	1.60	1.68	1.66
18:2	1.07	1.08	1.08	1.13	1.11	1.00	1.24
20:4	6.33	6.67	6.64	7.64	6.63	6.23	8.57
20:5	21.89	23.88	22.75	30.60	23.10		26.10
22:5	22.27	22.68	28.16	34.29	26.33		33.77
22:6	33.99	34.94	50.39	75.42	43.52		60.41
	(32.29)	(32.34)	(29.34)	(30.57)	(29.92)	(27.40)	(32.30)

S2 liposomes/sonicated

OI = 16:0 + 18:0/x

	FAME (X)		time (hrs)				
	0	3	7	22.5	30.17	49.5	75.5
18:1	1.54	1.57	1.60	1.67	1.64	1.75	1.76
18:2	1.07	1.10	1.08	1.06	1.32	1.66	1.72
20:4	6.33	6.26	6.92	6.30	10.87	22.80	79.10
20:5	21.89	21.05	23.02	20.60			
22:5	22.27	24.48	19.64	23.84			
22:6	33.99	38.48	48.93	51.88			
	(32.29)	(32.31)	(30.39)	(28.54)	(33.64)	(36.02)	(42.84)

## S3 liposomes/sonicated/diluted x 2

$$OI = 16:0 + 18:0/x$$

	FAME (X)		time (hrs)				
	0	4	8	23.5	30.75	50	78.5
18:1	1.54	1.52	1.61	1.63	1.67	1.70	1.77
18:2	1.07	1.06	0.95	1.24	1.30	1.73	3.02
20:4	6.33	6.06	5.93	10.46	12.34	25.78	
20:5	21.89	20.68	18.64	42.60			
22:5	22.27	23.69	17.47	43.73			
22:6	33.99	36.94	56.09	110.28			
	(32.29)	(31.72)	(26.64)	(32.06)	(32.66)	(37.85)	(42.60)

## S4 liposomes/sonicated/diluted x 4

$$OI = 16:0 + 18:0/x$$

	FAME (X)		time (hrs)				
	0	4	8	23.5	30.75	50	78.5
18:1	1.54	1.56	1.58	1.66	1.70	1.68	1.77
18:2	1.07	1.07	0.95	1.20	1.40	1.82	2.97
20:4	6.33	6.26	5.61	9.70	14.34	59.35	
20:5	21.89	21.23	17.74	35.76			
22:5	22.27	20.04		43.21			
22:6	33.99	47.90		91.50			
	(32.29)	(32.10)	(25.97)	(31.11)	(33.90)	(36.61)	(44.62)

## APPENDIX 4.7

Cu(II) Temperature = 42°C

$$OI = (16:0 + 18:0)/x$$

FAME (X)	time (hrs)					
	0	2.5	5	9	23	30
18:1	1.68	1.65	1.62	1.64	2.23	1.75
18:2	0.94	1.14	1.32	2.11	6.09	7.66
20:4	5.75	9.52	12.71	40.20		
20:5	17.14	38.57	47.04			
22:5	19.54	36.28	44.82			
22:6	58.38	80.10	129.46			
	(27.73)	(30.84)	(32.90)	(39.39)	(48.74)	(40.39)

Cu(II) Temperature = 4°C

$$OI = (16:0 + 18:0)/x$$

FAME (X)	time (hrs)					
	0	5	9	23	30	46
18:1	1.68	1.59	1.66	1.54	1.56	1.78
18:2	0.94	1.20	1.24	2.46	4.72	9.64
20:4	5.75	8.26	11.04	85.60		
20:5	17.14	32.16	47.28			
22:5	19.54	35.59	51.35			
22:6	58.38	66.72	124.10			
	(27.73)	(30.02)	(29.78)	(30.19)	(41.84)	(50.79)

Cu(II) Temperature = 4°C

$$OI = (16:0 + 18:0)/x$$

	FAME (X)						
	0	4	8	23.5	30.75	50	78.5
18:1	1.68	1.64	1.58	1.61	1.59	1.54	1.62
18:2	0.94	1.08	1.07	1.08	1.48	1.31	2.44
20:4	5.75	6.42	7.14	7.78	20.57	20.22	
20:5	17.14	21.34	27.34	19.34			
22:5	19.54	28.42	34.14	44.46			
22:6	58.38	46.92	62.02	96.44			
	(27.73)	(28.86)	(28.84)	(28.45)	(35.68)	(29.78)	(38.32)

Cu(II) Temperature = -13°C

(16:0 + 18:0)/x

FAME(x)	0	1.06	2.06	4	6	8	10	13	18
18:1	1.68	1.54	1.62	1.56	1.59	1.56	1.63	1.56	1.70
18:2	0.94	1.04	1.01	1.26	1.23	1.30	1.32	1.26	1.29
20:4	5.75	5.26	5.90	7.90	8.18	8.17	8.37	7.90	9.10
20:5	17.14	16.32	17.93	33.96	33.02	34.43	34.92	33.96	36.30
22:5	19.54	28.54	26.85	30.87	32.31	31.34	34.57	30.87	39.06
22:6	58.38	60.36	45.18	53.05	56.34	57.18	63.37	53.06	73.05
	(27.73)	(27.51)	(29.25)	(33.96)	(32.18)	(34.30)	(34.10)	(33.96)	

Cu(II) Temperature = -22°C

(16:0 + 18:0)/x

	0	1.06	4	6	8	10	13	18
18:1	1.68	1.70	1.60	1.60	1.59	1.68	1.78	1.70
18:2	0.94	0.98	1.18	1.12	1.24	1.20	1.06	1.22
20:4	5.75	5.13	6.96	6.60	7.69	7.70	6.84	7.40
20:5	17.14	12.72	27.47	23.60	33.12	28.50	22.84	27.81
22:5	19.54	27.54	27.66	26.04	30.69	33.74	34.56	32.84
22:6	58.38	40.64	44.93	44.82	54.68	62.72	61.34	59.00
	(27.73)	(26.14)	(32.35)	(31.38)	(33.61)	(32.48)	(26.36)	(31.84)

APPENDIX 4.8

S1 Temperature = 4°C

FAME	time (hours)							
	0	1	4	14	22	33	53	83
18:1	2.12	1.96	1.98	1.88	1.90	2.30	1.96	1.94
18:2	0.95	0.96	1.04	0.87	0.89	1.08	0.95	1.01
20:4	3.08	3.04	3.41	2.69	2.76	3.60	3.38	3.68
20:5	21.08	22.70	31.30	17.79	17.31	21.48	21.97	28.10
22:5	21.30	15.86	20.02	16.58	15.14	21.17	19.96	19.70
22:6	31.50	28.96	35.82	27.37	25.93	32.61	33.86	53.37
	(30.91)	(30.85)	(32.24)	(28.18)	(28.91)	(32.46)	(33.13)	(32.02)

S2 Temperature = 4°C Cu(II)

	time (hours)									
FAVE	0	2.5	19.75	26	44	49.5	69.75	92.5	117	
18:1	2.12	1.94	1.97	2.06	1.98	1.85	1.98	2.04	2.04	2.04
18:2	0.95	0.89	1.17	0.98	1.20	1.10	1.32	1.86	1.86	1.01
20:4	3.08	2.92	3.90	4.34	7.21	7.62	11.77	33.74	33.74	172.36
20:5	21.08	18.58	35.86	29.05	83.85	65.22	156.78			
22:5	21.30	18.25	27.15	18.45	57.08	24.18				
22:6	31.50	29.35	50.90	39.91	166.21	305.16				
	(30.91)	(29.63)	(32.82)	(32.13)	(37.39)	(32.42)	(40.10)	(46.38)	(46.08)	

S3 Temperature = 4°C Cu(II)/NaCl

	time (hours)									
FAVE	0	3.5	21	27	45.5	51	70.55	93.75	120	
18:1	2.12	1.87	1.96	2.09	1.94	1.92	2.16	1.96	1.93	
18:2	0.95	0.90	0.94	1.00	1.02	1.06	1.29	1.14	1.40	
20:4	3.08	2.82	3.23	3.86	4.20	4.54	8.67	9.16	16.09	
20:5	21.08	19.73	23.65	25.16	33.26	37.64	80.77			
22:5	21.30	14.50	20.38	16.70	24.15	20.49				
22:6	31.50	31.26	33.13	25.94	64.76	507.70				
	(30.91)	(29.48)	(30.97)	(32.48)	(32.96)	(33.13)	(35.54)	(38.61)	(37.38)	

S4 Temperature = 4°C Cu(II)/NaCl/Ascorbic Acid

time (hours)

FAME	0	2.5	19.75	26	44	49.5	69.75	92.5	117
18:1	2.12	1.87	1.94	1.96	1.97	2.00	2.02	2.08	2.04
18:2	0.95	0.89	0.96	0.92	1.03	1.02	1.39	1.30	1.62
20:4	3.08	2.85	3.38	3.58	4.76	5.32	9.02	11.24	21.70
20:5	21.08	20.67	22.07	22.36	39.56	36.69	11.54		
22:5	21.30	17.52	19.26	24.29	22.90	30.90	65.84		
22:6	31.50	28.01	46.56	38.28	64.62	113.84	227.22		
	(30.91)	(20.75)	(31.48)	(31.66)	(33.62)	(33.48)	(39.80)	(37.55)	(37.65)

S5 Temperature = 4°C Cu(II)/NaCl/NaNO<sub>2</sub><sup>-</sup>

time (hours)

FAME	0	3.5	22	47.5	94.5	5	11	13	21	53	102
18:1	2.12	1.82	1.92	2.20	1.95	2.04	1.82	2.00	2.22	1.84	1.86
18:2	0.95	0.86	0.86	1.01	0.91	0.84	0.83	0.80	0.88	0.80	0.79
20:4	3.08	2.86	2.72	2.95	3.26	2.87	2.60	2.38	2.84	2.84	3.03
20:5	21.08	17.88	16.39	21.70	23.90	26.80	17.07	12.45	15.54	17.71	20.26
22:5	21.30	14.59	14.48	17.98	20.45	28.78	16.52	14.62	16.86	16.88	16.18
22:6	31.50	30.57	24.29	35.96	32.94	77.75	28.02	19.30	28.46	27.26	45.50
	(30.91)	(29.59)	(29.90)	(30.71)	(27.60)	(29.70)	(27.74)	(26.42)	(28.90)	(27.94)	(27.71)

S6 Temperature = 4°C Cu(II)/NaCl/Ascorbic Acid/ $\text{NaNO}_2^-$

time (hours)

	0	3.5	21	37	45.5	5	11	22	53	53	83
18:1	2.12	1.94	1.94	2.00	2.04	1.85	1.87	2.02	1.88	1.87	1.84
18:2	0.95	0.92	0.93	0.96	0.97	0.98	0.88	0.95	0.89	0.98	0.98
20:4	3.08	2.98	2.99	3.00	3.29	3.10	3.04	3.04	3.14	3.58	3.72
20:5	21.08	20.90	20.94	21.32	22.21	27.48	25.32	19.06	20.92	28.18	29.56
22:5	21.30	15.74	17.60	14.74	16.02	18.78	23.30	17.34	17.76	19.21	22.16
22:6	31.50	27.19	34.40	25.40	26.66	31.76	46.10	26.65	36.29	38.63	46.72
	(30.91)	(30.41)	(30.78)	(30.70)	(31.41)	(29.40)	(29.46)	(30.08)	(29.40)	(31.00)	(31.02)

## APPENDIX 4.9

S1 Temperature = -8°C (Control)

 $(16:0+18:0)/x$ 

time (days)

FAVE(x0	0	56	61	85	103	133	196	454
18:1	2.12	1.93	1.94	1.92	1.89	1.90	1.59	1.92
18:2	0.95	0.88	0.94	0.90	0.92	0.91	1.06	1.20
20:4	3.08	2.94	3.16	3.10	3.21	3.30	3.42	4.72
20:5	21.08	17.45	19.78	20.74	22.34	19.95	24.42	36.55
22:5	21.30	17.62	18.21	16.34	13.00	22.07	18.52	25.64
22:6	35.10	29.87	33.30	35.06	37.84	39.05	27.36	39.64
	(30.91)	(26.88)	(29.78)	(27.94)	(28.90)	(29.78)	(30.02)	(34.36)

## S2 Temperature -8°C Cu(II)

(16:0+18:0)/x

time (days)

FAME(x)	0	21	56	68	169
18:1	2.12	2.02	2.08	2.20	1.93
18:2	0.95	1.10	1.09	1.16	1.24
20:4	3.08	3.25	3.44	3.66	3.97
20:5	21.08	23.05	20.42	22.05	28.70
22:5	21.30	16.96	19.90	22.70	21.64
22:6	35.10	35.28	32.74	35.13	34.44
	(30.91)	(30.46)	(32.71)	(33.02)	(33.11)

## S3 Temperature -8°C Cu(II)/NaCl

(16:0+18:0)/x

time (days)

FAME	0	21	56	68	169
18:1	2.12	1.88	2.12	2.12	2.25
18:2	0.95	0.92	1.44	1.81	2.45
20:4	3.08	3.77	9.70	18.47	47.53
20:5	21.08	29.29	75.26	144.40	
22:5	21.30	25.42	63.06		
22:6	35.10	44.30	76.44		
	(30.91)	(31.49)	(38.21)	(31.86)	(45.18)

## S4 Temperature -8°C Cu(II)/NaCl/Ascorbic Acid

(16:0+18:0)/x

<u>FAME (x)</u>	<u>0</u>	<u>7</u>	<u>21</u>	<u>56</u>
18:1	2.12	1.92	2.05	2.20
18:2	0.95	1.10	1.48	1.98
20:4	3.08	5.89	11.29	24.43
20:5	21.08	62.35		
22:5	21.30	38.71		
22:6	35.10	79.73		
	(30.91)	(34.01)	(38.91)	(42.10)

---

S5 Temperature = -8°C Cu(II)/NaCl/NaNO<sub>2</sub><sup>-</sup>

(16:0+18:0) / x

FAME(x)	0	32	61	85	103	133	196	454
18:1	2.12	2.02	1.87	1.82	1.86	1.86	1.77	1.91
18:2	0.95	0.82	0.91	0.88	0.89	0.89	1.08	1.28
20:4	3.08	2.54	2.93	2.95	3.04	3.30	4.01	6.48
20:5	21.08	13.58	20.08	20.70	21.04	23.13	31.61	57.19
22:5	21.30	14.72	16.40	16.67	14.54	23.72	20.66	35.67
22:6	35.10	22.37	29.30	29.38	35.00	47.06	38.69	67.98
	(30.91)	(27.02)	(29.42)	(29.08)	(28.56)	(29.40)	(25.92)	(36.03)

S6 Temperature =

<u>FAME(x)</u>	<u>0</u>	<u>21</u>	<u>32</u>
18:1	2.12	1.96	1.98
18:2	0.95	1.00	1.02
20:4	3.08	3.10	3.24
20:5	21.08	21.13	22.42
22:5	21.30	17.14	18.46
22:6	35.10	28.06	29.06
	(30.91)	(30.85)	(31.27)

---

-8°C Cu(II)/NaCl/NaNO<sub>2</sub><sup>-</sup>/Ascorbic Acid

(16:0+18:0)/x

time (days)

61	85	103	133	196	454
1.84	1.82	1.86	1.87	1.59	1.93
1.00	0.91	1.00	1.00	1.14	1.81
3.59	3.13	3.74	3.74	5.32	21.65
27.90	22.62	32.84	34.02	50.12	
20.20	17.89	19.94	22.00	27.28	
41.60	33.88	57.90	65.92	61.72	
(30.87)	(29.97)	(31.50)	(31.98)	(29.74)	(39.63)

## APPENDIX 4.10

## S1 Temperature - 20°C Control

(16:0+18:0)/x

time (days)

FAME (x)	0	85	164	217
18:1	2.12	1.90	1.66	1.88
18:2	0.95	0.88	0.98	1.10
20:4	3.08	2.71	2.84	3.42
20:5	21.08	15.72	19.19	23.74
22:5	21.30	15.12	16.05	16.70
22:6	35.10	24.01	23.13	29.28
	(30.91)	(28.47)	(28.56)	(25.55)

## S2 Temperature -20°C

(16:0+18:0)/x

FAME (X)	time (days)					
	0	6	19	34	102	217
18:1	2.12	1.87	2.13	2.14	2.10	1.20
18:2	0.95	0.84	1.00	1.09	1.18	1.26
20:4	3.08	3.22	3.12	3.42	3.67	3.86
20:5	21.08	22.14	18.12	21.52	24.68	24.35
22:5	21.30	17.94	16.34	32.31	21.44	19.38
22:6	35.10	31.40	31.08	35.19	35.72	35.17
	(30.91)	(28.90)	(30.60)	(32.16)	(33.04)	(18.99)

APPENDIX 4.10

S3 Temperature -20°C Cu(II)/NaCl

(16:0+18:0)/x

time (days)

FAME (x)	0	6	19	35	57	67	81	102	217
18:1	2.12	1.97	1.96	2.36	2.14	2.42	2.16	2.29	2.37
18:2	0.95	1.02	1.06	1.62	1.64	1.97	1.76	2.26	5.34
20:4	3.08	3.32	3.37	5.73	6.07	6.71	5.55	8.02	19.04
20:5	21.08	29.78	21.26	38.30	37.92	38.18	32.21	55.52	303
22:5	21.30	21.30	19.82	38.93	32.91	34.02	26.32	32.71	
22:6	35.10	32.06	30.69	42.21	51.93	57.66	40.70	102.77	
	(30.91)	(30.85)	(32.70)	(30.96)	(37.72)	(35.68)	(40.15)	(30.08)	(41.58)

S4 Temperature -20°C CuII/NaCl/Ascorbic acid

(16:0+18:0)/x

time (days)

FAME	0	7	19	35	67	81	102	164
18:1	2.12	1.89	2.00	2.34	2.00	1.96	2.31	2.40
18:2	0.95	0.91	1.15	1.22	1.12	1.07	2.38	6.98
20:4	3.08	3.38	3.94	4.05	3.74	3.59	7.82	31.88
20:5	21.08	27.81	25.62	24.38	24.16	23.02	54.06	
22:5	21.30	21.84	21.78	24.90	22.35	22.62	30.67	
22:6	35.10	35.10	36.50	36.71	30.90	36.94	96.11	67.98
	(30.91)	(29.48)	(32.98)	(34.57)	(32.85)	(31.22)	(43.25)	(51.33)

## APPENDIX 4.11

S1 NaCl (30g/100 ml)

(16:0 + 18:0)/x

FAME (X)	time (days)					
	0	2	5	8	12	15
18:1	2.18	2.10	2.08	2.04	1.98	2.07
18:2	0.92	0.95	1.08	1.06	1.33	3.46
20:4	1.78	1.96	2.78	3.42	7.50	119.58
20:5	5.96	7.16	11.84	14.74	38.27	
22:5	8.19	8.92	13.38	17.45	51.34	
22:6	7.63	8.70	14.81	20.60	87.20	
	(25.08)	(26.18)	(29.36)	(28.02)	(32.84)	

## S2 NaCl (15g/100ml)

(16:0+18:0) / x

FAME(x)	time (days)									
	0	2	5	8	16	20	26	49		
18:1	2.18	2.04	2.05	1.96	1.88	1.99	2.00	2.14		
18:2	0.92	0.94	1.02	0.95	1.11	1.17	1.54	4.60		
20:4	1.78	1.94	2.32	2.37	3.72	4.76	10.88	154.89		
20:5	5.96	7.88	10.23	8.77	15.40	18.98	53.45			
22:5	8.19	8.48	10.45	11.18	15.91	21.47				
22:6	7.63	8.38	10.78	11.46	25.94	31.04				
	(25.08)	(26.32)	(27.47)	(27.08)						

## S3 NaCl (7.5g/100ml)

(16:0+18:0)/x

	time (days)									
FAME (x)	0	2	5	8	16	20	26	49		
18:1	2.18	2.10	1.98	1.97	2.00	2.01	2.02	2.15		
18:2	0.92	0.98	0.89	1.00	1.08	1.10	1.28	3.99		
20:4	1.78	2.12	2.09	2.38	2.81	4.37	60.31	154.89		
20:5	5.96	7.98	7.17	9.20	11.37	13.12	18.04			
22:5	8.19	9.64	10.07	10.77	12.95	10.86	20.18			
22:6	7.63	9.69	9.70	11.16	14.60	21.70	23.06			
	(25.08)	(27.00)	(23.76)	(28.00)	(28.41)		(35.51)	(50.02)		

S4 NaCl (3.75g/100ml)

(16:0+18:0)/x

time (days)

FAME (x)	0	2	5	8	16	20	26	49
18:1	2.18	2.02	2.00	2.04	2.08	2.07	2.05	2.12
18:2	0.92	0.96	0.86	0.96	1.10	1.30	1.25	2.14
20:4	1.78	2.06	1.92	2.19	2.80	3.32	3.52	12.77
20:5	5.96	7.73	6.12	7.84	10.74	12.54	14.38	41.28
22:5	8.19	9.40	9.73	10.26	8.80	12.78	14.58	34.50
22:6	7.63	9.52	9.30	10.28	16.24	15.54	19.88	103.72
	(25.08)	(26.97)	(22.93)	(25.70)	(28.93)	(30.64)	(31.86)	(42.08)

S5 Temperature -20°C Cu(II)/NaCl/NO<sub>2</sub>

(16:0+18:0)/x

FAME (X)	time (days)					
	0	19	82	125	164	217
18:1	2.12	1.97	1.98	1.89	1.80	1.96
18:2	0.95	1.00	1.08	1.08	1.38	1.52
20:4	3.08	3.15	3.63	3.74	4.72	5.64
20:5	21.08	20.24	23.40	27.18	34.40	41.77
22:5	21.30	16.64	22.62	21.72	25.30	27.69
22:6	35.10	28.88	37.26	50.98	41.29	(50.66)
	(30.91)	(30.45)	(32.27)	(33.10)	(33.65)	(23.81)

S6 Temperature -20°C Cu(II)/NaCl/NO<sub>2</sub><sup>-</sup>/Ascorbic Acid

(16:0+18:0)/x

FAME (X)	time (days)					
	0	19	82	125	164	217
18:1	2.12	1.79	1.88	1.85	1.70	1.86
18:2	0.95	0.92	0.96	0.94	0.89	1.04
20:4	3.08	2.63	2.92	3.10	2.68	3.31
20:5	21.08	16.10	2.89	25.00	17.76	22.66
22:5	21.30	14.28	15.12	16.62	16.35	16.01
22:6	35.10	24.85	32.62	53.97	19.70	27.61
	(30.91)	(28.56)	(29.97)	(30.50)	(21.94)	(24.02)

## APPENDIX 4.12

Control

$$OI = (16:0 + 18:0)/x$$

FAME (X)	time (hrs)						
	0	2	5	24.5	30	53	85
18:1	1.95	1.97	1.96	1.97	1.98	2.07	2.16
18:2	1.19	1.21	1.23	1.32	1.37	1.68	2.21
20:4	3.10	3.25	3.45	4.50	4.94	8.55	19.78
20:5	17.76	18.44	19.76	29.32	30.63	58.65	203.23
22:5	13.30	13.67	15.07	19.44	21.22	38.58	72.11
22:6	15.44	16.30	18.71	27.68	30.90	72.25	111.78
	(31.80)	(32.27)	(32.40)	(34.60)	(35.90)	(39.74)	(44.71)

S1 (16:0+18:0)/x

FAME (x)	time (days)									
	0	2	4	6.5	11.5	24.5	30	53		
18:1	1.95	1.97	1.96	1.98	2.00	2.16	2.18	2.38		
18:2	1.19	1.23	1.26	1.32	1.45	2.15	2.40	5.00		
20:4	3.10	3.51	3.89	4.44	5.77	16.69	21.28			
20:5	17.76	20.33	23.32	27.24	39.31	151.93	151.83			
22:5	13.30	14.64	17.10	19.95	26.97	60.36	56.23			
22:6	15.44	18.29	22.08	27.02	41.99	176.24	175.19			
	(31.80)	(32.93)	(33.34)	(34.32)	(36.95)	(44.06)	(45.55)	(54.91)		

S2 (16:0 + 18:0)/x

time (hours)

FAME (x)	0	0.75	3	4	6.5	10	25	30.5	53
18:1	1.95	1.95	1.96	1.98	1.98	2.00	2.11	2.11	2.42
18:2	1.19	1.21	1.26	1.28	1.31	1.39	1.74	1.92	5.89
20:4	3.10	3.40	3.84	3.88	4.18	4.82	7.93	11.60	
20:5	17.76	19.65	21.53	23.62	25.05	28.92	60.03	77.54	
22:5	13.30	14.74	16.56	17.09	17.56	21.00	24.52	44.07	
22:6	15.44	17.52	20.97	21.81	23.34	28.68	54.35	87.23	
	(31.80)	(32.42)	(33.13)	(34.02)	(34.07)	(35.28)	(40.22)	(41.87)	(52.27)

S3 (16:0 + 18:0)/x

time (hours)

FAME (x)	0	0.75	3	5	7	10	25	30.5	53	83
18:1	1.95	1.95	1.96	1.99	1.97	1.98	2.06	2.06	2.06	2.36
18:2	1.19	1.21	1.26	1.34	1.34	1.35	1.47	1.46	1.49	5.51
20:4	3.10	3.40	3.94	4.37	4.52	4.47	5.04	4.82	5.04	159.00
20:5	17.76	19.51	23.48	26.66	26.78	25.26	31.48	30.33	30.97	
22:5	13.30	14.52	16.96	19.36	19.13	19.15	21.87	20.86	18.97	
22:6	15.44	17.32	21.66	25.87	25.98	25.82	30.18	26.34	24.65	
	(31.80)	(32.39)	(33.58)	(34.66)	(34.82)	(34.86)	(36.52)	(36.09)	(36.24)	(53.31)

## S4 (16:0 + 18:0) x

	time (hours)							
<u>FAME (x)</u>	<u>0</u>	<u>1.50</u>	<u>3.5</u>	<u>5</u>	<u>10.75</u>	<u>26</u>	<u>57</u>	<u>83</u>
18:1	1.95	1.96	1.96	1.97	1.99	2.04	2.06	2.03
18:2	1.19	1.23	1.28	1.31	1.34	1.43	1.47	1.44
20:4	3.10	3.55	3.99	4.29	4.27	4.68	4.84	4.75
20:5	17.76	20.29	23.78	25.33	31.41	28.72	29.67	28.50
22:5	13.30	15.27	17.35	18.69	17.79	19.94	18.80	19.36
22:6	15.44	18.78	22.37	24.26	23.09	27.20	25.46	24.40
	(31.80)	(32.67)	(34.00)	(34.20)	(34.87)	(35.90)	(35.90)	(35.62)

S5 (16:0 + 18:0)/x

	time (hours)										
FAME (x)	0	1.50	3.5	5.5	7.5	10.75	26	57			
18:1	1.95	1.94	1.97	1.98	1.99	2.01	2.00	2.02			
18:2	1.19	1.23	1.29	1.32	1.33	1.38	1.36	1.39			
20:4	3.10	3.55	4.06	4.30	4.20	4.78	4.48	4.55			
20:5	17.76	20.99	25.48	26.30	25.94	29.92	27.86	28.81			
22:5	13.30	15.45	17.25	19.14	18.69	21.12	19.46	19.53			
22:6	15.44	19.00	22.32	24.96	24.48	29.20	26.19	25.66			
	(31.80)	(32.75)	(34.15)	(34.45)	(34.76)	(35.91)	(34.83)	(35.14)			

## APPENDIX 6.1

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
4 weeks	18:1	0.97	0.96	0.96	0.98	0.97	0.96	0.96
	18:2	7.32	8.49	8.20	8.32	8.04	7.73	7.30
	18:3	21.82	28.22	28.14	29.76	26.16	31.32	29.84
		(43.00)	(44.00)	(43.86)	(44.44)	(43.80)	(43.99)	(43.40)

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
7 weeks	18:1		0.94	1.00	0.97	0.97	0.97	0.97
	18:2		8.16	8.02	7.81	7.62	7.30	7.11
	18:3		21.40	20.75	19.77	19.25	19.12	18.11
			(41.73)	(42.64)	(41.80)	(41.68)	(41.66)	(41.40)

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
16 weeks	18:1			0.92	0.94	0.92	0.93	0.89
	18:2			8.33	7.81	7.86	8.15	7.10
	18:3			20.78	20.50	20.53	20.55	19.20
				(40.60)	(41.32)	(40.95)	(41.12)	(40.12)

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
22 weeks	18:1			0.99	1.23	0.97	0.96	0.96
	18:2			9.69	9.78	7.97	7.66	7.77
	18:3			23.52	28.35	21.37	19.90	20.38
				(44.21)	(44.43)	(43.38)	(43.00)	(42.80)

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
33 weeks	18:1			1.00	1.02	1.01	1.02	1.03
	18:2			9.04	8.55	8.46	8.15	8.35
	18:3			22.56	20.28	23.13	21.12	21.69
				(43.42)	(43.58)	43.60	(43.30)	(43.70)

(16:0+18:0)/x

	FAME(x)	+ = 0	A	B	C	D	E	F
38 weeks	18:1			1.10	1.07	1.06	1.05	1.03
	18:2			10.84	9.22	8.95	8.80	8.35
	18:3			27.32	25.38	24.57	24.36	22.44
				(45.60)	(44.30)	(44.20)	(44.08)	(43.40)

## APPENDIX 6.2

 $(16:0+18:0)/x$ 

Sample

FAME(x)	+ = 0	A	B	C	D	E	F
18:1	1.42	1.29	1.28	1.28	1.28	1.36	1.40
18:2	1.16	1.36	1.33	1.21	1.16	1.07	1.16
20:4	3.74	4.44	4.41	3.91	3.82	3.34	3.76
20:5	17.33	20.11	21.13	21.14	21.20	17.88	17.54
22:5	17.70	23.52	24.52	20.03	17.09	15.28	18.20
22:6	25.43	30.91	31.91	31.25	25.00	21.72	26.19
	(28.76)	(28.60)	(28.59)	(29.07)	(28.63)	(26.61)	(28.85)

(16:0+18:0)/x

Sample

FAME(x) + = 0	A	B	C	D	E	F
18:1	1.26	1.25	1.36	1.28	1.36	1.35
18:2	1.48	2.18	1.30	1.16	1.07	1.25
20:4	5.26	9.07	4.22	3.82	3.34	2.00
20:5	21.18	46.29	24.32	21.21	17.18	24.46
22:5	24.38	17.84	16.24	17.09	15.22	13.12
22:6	37.76	30.72	27.86	25.00	21.62	23.54
	(21.32)	(35.34)	(31.55)	(33.37)	(32.40)	(30.94)

$$(16:0+18:0)/x$$

Sample

<u>FAME(x)</u> + = 0	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>
18:1		0.92	1.33	1.33	1.36	1.36
18:2		2.58	1.28	1.44	1.24	1.24
20:4		8.43	4.34	4.64	4.06	4.60
20:5			25.01	28.84	22.76	29.91
22:5			17.78	24.06	16.77	13.26
22:6			24.07	31.34	31.52	37.94
		(26.99)	(31.74)	(32.60)	(31.52)	(31.40)

(16:0+18:0)/x

Sample

<u>FAME(x)</u>	<u>+ = 0</u>	<u>A</u>	<u>B</u>	<u>C</u>
18:1			1.34	1.40
18:2			1.84	1.31
20:4			8.58	5.06
20:5				44.38
22:5				37.22
22:6				71.48
			(30.72	(29.72)

<u>D</u>	<u>E</u>	<u>F</u>
1.31	1.40	1.48
1.23	1.24	1.21
4.36	4.64	4.84
23.34	34.35	39.07
17.23	31.18	30.14
28.05	55.36	193.79
(25.79)	(28.44)	(27.31)

(16:0+18:0) / x

Sample

FAME(x) + = 0	A	B	C	D	E	F
18:1		1.24	1.46	1.45	1.18	1.33
18:2		1.90	1.74	1.46	1.18	1.15
20:4		7.34	6.32	4.64	4.71	4.09
20:5			35.42	25.90	30.39	23.94
22:5			35.46	24.18	24.84	21.67
22:6			49.10	32.22	47.36	32.23
		(28.53)	(30.14)	(27.26)	(30.54)	(25.14)

(16:0+18:0)/x

Sample

	A	B	C	D	E	F
<u>FAME(x)</u> + = 0						
18:1		1.23	41.09	2.57	2.16	2.20
18:2		1.77	4.53	2.50	1.94	1.72
20:4		8.41	31.09	10.05	6.48	5.73
20:5		36.18	178.44	57.60	37.22	38.14
22:5		36.18	136.80	150.61	40.86	38.14
22:6		48.89	195.43	58.14	55.28	56.48
		(18.09)	(41.04)	(35.88)	(37.51)	(21.68)

## APPENDIX 6.3

(16:0+18:0)/x

		Sample				
FAME (x)	+=0	0	G	H	I	J
4 weeks	18:1	0.80	0.76	0.74	0.74	0.76
	18:2	3.38	3.28	2.97	3.02	3.20
	18:3	10.46	12.44	12.54	13.70	13.79
		(36.450)	(34.21)	(31.23)	(33.62)	(34.20)

(16:0+18:0)/x

		Sample			
FAME (x)		G	H	I	J
7 weeks	18:1			0.80	0.79
	18:2			3.52	3.52
	18:3			13.10	10.24
				(35.21)	(34.40)

(16:0+18:0)/x

		Sample			
FAME (x)		G	H	I	J
12 weeks	18:1	0.85	0.87	0.85	0.90
	18:2	3.75	3.69	3.35	4.30
	18:3	15.55	16.40	15.45	18.20
		(38.40)	(38.780)	(37.70)	(40.20)

(16:0+18:0)/x

		Sample			
FAME(x)		G	H	I	J
16 weeks	18:1	0.80	0.85		
	18:2	3.38	3.73		
	18:3	10.46	10.47		
		(34.59)	(36.16)		

(16:0+18:0)/x

		Sample			
FAME(x)		G	H	I	J
20 weeks	18:1	0.81	0.92	0.85	0.88
	18:2	3.82	4.26	3.80	4.00
	18:3	12.96	15.54	13.77	12.13
		(36.77)	(37.76)	(37.08)	(38.14)

(16:0+18:0)/x

		Sample			
FAME(x)		G	H	I	J
33 weeks	18:1	0.90	0.86	0.89	0.89
	18:2	4.17	4.13	4.14	4.00
	18:3	11.85	12.03	12.74	13.19
		(37.68)	(36.69)	(38.00)	(38.21)

$(16:0+18:0)/x$ 

		Sample			
	FAME (x)	G	H	I	J
38 weeks	18:1	0.88	0.90	0.87	0.92
	18:2	4.04	4.52	4.34	4.46
	18:3	12.49	12.62	15.12	14.48
		(36.54)	(37.58)	(38.03)	(38.58)

## APPENDIX 6.4

(16:0+18:0)/x

		Sample				
	FAME (x)	G	H	I	J	
4 weeks	18:1	1.65	1.65	1.65	1.65	1.70
	18:2	0.84	0.84	0.85	0.91	1.09
	20:4	2.60	2.51	2.53	2.46	3.27
	20:5	19.99	20.36	20.35	22.54	32.44
	22:5	15.12	14.88	14.91	16.34	18.58
	22:6	35.18	34.38	35.61	29.26	43.48
		(27.94)	(27.68)	(27.72)	(28.52)	(30.00)

(16:0+18:0)/x

		Sample			
	FAME (x)	G	H	I	J
7 weeks	18:1			1.72	1.70
	18:2			1.04	1.16
	20:4			2.73	4.02
	20:5			23.39	41.64
	22:5			17.74	31.68
	22:6			32.89	82.91
				(23.52)	(30.26)

(16:0+18:0)/x

		Sample			
	FAME(x)	G	H	I	J
12 weeks	18:1	1.78	1.84	1.69	1.72
	18:2	1.02	1.12	1.18	1.26
	20:4	2.82	2.76	3.38	4.18
	20:5	26.96	27.06	33.13	26.96
	22:5	17.33	22.15	22.72	37.83
	22:6	40.46	32.62	48.68	67.83
			(28.94)	(30.01)	(30.42)

(16:0+18:0)/x

		Sample			
	FAME(x)	G	H	I	J
16 weeks	18:1	1.84	1.82		
	18:2	1.14	1.04		
	20:4	2.70	3.00		
	20:5	25.40	28.29		
	22:5	19.80	18.82		
	22:6	36.32	42.44		
			(29.18)	(28.42)	

(16:0+18:0)/x

		Sample			
	FAME (x)	G	H	I	J
20 weeks	18:1	1.84	1.74	1.55	1.86
	18:2	1.02	1.06	1.41	1.40
	20:4	2.92	3.20	4.17	4.12
	20:5	26.96	29.48	39.69	36.47
	22:5	17.57	21.26	25.28	25.86
	22:6	40.09	42.11	37.50	56.80
		(31.27)	(28.56)	(34.130)	(34.65)

(16:0+18:0)/x

		Sample			
	FAME (x)	G	H	I	J
33 weeks	18:1	1.78	1.62	1.74	1.64
	18:2	1.01	1.34	1.26	1.64
	20:4	2.82	3.77	3.23	5.56
	20:5	24.00	40.06	30.70	47.68
	22:5	14.72	24.56	18.30	28.72
	22:6	37.32	30.58	24.66	49.59
		(30.60)	(33.640)	(33.00)	(37.18)

(16:0+18:0)/x

		Sample			
	FAME (x)	G	H	I	J
38 weeks	18:1	1.74	1.64	1.54	1.22
	18:2	0.98	1.28	1.58	3.90
	20:4	2.81	3.30	4.43	40.03
	20:5	27.20	35.26	48.20	686.98
	22:5	18.80	16.32	23.87	26.12
	22:6	26.86	38.61	44.44	140.31
		(30.040	(32.26)	(35.08)	(40.86)

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## APPENDIX 6.5

 $(16:0+18:0)/x$ 

Sample

	FAME(x)	t = 0	K	L	M	N	O	P
3 weeks	18:1	0.84	0.88	0.92	0.88	0.92	0.87	0.86
	18:2	3.78	3.78	3.77	3.61	3.53	3.58	3.86
	18:3	11.26	15.90	15.28	14.09	16.08	16.27	16.60
		(34.71)	(39.18)	(39.58)	(38.54)	(39.23)	(38.56)	(38.84)

 $(16:0+18:0)/x$ 

Sample

	FAME(x)	t = 0	K	L	M	N	O	P
6 weeks	18:1				0.84	0.83	0.78	
	18:2				3.40	3.33	3.34	
	18:3				10.53	10.11	10.63	
					(35.01)	(34.94)	(34.22)	

(16:0+18:0)/x

Sample

FAME(x)	t = 0	K	L	M	N	O	P
11 weeks	18:1	0.76	0.80	0.78	0.76	0.74	0.76
	18:2	2.87	3.23	3.45	3.13	2.97	3.13
	18:3	10.54	12.25	11.68	12.76	11.88	12.76
		(33.14)	(32.32)	(34.50)	(33.50)	(33.08)	(34.01)

(16:0+18:0)/x

Sample

FAME(x)	t = 0	K	L	M	N	O	P
19 weeks	18:1	0.92	0.93	0.97	0.92	0.89	0.87
	18:2	4.00	4.14	4.14	4.15	4.17	3.98
	18:3	11.54	11.47	11.41	11.48	13.58	11.66
		(38.21)	(38.83)	(38.79)	(38.19)	(37.76)	(37.96)

(16:0+18:0)/x

		Sample						
FAME(x)	t = 0	K	L	M	N	O	P	
26 weeks	18:1	0.88	0.90	0.85	0.90	0.86		
	18:2	3.74	3.84	3.91	3.86	3.90		
	18:3	10.91	10.52	10.47	12.33	11.48		
		(37.61)	(38.30)	(37.26)	(38.34)	(37.67)		

(16:0+18:0)/x

		Sample						
FAME(x)	t = 0	K	L	M	N	O	P	
37 weeks	18:1	0.94	0.99	0.93	0.99	0.94	0.90	
	18:2	4.30	4.62	4.48	4.53	4.55	4.57	
	18:3	18.74	26.15	12.66	25.68	26.48	15.10	
		(39.20)	(40.92)	(38.70)	(40.43)	(39.72)	(38.50)	

APPENDIX 6.6

$$(16:0+18:0)/x$$

Sample

FAME(x)	t=0	K	L	M	N	O	P
3 weeks							
18:1	1.90	1.89	1.75	1.70	1.79	1.75	1.75
18:2	0.94	1.02	0.92	1.08	1.10	0.98	1.00
20:4	2.60	2.58	2.90	3.52	3.08	2.80	2.82
20:5	24.65	25.10	19.52	18.81	30.30	18.26	18.44
22:5	14.32	14.47	17.61	18.81	18.88	16.39	17.01
22:6	27.06	27.84	35.46	50.20	34.98	30.24	31.26
	(29.42)	(29.36)	(26.09)	(29.33)	(27.98)	(29.48)	(29.73)

(16:0+18:0)/x

6 weeks	FAME(x)		Sample					
	K	L	M	N	O	P		
18:1			1.84	1.86	1.88			
18:2			1.14	1.28	1.20			
20:4			3.76	4.06	3.48			
20:5			24.35	26.42	43.20			
22:5			36.84	46.22	30.60			
22:6			41.98	143.09	58.46			
			(31.73)	(31.17)	(29.20)			

(16:0+18:0) / x

	Sample						
	FAME(x)	K	L	M	N	O	P
11 weeks	18:1	1.92	1.78	1.49	1.62	1.53	1.79
	18:2	0.96	1.07	1.20	1.12	1.36	1.07
	20:4	2.82	3.36	4.07	3.71	3.86	3.37
	20:5	24.98	32.04	38.60	31.60	45.26	32.16
	22:5	16.82	20.38	30.04	23.98	27.44	20.51
	22:6	33.48	50.25	69.58	59.60	53.26	39.64
		(26.08)	(28.32)	(26.43)	(28.28)	(31.28)	(29.12)

(16:0+18:0)/x

Sample

	FAME(x)	K	L	M	N	O	P
19 weeks	18:1	1.93	1.77	1.48	1.49	1.50	1.93
	18:2	1.00	1.13	1.30	1.28	1.28	1.08
	20:4	3.02	3.74	4.82	4.70	4.36	3.49
	20:5	25.63	34.91	44.31	42.16	41.48	31.64
	22:5	18.29	22.85	31.21	29.45	28.26	23.26
	22:6	29.58	51.46	70.03	71.93	62.98	48.14
		(27.48)	(29.11)	(30.28)	(29.61)	(29.14)	(30.11)

(16:0+18:0)/x

Sample

FAME(x)	K	L	M	N	O	P
26 weeks						
18:1	1.95	1.74	1.43	1.39	1.39	1.97
18:2	1.12	1.24	1.40	1.51	1.46	1.14
20:4	3.14	4.40	5.22	5.01	4.49	3.52
20:5	31.65	39.51	54.36	58.86	45.80	33.96
22:5	21.57	26.93	32.21	39.38	27.48	25.21
22:6	30.33	59.67	82.83	105.93	42.94	50.01
	(29.12)	(30.43)	(34.79)	(30.72)	(34.35)	(31.11)

(16:0+18:0)/x

Sample

FAME(x)	K	L	M	N	O	P
31 weeks						
18:1	1.81	1.48	1.18	1.13	1.25	1.68
18:2	1.06	1.48	1.50	1.75	1.25	1.30
20:4	3.23	5.30	4.91	5.38	4.34	4.47
20:5	32.91	59.17	51.93	70.26	48.76	48.29
22:5	18.49	24.17	20.81	29.75	22.12	20.40
22:6	34.12	72.20	56.35	59.86	50.90	67.02
	(31.47)	(30.72)	(33.18)	(31.22)	(33.16)	(30.91)

APPENDIX 6.7

(16:0+18:0)/x

		Sample							
FAME(x)	t=0	Q	R	S	T	U	V		
3 weeks	18:1	0.97	0.96	0.83	0.95	0.89	0.89	0.89	0.89
	18:2	9.80	10.76	9.13	9.08	10.56	10.70	10.70	10.70
	18:3	21.03	21.08	14.57	16.15	19.50	19.42	19.42	19.42
		(42.96)	(42.35)	(38.96)	(42.72)	(41.78)	(41.79)	(41.79)	(41.79)

(16:0+18:0)/x

		Sample							
FAME(x)	t=0	Q	R	S	T	U	V		
11 weeks	18:1	0.96	0.95	0.95	0.96	0.96	0.96	0.96	0.96
	18:2	9.83	10.10	10.03	9.76	10.12	10.12	10.12	10.12
	18:3	20.16	21.16	22.00	20.02	21.48	21.48	21.48	21.48
		(43.96)	(42.45)	(44.11)	(42.91)	(44.31)	(43.40)	(43.40)	(43.40)

(16:0+18:0)/x

15 weeks	FAME(x) t=0		Sample					
	18:1	18:2	Q	R	S	T	U	V
	18:1	18:2	0.99	0.94	0.88			0.94
	18:2	18:3	7.99	8.30	7.18			8.17
	18:3		15.04	14.60	12.57			17.02
			(41.23)	(40.58)	(38.96)			(40.91)

(16:0+18:0)/x

19 weeks	FAME(x) t=0		Sample					
	18:1	18:2	Q	R	S	T	U	V
	18:1	18:2	1.03	0.95	0.94	1.06	0.94	
	18:2	18:3	8.25	8.19	7.84	7.73	8.37	
	18:3		16.60	14.52	14.35	18.60	14.23	
			(42.84)	(41.10)	(40.48)	(45.53)	(40.85)	

(16:0+18:0)/x

Sample

	FAME(x)	t=0	Q	R	S	T	U	V
21 weeks	18:1		1.06	1.02	0.93			0.95
	18:2		9.02	10.10	8.07			9.14
	18:3		25.62	19.63	15.40			16.32
			(43.46)	(42.60)	(40.10)			(43.17)

(16:0+18:0)/x

Sample

	FAME(x)	t=0	Q	R	S	T	U	V
36 weeks	18:1		1.17	1.10	1.06	1.05	1.06	
	18:2		12.08	12.68	11.96	11.46	11.84	
	18:3		23.60	22.58	20.60	19.17	20.13	
			(48.16)	(45.48)	(45.84)	(44.91)	(44.79)	

APPENDIX 6.8

$$(16:0+18:0)/x$$

3 weeks	Sample									
	FAME(x)	t=0	Q	R	S	T	U	V		
18:1	1.19	1.20	1.15	1.14	1.33	1.21	1.20			
18:2	1.18	1.20	1.19	1.21	1.10	1.52	1.18			
20:4	4.60	4.85	4.93	4.91	3.79	4.29	4.81			
20:5	40.21	41.76	26.42	26.39	21.80	24.31	38.42			
22:5	28.04	30.01	18.97	19.52	17.00	19.72	29.19			
22:6	34.96	42.13	37.91	38.00	31.00	41.36	40.86			
	(21.31)	(20.15)	(28.24)	(27.94)	(28.44)	(28.10)	(29.12)			

(16:0+18:0)/x

11 weeks	FAME(x)		Sample						
	t=0	t=0	Q	R	S	T	U	V	
18:1	1.44	1.38	1.24	1.42	1.42	1.42	1.42	1.42	
18:2	1.30	1.29	1.25	1.16	1.16	1.16	1.16	1.35	
20:4	4.28	4.18	4.17	4.02	4.02	4.02	4.02	4.48	
20:5	25.64	23.29	24.52	22.60	22.60	22.60	22.60	22.07	
22:5	20.56	21.84	18.82	19.46	19.46	19.46	19.46	22.70	
22:6	38.71	42.52	34.96	33.62	33.62	33.62	33.62	38.19	
	(26.52)	(26.76)	(25.88)	(26.56)	(26.56)	(26.56)	(26.56)	(24.06)	

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	FAME(x)	t=0	Q
15 weeks	18:1		1.30
	18:2		1.26
	20:4		4.10
	20:5		24.80
	22:5		19.60
	22:6		37.60
			(28.02)

---

(16:0+18:0)/x

Sample

R	S	T	U	V
1.15	1.26			1.15
1.26	1.32			1.31
4.24	4.14			4.32
21.13	26.90			25.91
18.96	19.97			23.46
34.70	34.98			40.21
(28.62)	(30.26)			(31.13)

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	FAME(x)	t=0	Q
19 weeks	18:1		1.24
	18:2		1.30
	20:4		4.74
	20:5		30.76
	22:5		19.38
	22:6		36.08
			(30.19)

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(16:0+18:0)/x

Sample

R	S	T	U	V
1.08	1.07	1.22	1.11	
1.50	1.25	1.26	1.24	
5.02	5.02	4.55	4.63	
36.63	27.06	29.92	28.78	
126.52	18.36	19.18	17.71	
42.22	34.00	52.08	38.38	
(30.40)	(29.77)	(30.52)	(29.81)	

(16:0+18:0)/x

Sample

	FAME(x)	t=0	Q	R	S	T	U	V
21 weeks	18:1	1.22	1.20	1.12	1.20	1.12	1.20	1.20
	18:2	1.21	1.31	1.24	1.31	1.24	1.31	1.32
	20:4	4.32	4.18	4.70	4.18	4.70	4.21	4.21
	20:5	28.64	31.64	29.74	31.64	29.74	28.65	28.65
	22:5	19.46	18.55	18.70	18.55	18.70	19.21	19.21
	22:6	37.50	33.33	33.86	33.33	33.86	37.51	37.51
		(30.94)	(21.16)	(30.48)	(21.16)	(30.48)	(30.86)	(30.86)

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	FAME (x)	t=0	Q
36 weeks	18:1		1.54
	18:2		1.54
	20:4		5.39
	20:5		36.48
	22:5		26.26
	22:6		49.51
			(34.66)

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(16:0+18:0)/x

Sample

R	S	T	U	V
1.54	1.43	1.12	1.13	1.53
1.55	1.40	1.25	1.26	1.52
5.43	5.19	4.74	4.76	5.20
36.91	28.82	28.84	28.83	35.96
27.29	27.21	18.36	18.46	25.42
50.25	52.32	35.21	37.83	47.64
(35.16)	(34.01)	(30.19)	(30.27)	(34.56)

## APPENDIX 6.9

Chroma (C)

	time (weeks)						
	0	3	9	18	23	29	38
A	18.95	18.10					
B	18.13	17.63	21.06		21.36		
C	16.84	17.75	17.88		16.49		19.22
D	17.84	16.79			15.15		17.25
E	19.37	18.59			18.66		17.86
F	18.85	17.92			17.33		18.73
G	20.48		19.19	17.18	18.92		18.71
H	19.92		17.84	18.37	17.85		17.80
I	18.41	17.17	15.93	18.78			17.65
J	16.60		15.74	15.72			14.71
K	21.43		19.42	21.14		18.44	17.66
L	19.51		18.02			17.82	616.73
M	16.80	14.73	16.07	16.16		15.54	15.79
N	16.04	15.57	16.09	14.67		15.63	15.92
O	15.10	14.65	14.57	14.82		16.49	14.97
P	19.88		18.93	18.10		18.45	18.27
Q	17.02		17.95	18.69			16.60
R	18.37		17.89	17.85	17.76		18.29
S	17.44		16.58	17.76	17.55		14.86
T	18.07		17.51	19.52			17.59
U	18.19	18.17		16.25			15.91
V	17.74		18.42		19.18		17.94

## APPENDIX 6.10

Lightness (L)

	time (weeks)						
	0	3	9	18	23	29	38
A	62.92	64.84					
B	63.72	64.77	65.59		69.11		
C	70.82	65.64	64.71		70.82		
D	67.29	67.15			73.79		65.64
E	65.60	65.64			66.67		63.83
F	70.34	68.77			72.74		68.62
G	67.37		58.03	64.31	59.81		63.86
H	61.40		63.86	61.51	65.15		65.35
I	63.02	65.18	64.75	60.95			65.73
J	65.09		64.98	63.52			68.57
K	65.27		66.91	65.35		67.66	65.99
L	65.38		66.51			67.01	67.72
M	67.22	69.09	70.53	68.18		70.97	71.00
N	68.00	71.55	69.31	72.37		71.57	70.75
O	68.57	69.75	69.54	70.66		69.63	70.21
P	67.71		66.46	68.44		67.96	66.94
Q	70.32		66.72	69.09			71.17
R	65.67		65.40	66.36	66.44		63.54
S	65.69		64.89	63.24	63.99		67.04
T	64.50	61.12	62.42	65.89			62.71
U	62.32	63.27		64.36			65.62
V	62.30		67.45		66.20		66.30

## APPENDIX 6.11

Hue (H)	time (weeks)						
	0	3	9	18	23	29	38
A	71.32	76.09					
B	74.38	75.93	82.32		79.51		
C	77.19	73.81	74.60		81.18		79.48
D	75.05	75.10			83.71		75.92
E	66.61	68.96			73.20		75.92
F	66.68	71.80			71.10		72.30
G	83.34		77.09	82.52	77.71		81.40
H	78.82		80.47	80.62	81.72		83.44
I	82.09	81.44	81.96	82.45			87.32
J	81.69		83.41	82.83			86.62
K	61.11		65.76	64.04		68.25	67.91
L	69.06		76.60			80.13	82.11
M	77.47	78.98	8.97	82.85		84.91	87.03
N	87.19	82.55	83.01	84.84		84.71	87.40
O	80.51	81.38	85.65	85.04		86.53	87.67
P	64.04		66.26	74.78		69.68	73.56
Q	75.73		74.99	77.23			83.82
R	70.44		68.09	67.30	68.19		68.90
S	69.65		64.75	67.93	65.22		66.74
T	63.33		61.48	58.88			68.08
U	61.51	60.51		65.18			71.40
V	60.66		79.12		79.55		80.51

## Appendix 6.12

Experiment 1 (Raw Pork)

O/take	Sample	Temp	Salt	AROMA			FLAVOUR							Overall Qual.				
				Overall			Overall			Overall					Stale			
				Str.	Pork	Other	Str.	Pork	Smokey	Bacon	Fatty	Salt	Sweet		Other	Rancid	Musty	Other
1	A	1	1%	5.741	4.720	2.013	5.664	4.300	1.334	1.613	3.879	4.081	2.962	1.588	1.820	1.750	1.720	3.960
1	B	2	1%	4.629	4.032	1.757	5.344	4.212	1.287	1.695	3.807	3.798	2.750	1.310	1.671	1.269	0.887	4.153
1	C	3	1%	4.885	4.599	1.572	5.464	4.755	0.865	1.637	3.933	3.494	2.621	1.196	1.623	1.445	0.903	4.922
1	D	4	1%	5.167	4.570	1.464	5.492	4.456	1.213	1.704	3.654	3.955	2.578	1.135	1.379	1.478	1.371	4.380
1	E	5	1%	5.364	4.645	1.655	5.610	5.150	1.155	1.330	3.506	3.930	2.901	1.396	1.208	1.458	1.403	4.255
1	F	4	0%	4.858	4.911	1.435	5.241	5.283	0.829	0.836	3.542	2.127	2.437	1.054	1.017	1.677	1.132	5.007
	Sig.diff (P=0.05)			0.855	1.030	0.769	0.762	1.169	0.449	0.958	1.128	0.513	0.508	0.503	0.698	0.595	0.718	1.200
2	A	1	1%	5.032	3.280	2.489	5.414	2.692	1.264	2.413	3.618	3.897	2.311	2.238	2.587	2.069	1.761	3.219
2	B	2	1%	4.962	3.915	1.776	5.168	4.017	1.291	1.933	3.029	4.064	2.411	1.389	1.186	1.529	1.353	4.269
2	C	3	1%	4.987	3.481	2.139	5.250	3.241	1.641	2.511	3.034	3.883	2.425	1.365	1.300	1.243	1.285	4.826
2	D	4	1%	4.990	3.957	1.805	5.166	4.423	1.328	1.864	2.920	3.904	2.055	1.153	1.213	1.457	1.180	4.886
2	E	5	1%	4.674	3.783	1.709	5.227	3.680	1.527	2.217	3.197	4.190	1.981	1.192	1.179	1.565	1.241	4.238
2	F	4	0%	4.788	4.086	1.445	4.731	3.721	1.270	1.847	2.866	3.254	2.420	1.168	0.950	1.352	1.453	4.500
	Sig.diff (P=0.05)			0.687	0.579	0.580	0.692	1.054	0.526	0.807	0.562	0.641	0.510	0.866	0.994	0.889	0.720	1.129
3	C	3	1%	5.026	3.745	2.080	5.523	3.802	1.048	1.942	3.468	3.690	2.622	1.416	1.590	1.609	1.298	4.495
	Sig.diff (P=0.05)			0.633	1.156	0.803	0.850	1.145	0.593	1.122	0.618	1.444	0.443	0.544	0.633	0.526	0.613	0.928
4	C	3	1%	4.869	3.756	2.880	5.527	3.601	1.431	2.621	4.058	3.721	2.521	1.371	1.678	1.260	1.737	3.901
4	D	4	1%	5.148	4.306	2.365	5.915	3.980	1.208	2.617	3.991	4.145	2.092	1.100	1.336	1.099	1.239	4.479
4	E	5	1%	4.811	3.941	2.187	5.674	4.219	1.043	1.557	3.840	3.494	2.244	1.639	1.787	1.638	1.822	3.912
4	F	4	0%	4.784	4.117	2.127	5.424	5.113	1.072	0.847	3.826	2.241	2.526	1.603	1.723	1.666	1.528	4.261
	Sig.diff (P=0.05)			0.418	0.605	0.682	0.488	0.598	0.373	0.663	0.638	0.451	0.528	0.458	0.406	0.252	0.706	0.696
6	C	3	1%	4.819	3.228	2.833	5.924	3.292	1.361	2.611	3.618	4.257	2.263	2.053	2.521	2.063	2.125	3.375
6	D	4	1%	4.903	4.217	2.000	5.910	3.875	1.306	2.556	2.993	4.410	2.071	1.114	1.979	1.771	2.097	4.403
6	E	5	1%	4.481	4.130	1.935	5.741	3.991	1.139	2.185	3.407	4.333	1.935	1.348	2.343	1.778	1.491	4.065
6	F	4	0%	4.815	4.363	1.750	5.657	5.194	1.120	1.315	2.935	2.731	2.307	1.148	1.259	1.250	1.880	5.972
	Sig.diff (P=0.05)			0.651	0.747	0.720	0.528	0.632	0.159	0.900	0.424	0.783	0.398	0.906	0.731	0.530	1.112	0.888
8	D	4	1%	4.761	3.615	2.285	5.844	3.286	1.747	3.485	3.916	4.500	2.262	1.796	2.775	2.484	1.799	3.201
8	E	5	1%	4.864	3.705	2.518	6.094	4.269	1.368	2.590	3.988	4.263	2.015	1.488	1.997	2.097	2.156	3.521
8	F	4	0%	4.547	3.918	1.859	5.405	4.926	1.139	1.248	3.569	2.848	2.398	1.539	1.624	2.117	1.787	4.415
	Sig.diff (P=0.05)			0.504	0.731	0.727	0.339	0.925	0.285	0.924	0.495	0.652	0.452	0.734	0.802	0.891	0.934	1.046
9	D	4	1%	4.590	4.041	2.066	5.528	3.895	1.454	2.300	3.766	4.192	2.695	1.461	3.207	2.134	1.684	3.238
9	E	5	1%	4.859	3.022	2.881	5.980	3.605	1.443	3.127	3.965	4.558	2.819	2.396	2.317	2.340	2.182	4.152
9	F	4	0%	4.559	4.094	1.872	5.626	4.771	1.148	1.926	3.285	3.497	2.525	1.133	0.930	1.529	1.913	5.900
	Sig.diff (P=0.05)			0.838	1.073	0.973	0.636	1.344	0.504	1.661	0.798	1.135	0.722	0.988	1.111	1.058	1.194	1.317

		AROMA										FLAVOUR										Overall	
O/take	Sample	Temp	Salt	Overall		Pork	Other	Str.	Pork	Smokey	Bacon	Fatty	Salt	Sweet	Overall		Rancid	Stale		Other	Qual.		
				Str.	Other										Other	Musty							
1	G	4	0%	5.567	4.743	1.735	5.426	4.800	1.197	1.089	3.096	2.236	2.564	1.229	1.032	1.197	1.169	4.583					
1	H	4	1%	5.842	4.900	1.721	6.266	5.866	0.957	1.434	2.713	3.342	2.115	1.016	1.537	1.418	0.623	5.647					
1	I	4	2%	5.694	4.687	2.258	5.996	4.376	1.753	2.619	3.666	4.745	2.551	0.933	1.488	1.623	0.877	5.088					
1	J	4	3%	6.027	5.050	1.857	6.010	4.328	1.629	2.699	3.589	5.763	2.268	1.287	1.305	1.247	1.612	4.407					
	Sig.diff		(P=0.05)	0.855	1.030	0.769	0.762	1.169	0.449	0.958	1.128	0.513	0.508	0.503	0.698	0.595	0.718	1.200					
2	I	4	2%	5.099	4.931	1.433	6.092	4.621	1.562	2.053	2.604	4.656	2.000	1.377	1.070	1.120	1.378	5.401					
2	J	4	3%	5.328	4.457	2.012	6.374	4.543	1.857	2.472	2.450	5.681	1.930	1.090	1.169	1.456	1.500	4.791					
	Sig.diff		(P=0.05)	0.687	0.579	0.580	0.692	1.054	0.526	0.807	0.562	0.641	0.510	0.866	0.994	0.889	0.720	1.129					
3	G	4	0%	5.323	4.824	2.155	5.428	5.798	1.271	1.032	3.231	2.549	2.381	1.521	1.191	1.206	1.222	5.610					
3	H	4	1%	5.549	4.831	2.107	5.702	5.418	1.311	1.432	2.792	3.601	2.346	1.414	0.961	1.113	1.635	5.686					
3	I	4	2%	5.521	4.866	2.421	6.001	4.559	1.724	2.560	3.042	4.435	2.191	1.522	1.089	1.339	1.853	5.267					
3	J	4	3%	4.844	4.154	1.926	5.923	4.479	1.559	2.177	2.998	5.122	1.983	1.469	1.107	1.269	1.573	4.650					
	Sig.diff		(P=0.05)	0.633	1.156	0.803	0.850	1.145	0.593	1.122	0.618	1.444	0.443	0.544	0.633	0.526	0.613	0.928					
4	G	4	0%	5.366	5.071	2.121	6.010	5.487	0.985	1.152	2.990	2.471	1.968	1.513	1.066	1.109	1.859	5.654					
4	H	4	1%	5.344	4.834	2.511	5.679	5.606	0.939	0.945	2.782	3.017	2.447	1.382	0.906	1.120	1.753	5.867					
	Sig.diff		(P=0.05)	0.418	0.605	0.682	0.488	0.598	0.373	0.663	0.638	0.451	0.528	0.458	0.406	0.252	0.706	0.696					
5	G	4	0%	4.904	4.703	1.565	5.734	5.838	1.074	0.915	3.917	2.316	2.582	1.179	1.250	1.045	1.337	5.581					
5	H	4	1%	5.021	4.069	1.922	5.729	4.322	1.189	2.302	3.524	3.518	2.233	1.366	1.628	1.425	1.846	5.038					
5	I	4	2%	4.771	4.209	2.450	6.035	4.608	1.237	2.066	3.502	4.664	2.098	1.817	2.279	1.941	2.225	4.321					
5	J	4	3%	5.203	4.417	2.975	6.203	4.133	1.266	1.869	3.775	5.794	1.827	1.721	1.772	2.364	2.241	3.599					
	Sig.diff		(P=0.05)	0.627	0.740	0.799	0.476	0.905	0.380	0.820	0.505	0.602	0.558	0.719	0.929	0.825	1.071	1.069					
6	G	4	0%	5.074	4.581	1.861	5.954	5.806	1.046	1.130	3.009	2.713	2.249	1.241	1.074	1.194	1.565	6.806					
6	H	4	1%	5.370	4.752	1.861	5.769	4.806	1.176	1.759	2.880	3.398	1.863	1.804	1.426	1.694	1.713	5.139					
	Sig.diff		(P=0.05)	0.651	0.747	0.720	0.528	0.632	0.159	0.900	0.424	0.783	0.398	0.906	0.731	0.530	1.112	0.888					
8	G	4	0%	5.337	4.477	2.287	5.631	4.955	1.255	1.571	3.188	2.712	1.857	1.401	1.265	1.859	1.717	4.928					
8	H	4	1%	5.282	3.984	2.765	5.844	4.365	1.447	2.151	3.714	3.845	1.814	1.448	1.879	3.282	1.837	3.703					
8	I	4	2%	5.075	3.702	3.167	6.487	4.350	1.537	2.853	3.857	5.160	1.865	2.289	2.263	2.957	2.532	3.525					
8	J	4	3%	5.337	4.206	2.920	6.410	3.741	1.652	3.360	3.797	5.840	1.718	2.124	2.640	3.301	2.869	2.959					
	Sig.diff		(P=0.05)	0.504	0.731	0.727	0.339	0.925	0.285	0.924	0.495	0.652	0.452	0.734	0.802	0.891	0.934	1.046					
9	G	4	0%	5.233	4.715	2.117	5.567	5.297	0.963	0.977	3.366	2.413	2.527	1.223	0.842	1.188	2.020	6.377					
9	H	4	1%	4.840	4.140	2.263	5.543	4.942	1.695	1.874	2.636	3.450	2.409	1.312	1.385	2.961	1.592	4.515					
9	I	4	2%	4.690	3.498	2.910	5.969	4.094	1.364	2.217	3.588	4.989	1.992	1.976	2.610	3.352	2.341	2.918					
9	J	4	3%	5.066	3.665	3.108	6.225	3.459	1.788	3.382	3.542	5.644	2.015	2.517	2.595	3.242	2.751	3.278					
	Sig.diff		(P=0.05)	0.838	1.073	0.973	0.636	1.344	0.504	1.661	0.798	1.135	0.722	0.988	1.111	1.058	1.194	1.317					

O/take	Sample	Temp	Salt	AROMA			FLAVOUR							Overall			Overall Qual.	
				Overall Str.	Pork	Other	Overall Str.	Pork	Smokey	Bacon	Fatty	Salt	Sweet	Overall Other	Rancid	Stale Musty		Other
1	K	4	0%	5.030	4.748	1.591	4.894	4.876	1.034	0.789	3.308	2.378	2.609	1.060	0.956	2.058	1.247	4.198
1	L	4	1%	5.231	4.183	2.007	5.318	4.066	1.111	1.875	3.646	3.697	2.759	1.074	1.375	1.389	1.173	4.481
1	M	4	2%	5.462	4.471	2.029	5.963	3.928	1.857	2.345	3.629	5.267	2.378	1.171	1.626	1.334	1.456	3.840
1	N	4	3%	5.272	4.681	1.990	6.475	4.034	1.528	2.515	4.010	6.516	2.039	1.123	1.742	1.412	1.069	3.583
1	O	4	4%	6.031	4.282	2.483	6.420	3.488	1.481	3.029	4.011	6.598	2.280	1.212	1.867	1.398	1.891	3.627
1	P	4	1% K	5.683	4.923	1.546	5.699	4.803	1.091	1.138	3.248	3.028	2.290	1.472	1.926	2.004	2.425	3.511
	Sig.diff (P=0.05)			0.855	1.030	0.769	0.762	1.169	0.449	0.958	1.128	0.513	0.508	0.503	0.698	0.595	0.718	1.200
2	M	4	2%	5.197	4.234	1.864	5.741	3.920	1.575	2.409	3.267	5.271	1.645	1.180	1.418	1.581	1.480	3.958
2	N	4	3%	4.911	4.215	1.662	6.587	3.196	1.712	3.422	2.811	6.420	1.299	1.240	1.577	1.123	1.208	4.244
2	O	4	4%	5.078	4.219	1.839	6.294	3.555	1.954	2.400	3.373	7.162	1.265	1.387	2.058	1.391	1.153	3.172
	Sig.diff (P=0.05)			0.687	0.579	0.580	0.692	1.054	0.526	0.807	0.562	0.641	0.510	0.866	0.994	0.889	0.720	1.129
3	K	4	0%	5.140	4.158	2.029	5.259	4.554	1.260	1.952	3.407	3.133	2.528	1.537	1.077	1.511	1.700	4.492
3	L	4	1%	4.908	3.918	1.995	5.597	4.309	1.319	2.169	3.458	3.718	2.311	1.434	1.439	1.494	1.571	4.775
3	M	4	2%	5.248	4.347	2.188	6.230	3.892	1.672	3.007	3.581	5.692	2.275	1.333	1.410	1.520	1.376	4.305
3	N	4	3%	5.136	4.177	2.148	6.081	3.708	1.945	3.122	3.178	5.349	2.172	1.149	1.302	1.352	1.412	4.199
3	O	4	4%	5.311	4.043	2.510	6.209	3.171	2.324	3.485	3.671	5.877	2.462	1.528	1.497	1.249	1.703	3.826
3	P	4	1% K	5.135	3.978	2.180	5.382	4.128	1.148	1.847	3.484	3.231	2.452	1.835	1.956	1.818	1.965	3.552
	Sig.diff (P=0.05)			0.633	1.156	0.803	0.850	1.145	0.593	1.122	0.618	1.144	0.443	0.544	0.633	0.526	0.613	0.928
5	K	4	0%	4.772	4.239	2.528	5.315	4.816	0.962	1.418	3.665	2.499	2.178	1.975	1.864	1.619	1.672	4.373
5	L	4	1%	5.115	3.505	3.053	5.843	4.256	1.423	2.572	3.916	4.094	2.530	1.493	1.934	2.031	1.635	3.824
5	M	4	2%	5.556	3.536	3.560	6.356	3.505	1.429	3.237	4.348	5.966	1.925	1.955	2.508	2.207	1.684	3.352
5	N	4	3%	5.349	3.913	2.947	6.749	3.357	1.799	3.490	4.199	6.661	1.775	2.222	3.072	2.440	2.175	2.860
5	O	4	4%	5.264	3.375	3.989	6.801	2.813	1.668	3.599	4.295	7.020	1.794	1.536	2.604	2.504	2.695	2.151
5	P	4	1% K	5.368	3.841	3.056	5.886	4.116	1.115	1.182	3.807	4.178	2.341	1.902	2.326	1.965	2.762	3.887
	Sig.diff (P=0.05)			0.627	0.740	0.799	0.476	0.905	0.380	0.820	0.505	0.602	0.558	0.719	0.929	0.825	1.071	1.069
7	K	4	0%	4.421	3.893	1.593	5.460	5.726	1.184	1.041	3.698	2.813	2.336	1.198	1.165	1.427	1.328	6.012
7	L	4	1%	5.270	3.433	2.899	5.340	4.161	1.804	3.203	3.563	3.713	2.172	1.462	1.683	2.221	1.423	4.317
7	M	4	2%	5.175	3.743	3.016	6.508	4.162	1.815	3.565	3.921	5.737	1.814	2.556	2.406	2.322	2.081	3.346
7	N	4	3%	5.580	3.637	3.346	7.136	3.411	2.259	4.047	3.807	6.451	1.770	1.857	2.565	2.482	1.886	3.465
7	O	4	4%	5.353	3.005	3.957	7.031	3.117	2.051	4.250	3.978	6.909	1.885	2.147	2.671	2.622	2.431	2.537
7	P	4	1% K	5.190	4.254	2.260	6.293	4.421	1.667	1.689	3.756	4.083	1.866	1.902	1.948	2.800	2.970	3.393
	Sig.diff (P=0.05)			0.529	1.090	1.130	0.786	0.869	0.471	0.789	0.373	1.082	0.865	0.834	0.668	1.083	1.571	1.267
9	K	4	0%	4.561	3.951	1.935	5.661	5.057	1.091	0.853	3.570	2.692	2.425	2.179	1.589	2.084	1.942	4.766
9	L	4	1%	5.044	3.728	2.889	5.765	4.038	2.087	3.040	3.322	4.324	2.243	2.115	1.647	1.992	1.884	4.293
9	M	4	2%	5.114	4.107	2.352	5.943	3.898	1.719	3.109	3.440	4.662	2.531	2.584	1.913	2.208	2.011	4.238
9	N	4	3%	5.054	3.156	3.153	6.561	2.885	1.932	3.652	3.739	6.365	2.136	3.352	3.194	2.869	1.914	2.253
9	O	4	4%	5.231	2.875	3.444	6.897	2.709	1.707	4.657	3.719	6.770	1.513	2.680	3.300	3.994	3.988	2.002
9	P	4	1% K	5.017	4.234	2.630	6.321	4.726	1.292	1.665	3.848	4.280	2.172	2.433	2.928	2.593	2.381	3.006
	Sig.diff (P=0.05)			0.838	1.073	0.973	0.636	1.344	0.504	1.661	0.798	1.135	0.722	0.988	1.111	1.058	1.194	1.317

O/take	Sample	Temp	Salt	AROMA				FLAVOUR				Overall											
				Overall Str.	Pork	Other	Overall Str.	Pork	Smoky	Bacon	Fatty	Salt	Sweet	Overall Other	Rancid	Stale	Musty	Other	Overall Qual.				
1	Q	4	0%	5.090	3.967	1.822	3.571	3.294	0.936	1.046	3.079	1.970	2.636	1.050	1.103	1.748	1.168	3.555					
				4.267	3.512	1.821	4.647	3.578	1.805	1.813	3.281	3.628	2.511	1.149	0.885	1.129	1.198	4.482					
				4.980	4.999	2.810	6.429	2.414	1.806	4.357	4.254	6.868	2.405	1.135	1.890	1.673	1.095	3.515					
				5.035	3.837	2.272	5.805	2.793	1.790	3.215	4.275	6.015	2.006	1.417	1.639	1.657	1.600	3.254					
				5.080	3.528	2.345	6.358	2.634	2.240	3.474	4.199	6.827	1.972	1.972	1.142	1.607	1.473	3.234					
				4.511	3.286	2.080	4.980	2.261	1.394	3.213	3.293	4.695	2.797	1.396	1.493	1.610	1.435	3.796					
				Sig.diff (P=0.05)																			
				0.855	1.030	0.769	0.762	1.169	0.449	0.958	1.128	0.513	0.508	0.503	0.698	0.595	0.718	1.200					
				2	T	4	3%	4.805	2.758	2.777	6.174	1.793	2.266	4.896	3.785	6.233	1.702	1.099	1.869	1.776	1.160	3.596	
								4.766	2.920	2.696	6.702	1.575	2.583	4.358	3.753	7.389	1.590	1.275	2.099	1.290	1.610	3.247	
Sig.diff (P=0.05)																							
0.687	0.579	0.580	0.692					1.054	0.526	0.807	0.562	0.641	0.510	0.866	0.994	0.889	0.720	1.129					
3	Q	4	0%					5.135	3.734	2.581	5.079	3.926	1.239	1.590	2.840	2.535	2.459	1.206	1.269	1.257	1.830	4.489	
								4.916	3.713	2.219	5.539	3.490	1.351	3.187	3.409	4.256	2.425	1.210	1.441	1.475	1.504	4.558	
								5.124	3.406	2.917	6.192	2.669	1.889	3.704	3.834	3.704	6.069	1.844	1.378	1.701	1.494	2.184	3.704
								4.862	2.946	2.824	5.849	1.999	2.150	3.993	3.895	5.971	2.146	1.374	1.975	1.315	1.691	3.777	
								4.919	3.564	2.492	5.648	2.913	1.687	2.735	3.451	4.407	1.847	1.493	1.814	1.705	1.823	3.843	
								Sig.diff (P=0.05)															
				0.633	1.156	0.803	0.850	1.145	0.593	1.122	0.618	1.444	0.443	0.544	0.633	0.526	0.613	0.928					
				4	Q	4	0%	4.570	3.047	2.699	4.411	3.412	1.023	1.761	3.122	2.307	2.262	1.345	1.340	1.433	1.680	3.773	
								4.904	3.203	3.058	5.278	2.663	1.407	3.348	3.935	3.650	1.938	1.069	1.209	1.189	1.542	4.386	
								4.964	3.034	3.214	6.223	2.562	1.489	3.923	3.860	5.593	1.808	1.615	1.543	1.338	1.795	4.187	
5.157	2.650	3.860	6.318					1.898	1.611	4.063	4.221	7.027	1.568	1.446	1.367	1.050	1.537	3.197					
Sig.diff (P=0.05)																							
0.418	0.605	0.682	0.488					0.598	0.373	0.663	0.638	0.451	0.528	0.458	0.406	0.252	0.706	0.696					
5	Q	4	0%					4.432	2.740	3.128	5.062	3.633	1.120	1.966	3.735	2.482	2.341	1.371	1.464	1.944	2.143	3.938	
								4.854	2.528	3.491	5.458	2.747	1.300	3.315	4.052	4.442	2.416	1.615	1.999	2.153	1.387	3.861	
								4.600	2.698	3.233	5.801	2.414	1.364	3.656	4.229	5.221	2.085	1.383	1.728	2.086	1.606	3.635	
								5.243	2.630	3.956	6.410	2.974	1.402	3.626	4.639	5.953	2.078	1.835	2.081	2.111	1.571	3.238	
				4.575	2.760	3.401	6.845	1.815	1.540	4.274	4.390	7.586	1.888	1.495	1.931	2.164	1.727	2.382					
				4.072	2.711	2.695	5.063	2.710	1.127	2.653	4.263	3.781	2.040	2.176	3.307	2.742	3.100	2.593					
				Sig.diff (P=0.05)																			
				0.627	0.740	0.799	0.476	0.905	0.380	0.820	0.505	0.602	0.558	0.719	0.929	0.825	1.071	1.069					
				6	Q	4	0%	4.463	2.304	3.417	4.565	3.083	1.269	2.296	3.120	2.491	1.749	1.630	1.130	1.694	1.787	4.028	
								4.741	2.247	3.731	5.315	2.454	1.639	3.870	3.426	4.056	1.954	1.208	1.491	1.611	1.676	4.380	
4.593	2.130	3.380	6.241					2.213	1.750	4.519	3.574	5.556	1.935	1.514	2.009	1.556	1.602	4.065					
4.019	1.870	3.287	5.259					2.343	1.306	2.815	3.148	3.556	1.778	1.652	2.769	1.944	2.509	2.880					
Sig.diff (P=0.05)																							
0.651	0.747	0.720	0.528					0.632	0.159	0.900	0.424	0.783	0.398	0.906	0.731	0.530	1.112	0.888					
9	Q	4	0%					4.501	2.652	3.379	4.803	3.985	1.147	2.197	3.860	2.120	2.423	1.249	1.361	1.656	1.308	4.176	
								4.595	3.007	2.531	5.037	2.739	1.356	3.347	3.483	4.091	2.491	1.188	1.125	1.845	0.616	4.158	
								5.268	3.007	3.892	5.837	2.564	2.122	4.780	4.314	5.336	2.055	1.781	1.721	2.512	1.893	3.421	
								4.751	4.33	2.834	6.672	2.382	1.716	4.606	4.658	7.245	1.670	1.403	2.667	2.210	1.726	3.097	
				4.591	2.743	2.754	6.832	2.342	2.133	5.558	4.451	4.451	1.808	1.882	2.437	2.224	2.024	2.855					
				4.259	3.506	2.172	5.211	3.637	1.467	2.153	3.758	3.694	2.087	1.879	2.760	2.606	2.053	2.790					
				Sig.diff (P=0.05)																			
				0.838	1.073	0.973	0.636	1.344	0.504	1.661	0.798	1.135	0.722	0.988	1.111	1.058	1.194	1.317					