RADIATION-INDUCED CELLULAR CHANGES; THE ROLE OF DNA AND MEMBRANES IN CELL KILLING.

A thesis submitted to the University of London for the Degree of Doctor of Philosophy in Radiobiology

by

ANDREW MICHAEL GEORGE

ROYAL POSTGRADUATE MEDICAL SCHOOL
TO MY FRIENDS,

MARTIN, HELEN, ROS, JOHN AND PAULINE, CHRIS,
KAYE AND GEOFF, IAN, BARRY, MIKE, BILL AND
MALCOLM
TO

Maculinea arion Linnaeus

The Large Blue

May she find safe refuge in France.
ABSTRACT

DNA is the most likely site for the lethal radiation event. However, doses used to measure DNA damage are often higher than those required for biological inactivation. Also the oxygen enhancement of radiation effects on DNA synthesis, DNA strand-breaks and DNA degradation, is often less than its enhancement of lethality. Since RNA, protein, and membrane could all be involved with DNA structure and function, it is feasible that such macromolecules could be involved in radiation lethality. Indeed, Alper (1963) postulated that membranes would be a target particularly sensitive to the presence of oxygen during irradiation.

Following work by McGrath and Williams (1966) it became customary to associate the ability to repair strand breaks with radio-resistance, and the designation of unrepaired breaks, particularly double-strand breaks, as the vital damage leading to cell death. However, DNA degradation was overlooked by these authors and this led to a misinterpretation of their results. I have reassessed their work on strand break production and repair in E.coli B/r and B5-1, using hydroxyapatite chromatography. I found that within the sensitivity of this technique, these strains were equally repair proficient.

Published literature implicates that DNA-membrane interaction could be an important target, and that lipid peroxidation could explain the increased cell killing in the presence of oxygen. I have therefore studied the possible role of these two criteria by altering the membrane fatty acid composition of an E.coli unsaturated fatty acid auxotroph (K1060) and a cultured human cell line (LDV). The results did not support a significant role for radiation induced lipid peroxidation in cell killing. However, in bacterial studies alone, some relationship
between radiation sensitivity and membrane structure, probably dependent on membrane fluidity, was established. Changes in membrane fluidity were evidently not able to influence the initiation or repair of DNA strand-breaks.
ACKNOWLEDGEMENTS

I would like to thank:

Dr. William A. Cramp for his constant help and patience over the last four years;

Dr. Shirley Hornsey for her encouragement;

Dr. John Lunec for stimulating discussions and his assistance with the X-irradiations in Chapter 4;

Miss Hilary Walker for estimating non-protein bound SH levels in Chapter 4; 

Mrs. Gail Rowlinson for her assistance with oxygen measurements in Chapter 4;

Profs. M.B. Yatvin and G. Ahnstrom for their advice and support. Mr. Tom Liddy and Mr. Lee Wardlaw for their services on the Linear Accelerator, particularly on those occasions when I overran my allocated time by up to an hour;

Mr. John Ledda for help with some of the figures, and Mr. Fred Paice for photography;

Dr. Geoff. Gibbons, Dr. Soundararajan Venkatesan and Mr. Clive Pullinger for their advice and use of their facilities for fatty acid analyses;

All my colleagues past and present in the Cyclotron Unit, and Lipid Metabolism Unit.

Especial thanks are due to:

Mrs. Doreen Wishart for typing the manuscript;

The Medical Research Council for providing the opportunity for this work.
# CONTENTS

<table>
<thead>
<tr>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>6</td>
</tr>
<tr>
<td>Contents</td>
<td>7</td>
</tr>
<tr>
<td>List of Figures</td>
<td>8</td>
</tr>
<tr>
<td>List of Tables</td>
<td>16</td>
</tr>
</tbody>
</table>

## CHAPTER 1. Historical

1.1. Effect of radiation on DNA
   1.1.1. DNA synthesis 17
   1.1.2. Degradation 18
   1.1.3. DNA Strand Breaks 22
   1.1.3.a. Single-strand Breaks 23
   Anomalies of strand-break analysis 25
   1.1.3.b. Alkali-labile sites 29
   1.1.3.c. The effect of oxygen on strand-break production 30
   1.1.3.d. Rapid lysis techniques 31
   1.1.3.e. Double-strand breaks: production, repair and relevance to cell lethality 33

1.2. Effects of radiation on membrane or membrane constituents 41

1.3. Evidence for two or multiple target interaction 44
   1.3.1. Effect of lipid peroxidation on other macromolecules 44
   1.3.2. Membrane complexes 45
   1.3.2.a. Bacteria 45
   1.3.2.b. Mammalian cells 48

## CHAPTER 2. Strand break repair in E.coli B/r and B<sub>s-1</sub> as detected by hydroxylapatite chromatography 50

<table>
<thead>
<tr>
<th>Summary</th>
<th>51</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Introduction</td>
<td>52</td>
</tr>
<tr>
<td>2.2. Materials and Methods</td>
<td>58</td>
</tr>
<tr>
<td>2.2.1. Preparation of bacteria</td>
<td>58</td>
</tr>
<tr>
<td>2.2.2. Irradiation</td>
<td>60</td>
</tr>
<tr>
<td>2.2.3. Dosimetry</td>
<td>62</td>
</tr>
</tbody>
</table>
2.2.4. Post-irradiation Incubation 62
2.2.5. Alkaline lysis 64
2.2.6. Hydroxylapatite Chromatography 64
2.2.7. DNA degradation 66
2.3. Results 66
2.3.1. Post-irradiation incubation in non-nutrient 0.39% NaCl 66
2.3.2. Post-irradiation incubation in minimal-growth medium 70
2.3.3. Results following irradiation in cold chase medium. 73
2.4. Discussion 78

CHAPTER 3. The influence of membrane fluidity on the induction and repair of DNA strand breaks in the unsaturated fatty acid auxotroph E.coli K1060 83
Summary 84
3.1. Introduction 85
3.2. Materials and Methods 89
3.2.1. Growth and radioisotope labelling of K1060 89
3.2.2. Irradiation 90
3.2.3. Strand-break determination and DNA degradation 90
3.2.4. Survival 90
3.3. Results 93
3.3.1. Harvesting and Cooling 93
3.3.2. Survival 94
3.3.3. DNA degradation 100
3.3.4.1. Strand-breaks in non-nutrient conditions 100
3.3.4.2. Strand-breaks in growth medium 104
3.4. Discussion 109

CHAPTER 4. The influence of changes in the unsaturated fatty acid composition of membranes on the radiosensitivity of the human lymphoid cell line, LDV 116
Summary 117
4.1. Introduction 118
4.1.1. The alteration of mammalian cell membrane fatty acid composition 118
4.1.2. Effects of membrane fatty acid composition on radiation sensitivity 121
4.1.3. Membrane alterations and membrane fluidity
  4.1.3.i. Measurements of membrane fluidity
  4.1.3.ii. Effect of membrane fatty acid alterations on membrane fluidity

4.1.4. Other effects of fatty acid alterations

4.2. Materials and Methods
  4.2.1. Maintenance of LDV cells
    4.2.1.i. Storage and growth of LDV cells
    4.2.1.ii. Serum delipidation
    4.2.1.iii. Freezing of LDV cells
    4.2.1.iv. LDV growth characteristics
  4.2.2. Lipid Analysis
    4.2.2.i. Cell preparation procedure
    4.2.2.ii. Folch extraction of cells
    4.2.2.iii. Phospholipid/Neutral lipid separation
    4.2.2.iv. Transesterification
    4.2.2.v. Gas liquid chromatography
  4.2.3. Membrane Fluidity: Cell Preparation
  4.2.4. Cell survival
    4.2.4.i. Agar plates
    4.2.4.ii. Feeder cells
  4.2.5. Irradiation of Cells
    4.2.5.i. Electrons
    4.2.5.ii. X rays
    4.2.5.iii. Gassing vessels used for electron radiation
  4.2.6. Estimation of non-protein bound sulphydryl levels

4.3. Results
  4.3.1. Membrane fatty acid alteration
    4.3.1.i. Whole cells
    4.3.1.ii. Nuclei
    4.3.1.iii. Evidence of homeostasis
  4.3.2. Membrane fluidity
  4.3.3. Irradiation
  4.3.4. Non-protein bound SH

4.4. Discussion

Addendum
### CHAPTER 5. Conclusions and General Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1.</td>
<td>Introduction</td>
<td>189</td>
</tr>
<tr>
<td>5.2.</td>
<td>Repair of DNA strand breaks</td>
<td>191</td>
</tr>
<tr>
<td>5.3.</td>
<td>Membrane damage</td>
<td>194</td>
</tr>
<tr>
<td>5.3.1.</td>
<td>E.coli K1060</td>
<td>194</td>
</tr>
<tr>
<td>5.3.2.</td>
<td>Mammalian cells</td>
<td>202</td>
</tr>
<tr>
<td>5.4.</td>
<td>Sublethal damage (SLD) and potentially lethal damage (PLD)</td>
<td>205</td>
</tr>
<tr>
<td>5.5.</td>
<td>Future Research</td>
<td>206</td>
</tr>
<tr>
<td>5.6.</td>
<td>Addendum</td>
<td>209</td>
</tr>
</tbody>
</table>

### REFERENCES

<table>
<thead>
<tr>
<th>References</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>212</td>
</tr>
<tr>
<td>FIGURE 2 A</td>
<td>Schematic representation of weak alkali hydroxylapatite technique for determining strand breaks.</td>
</tr>
<tr>
<td>FIGURE 2 B</td>
<td>DNA degradation measured by the loss of TCA-precipitable material for <em>E. coli</em> B/r and B$_{S-1}$.</td>
</tr>
<tr>
<td>FIGURE 2 C</td>
<td>Cross-sectional diagram of the vessel designed for temperature controlled electron irradiations.</td>
</tr>
<tr>
<td>FIGURE 2 D</td>
<td>Levels of strand-breakage and the time course of strand-break repair at 37°C in <em>E. coli</em> B$_{S-1}$, following electron irradiation and post-irradiation incubation in M/15 saline.</td>
</tr>
<tr>
<td>FIGURE 2 E</td>
<td>Dose-effect curves of initial strand-breakage in B/r and B$_{S-1}$ following irradiation in M/15 saline at 4°C with aerobic and anoxic gassing conditions. Weak alkali.</td>
</tr>
<tr>
<td>FIGURE 2 F</td>
<td>Dose-effect curves of strand-breakage in B/r and B$_{S-1}$ after maximum repair in M/15 saline at 37°C, following irradiation in aerobic and anoxic gassing conditions. Weak alkali.</td>
</tr>
<tr>
<td>FIGURE 2 G</td>
<td>Dose-effect curves of initial strand breakage obtained for <em>E. coli</em> B/r and B$_{S-1}$ following 4°C irradiation in minimal medium under aerobic and anoxic gassing conditions.</td>
</tr>
<tr>
<td>FIGURE 2 H</td>
<td>Dose-effect curves of strand breakage obtained for <em>E. coli</em> B/r and B$_{S-1}$ following irradiation in minimal medium and post radiation incubation at 37°C for 10 minutes.</td>
</tr>
<tr>
<td>FIGURE 2 I</td>
<td>Time course for post-irradiation degradation in B/r and B$_{S-1}$ following irradiation in minimal medium without interruption of growth.</td>
</tr>
<tr>
<td>FIGURE 2 J</td>
<td>Dose-effect curve displaying DNA degradation as the loss of only double-stranded eluting from hydroxylapatite columns.</td>
</tr>
</tbody>
</table>
FIGURE 2 K  Plot of strand-breakage versus incubation time in minimal medium for B/r and Bₛ₋₁, after a dose of 100 Gy in air.

FIGURE 2 L  Dose-effect curve for residual strand breakage in B/r and Bₛ₋₁ following irradiation at room temperature and post-irradiation incubation in growth medium at 37°C for 40 minutes.

FIGURE 2 M  DNA degradation in E.coli B/r and Bₛ₋₁ under the conditions described in figure 2 L.

FIGURE 2 N  Diagrammatic representation of DNA degradation proceeding at the same time as strand-break repair, without influencing the relative amounts of single-stranded and double-stranded DNA eluting from the hydroxylapatite columns.

FIGURE 3 a  Growth curve for E.coli K1060 at 37°C supplemented with oleic acid.

FIGURE 3 b  Growth curve for E.coli K1060 at 40°C supplemented with elaidic acid.

FIGURE 3 c  Survival curve for E.coli K1060 supplemented with oleic acid and irradiated with electrons, in 0.39% saline.

FIGURE 3 d  Survival curve for K1060 supplemented with oleic acid and irradiated in 0.39% saline.

FIGURE 3 e  Survival curve for K1060 supplemented with elaidic acid and irradiated in 0.39% saline.

FIGURE 3 f  Survival curves for oleic and elaidic acid grown E.coli K1060 irradiated in growth medium at room temperature and at ice temperature, followed by immediate dilution and plating.

FIGURE 3 g  Survival curves for oleic acid grown K1060 irradiated at ice and room temperature in growth medium. Samples were diluted and plated at 15 and 85 min. after post-irradiation holding at room temperature.

FIGURE 3 h  Post-irradiation DNA degradation of oleic grown K1060 irradiated in 0.39% saline or C/R buffer conditions, following post-irradiation incubation at 37°C for 30 minutes.
FIGURE 3 i Post-irradiation DNA degradation of oleic grown K1060 with time after irradiation (200 Gy) in fully supplemented growth medium.

FIGURE 3 j Graph representing the strand-breakage dose-effect curve for oleic grown K1060 following irradiation at ice temperature and 40°C in saline followed by a post-irradiation incubation time of 30 minutes at 37°C.

FIGURE 3 k Graph representing the strand-breakage dose-effect curve for elaidate grown K1060 following irradiation at ice-temperature and 40°C in saline, followed by a post-irradiation incubation time of 30 minutes at 37°C.

FIGURE 3 l Dose-effect curve of strand-breakage in oleic grown K1060 irradiated in growth medium at ice temperature or 40°C before and after repair at 37°C for 30 minutes.

FIGURE 3 m Dose-effect curve of strand-breakage in elaidate grown K1060 irradiated in growth medium at ice temperature or 40°C before and after repair at 37°C for 30 minutes.

FIGURE 3 n Dose-effect curve of strand-breakage in linoleate grown K1060 irradiated in saline at ice temperature or 37°C after post-irradiation incubation at 37°C for 30 minutes.

FIGURE 3 o Dose-effect curve of strand breakage in oleate or linolelaidate grown K1060 irradiated in saline at ice temperature or 37°C, before and after repair at 37°C for 30 minutes.

FIGURE 4 A Growth curves of LDV control cells.

FIGURE 4 B Growth curves of a 2nd passage of LDV cells grown on delipidated medium without fatty acid supplements.

FIGURE 4 C Growth curves of LDV cells at 1/1000 FCS following one passage in delipidated medium at 1/100 FCS without fatty acid supplements.

FIGURE 4 D Cross-sectional diagram of the vessel used to irradiate LDV cells with electrons.
14. FIGURE 4 E Circuit diagram for the oxygen electrode used for monitoring the oxygen levels inside the electron irradiation vessel.

FIGURE 4 F Oxygen-electrode chart recording illustrating the rate of oxygen removal from 4 ml of RPMI medium at 37°C in the electron irradiation vessel, during gassing and stirring with Nitrogen flowing over the medium surface at 2 litres/minute.

FIGURE 4 G Graphs obtained using the probe diphenylhexatriene in whole LDV cells. Plots of fluorescence polarization intensity (inversely proportional to fluidity) at 37°C versus optical density at 450 n.m.

FIGURE 4 H Further polarization versus optical density graphs using the fluorescent probe diphenylhexatriene. Measurements were made on whole cells and total cell membrane fractions at 4, 20 and 37°C.

FIGURE 4 I Graphs showing loss in viability of control LDV cells during nitrogen gassing at 2 litres/minute in the electron irradiation vessel.

FIGURE 4 I Graphs showing loss in viability of unsupplemented and oleic supplemented LDV cells. Conditions as for 1-4.

FIGURE 4 I Graphs showing loss in viability of linoleic supplemented LDV cells. Conditions as for 1-6.

FIGURE 4 J 1. Survival of control LDV cells following aerobic and anoxic electron irradiation in RPMI medium.

FIGURE 4 J 2. Survival of LDV cells supplemented with oleic acid, following aerobic and anoxic electron irradiation in RPMI medium.

FIGURE 4 J 3. Survival of LDV cells supplemented with linoleic acid, following aerobic and anoxic electron irradiation in RPMI medium.

FIGURE 4 K Graph demonstrating the effect of irradiation temperature on the survival of LDV cells supplemented with oleic acid and irradiated with electrons.
FIGURE 4 L Dose effect curves summarising the survival of LDV cells following electron irradiation.

FIGURE 4 M Graphs demonstrating the loss in viability of control and linoleic supplemented LDV cells due to the effects of rolling and anoxic gassing conditions inside the roller bottle used for X-irradiations.

FIGURE 4 N Survival of LDV cells (control, oleic and linoleic) following X-irradiation. Aerobic and anoxic gassing conditions.

FIGURE 4 O Dose-effect curve for the survival of LDV control cells irradiated with X-rays and gassed with 1% oxygen in nitrogen at room temperature.

FIGURE 4 P Dose-effect curve for the survival of LDV cells supplemented with oleic acid, irradiated with X-rays and gassed with 1% oxygen in nitrogen at room temperature.

FIGURE 4 Q X-ray survival curve for LDV cells supplemented with linoleic acid and irradiated in 1% oxygen in nitrogen at room temperature.

FIGURE 4 R Log./Log plot of non-protein sulphydryl levels per cell versus cell density of LDV cells, demonstrating the decrease in NPSH content with increased cell density.

FIGURE 5 A Diagrammatic representation of lipid separation and protein segregation below the membrane phase transition temperature.

PHOTOGRAPH P 1 Photograph of Electron irradiation vessel in situ.
<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Dosimetry of irradiation vessels (electrons)</td>
<td>63</td>
</tr>
<tr>
<td>3.1</td>
<td>Table of O.E.R. and $D_0$ values for <em>E.coli</em> K1060</td>
<td>99</td>
</tr>
<tr>
<td>4.A</td>
<td>Details of cell growth for Fatty Acid Analyses : Experiments 1 - 3.</td>
<td>134</td>
</tr>
<tr>
<td>4.B</td>
<td>Details of cell growth for Fatty Acid Analyses : Experiment 4.</td>
<td>135</td>
</tr>
<tr>
<td>4.C</td>
<td>Plating Efficiency of LDV Cells</td>
<td>140</td>
</tr>
<tr>
<td>4.D</td>
<td>Feeder Cell Experiment</td>
<td>141</td>
</tr>
<tr>
<td>4.E</td>
<td>Degassing half-times for the Electron Irradiation Vessel</td>
<td>148</td>
</tr>
<tr>
<td>4.F</td>
<td>Volume Changes occurring within the Electron Irradiation Vessel due to the</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>high gas flow rate of 2 litres per minute</td>
<td></td>
</tr>
<tr>
<td>4.G</td>
<td>Fatty Acid Analysis - Summary of Averages</td>
<td>151</td>
</tr>
<tr>
<td>4.H</td>
<td>Whole Cell Extract Fatty Acid Analysis (Experiment 1)</td>
<td>152</td>
</tr>
<tr>
<td>4.I</td>
<td>Whole Cell Extract Fatty Acid Analysis (Experiment 2)</td>
<td>154</td>
</tr>
<tr>
<td>4.J</td>
<td>Fatty Acid Analysis Nuclei only (Experiment 3)</td>
<td>156</td>
</tr>
<tr>
<td>4.K</td>
<td>Fatty Acid Analysis (Experiment 4)</td>
<td>157</td>
</tr>
<tr>
<td>4.L</td>
<td>Fatty Acid Analysis of Sera</td>
<td>160</td>
</tr>
<tr>
<td>4.M</td>
<td>O.E.R. and $D_0$ values for LDV cells irradiated with electrons</td>
<td>173</td>
</tr>
<tr>
<td>5.A</td>
<td>Fatty Acid Analysis. Nuclear Membrane Changes (Experiment 5).</td>
<td>211</td>
</tr>
</tbody>
</table>
CHAPTER 1.

Historical

1.1. EFFECT OF RADIATION ON DNA

1.1.1. DNA synthesis  Initial work in the late 1950's and early 1960's used a variety of precursors for studying post-irradiation DNA synthesis. They all concluded that DNA synthesis was inhibited. However, it soon became apparent (Smets 1966, Adelstein and Manasek 1967) that different precursors gave different results since the effects of radiation on the precursor metabolism affected DNA synthesis. In bacteria, Billen (1962) and Pollard and Achey (1966) had also demonstrated this apparent reduction in synthesis, but Stavric et al. (1968) considered that the lesser effects on the more radioresistant filamentous 6Y and 12Y E.coli strains might be due to a greater number of completed genomes present in these cells.

Later workers resorted to isolated nuclei, (Matsudaira and Furmo, 1971, Watkins 1972) bacterial-membrane complexes (Stratling and Knippers 1971) and permeabilized mammalian cells (Olson and Billen 1978) to bypass pool effects, and used nucleotide triphosphates as precursors. Matsudaira and Furmo (1971) found that post-irradiation DNA synthesis inhibition was enhanced in an oxygen-dependent and dose-dependent manner, in ascites cell nuclei. Olson and Billen (1978) concluded from precursor uptake and autoradiographic data that a dose of 1000 rads of X rays, delayed initiation of DNA synthesis in replicons while not measurably affecting DNA elongation in permeable Chinese hamster cells. Similar conclusions were drawn by Watanabe (1974) from autoradiographic data obtained in the murine L5178Y lymphoma cell. Precursor studies revealed a biphasic response curve with a sensitive component of $D_{37} = 1,300$ rads and a resistant component of $D_{37} = 10,500$ rads. The resistant component can probably
be related to the chain elongation. Again, essentially identical conclusions were obtained by Laughlin and Taylor (1980) and Painter and Young (1975) using ultracentrifugation techniques. Newly made DNA was of the same molecular weight in both irradiated and unirradiated cells implying a reduction in the number of sites of replication. Synthesis returned to near normal between 2 and 4 hours post-irradiation.

In bacterial DNA-membrane complexes isolated from E. coli B/r, Cramp et al. (1972) found a stimulation of DNA synthesis when isolated complexes were irradiated but a decrease in synthesis when whole cells were irradiated and extracted afterwards. An E. coli K12 strain did not give the same results however (Watkins 1980).

Billen (1969) obtained evidence in E. coli that after X-irradiation, the new initiation site(s) for the onset of DNA replication was induced at points on parent DNA not restricted to the fixed origin.

In summary, current views are that DNA synthesis initiation is temporarily halted at doses relevant to survival, but chain elongation itself is radioresistant.

1.1.2. DNA degradation

The phenomenon of DNA degradation is almost exclusive to micro organisms, and may obscure the process of DNA strand-break repair.

DNA degradation in micro organisms was first shown by Stuy (1961) using H. influenzae, and Miletic et al. (1964) in E. coli. Pollard and Achey (1964, 1966) demonstrated an OER of 4 for degradation in E. coli, and discovered that heating to 70°C before irradiation, prevented degradation. Degradation occurs randomly (Swez & Pollard 1966), leaving only double-stranded DNA (Pollard et al. 1966), is equal in all cells showing it, and involves the
whole DNA chain rather than simple base removal (Pollard and Achey 1967). Hildebrand and Pollard (1969) considered that since maximum degradation involved only 50% of bacterial DNA in bacteria containing the equivalent of two genomes, then only one genome per bacterium was degraded.

In 1968, Pollard and Weller discovered that the DNA degradation product was thymine and not smaller precursors, and that it began leaking out of the cells immediately after irradiation. No concomitant RNA or protein degradation occurred.

Town et al. (1970) demonstrated a greater X-ray sensitivity of log phase E.coli B/r compared to stationary phase. They correlated this with more extensive DNA degradation in the log phase cells. Differences in sensitivity between different phases of the cell cycle was reported earlier by Trgovcevic and Kucan (1969) but this was not always the case. For several strains of E.coli these authors showed a striking correlation between the extent of degradation and loss of colony forming ability. Miletic et al. (1963) and Stavric et al. (1968a) also showed a strain dependent degradation response.

In E.coli, degradation reaches a maximum with dose and then declines above a strain specific dose. This has been shown for low LET radiation (Stavric et al. 1968, Cramp and Watkins 1970), for neutrons (Cramp et al. 1976), and for protons (Huston and Pollard, 1967). Protons proved exceptional in producing up to 100% degradation. Cramp and Watkins (1970) also demonstrated that cell killing could be independent of DNA degradation. Irradiation in the presence of indane trione completely inhibited degradation but enhanced cell lethality. However, although indane trione needed to be present during irradiation to enhance cell killing, a preincubation for 30 minutes followed by extensive washing was still sufficient to inhibit degradation completely. This suggests that indane trione has two
independent effects. The first as a radiosensitizer, causing a greater cell death following irradiation, and the second as an enzyme inhibitor of exonuclease V. If the indane trione binds to the enzyme it would not then be washed out of the cell.

Most DNA degradation is caused by the ATP dependent exonuclease V (Youngs and Bernstein 1973) characterized by Goldmark and Linn (1972), who revealed its composition as two non-identical polypeptide chains. The enzyme is dependent on the presence of the rec B\(^+\) and rec C\(^+\) genes (each possibly coding for the two subunits) which also control recombination (Barbour et al. 1970). Residual DNA degradation in rec B or rec C mutants is believed to be an ATP independent nuclease (Barbour et al. 1970, Youngs and Bernstein 1973, Marsden et al. 1974), but no evidence of this nuclease was found by Coetzee and Pollard (1975) using the respiration inhibitors CO and KCN which completely stopped DNA degradation in the absence of alternative sources of ATP in the medium.

Green et al. (1973) demonstrated that chloramphenicol present 90 minutes before, during and after irradiation prevented DNA degradation to some extent in pol\(^+\) strains, and improved survival. They also showed that chloramphenicol did not act by preventing the induction of a nuclease post-irradiation. Indeed, radiation induces an inhibition of exonuclease V which is controlled by the exr and rec A genes, and which reduces the post-irradiation degradation by the rec B\(^+\) and rec C\(^+\) genes (Marsden et al. 1974, Pollard et al. 1974). This induction is RNA transcription dependent since it was inhibited by Actinomycin D (Grady and Pollard 1967), and also by starvation of an essential amino acid (Pollard et al. 1974) in *E.coli*.

This induced inhibition is responsible for the induced radioresistance first shown by Miletic et al. (1964) in *E.coli* B and confirmed by Pollard and Achey (1975) using UV, ionizing radiation, and
nalidixic acid preincubation, and by Smith and Martignoni (1976). Trgovcevic and Rupp (1974) also demonstrated that a short exposure to heat also induced radioresistance. Although the induction of inhibition of post-irradiation degradation and induction of radioresistance has been demonstrated for E. coli Pollard and Snipes (1977) obtained no evidence of these systems in two lex A\textsuperscript{+} and rec A\textsuperscript{+} Pseudomonas species. Pollard et al. (1975) finally demonstrated that the induction of the inhibitor of DNA degradation, long thought to be associated with the induction of a prophage, was after all, independent of such an event.

The existence of DNA degradation implies that one of the steps associated with the repair of DNA is occurring (such as removal of damaged DNA prior to new synthesis) but gives no good indication of whether or not such repair is biologically effective (Setlow and Setlow 1972). In M. radiodurans the repair of X-ray damage is associated with the breakdown of DNA. At 100% survival there is appreciable breakdown (several percent) of the DNA in irradiated cells before synthesis resumes. The correlation of DNA breakdown with successful repair is also indicated by the effects of incorporated 5-bromo deoxyuridine B\textsubscript{U}d\textsubscript{r} on degradation and survival. The analog has little effect on the production of single-strand breaks or on the rejoining of breaks but it does interfere with the degradation of DNA (Lett et al. 1970). A progressive increase in substitution with B\textsubscript{U}d\textsubscript{r} led to a progressive decrease of degradation and loss of the shoulder, and by 40% substitution the degradation after anoxic radiation is zero. The large shoulder of the survival curve had gone without a change in the survival curve slope.

Literature evidence for mammalian cell DNA degradation is limited. Kuzin (1963) showed an intense depolymerisation of DNA, according to loss of viscosity, in spleen tissue following whole body
irradiation of mice. They considered this to be due to DNase action since by extracting DNA from the irradiated spleen cells with phenol, the drop in viscosity post-irradiation in the extracted DNA did not occur until after two hours, pointing to enzyme interference rather than the direct action of radiation on DNA.

Other attempts to demonstrate mammalian cell DNA degradation have been tried. Dalrymple et al. (1969) at a dose of 1000 rads of X rays on mouse L cells which produced 99% cell killing, could find no evidence for DNA degradation during the 6 hours post-irradiation incubation when sublethal damage was fully repaired. They also noted that the intracellular concentrations of alkaline deoxyribonuclease and DNA polymerase were not increased after irradiation. No DNA degradation could be found by Painter (1968) using doses up to 100 Krad in HeLa cells. A dose of 1 Krad produced no degradation in human amnion cells (Little 1967). However, Ben-Hur and Elkind (1974) following a dose of 10.4 Krad to cultured Chinese hamster cells noted a 6% loss in TCA precipitable DNA after 5 hours post-irradiation incubation at 37°C. This degradation was enhanced by elevated temperatures post-irradiation and 10% degradation was recorded after 5 hours at 42°C for the same dose. This degradation was demonstrable on sucrose gradients at pH 8.6 and was considered not to be related to alkali-labile sites of damage to the DNA. This post-irradiation temperature when continued for more than an hour, enhances X-ray cell killing (Ben-Hur et al. 1974).

In summary, although the extent of DNA degradation has been implicated with cell killing in some bacterial mutants, there is in general a poor correlation with cell survival.

1.1.3. DNA Strand Breaks

Until McGrath and Williams (1966) lysed cells on top of alkaline sucrose gradients, damage to DNA of cells and bacteria had
been studied using DNA extracted by various chemical methods prior to ultra centrifugation techniques. These extraction procedures themselves certainly led to a reduction in size of the DNA. However, phage particles, which have a low molecular weight, could be studied quite readily on neutral sucrose gradients (Freifelder 1965). Freifelder observed breaks in double and single strands of bacteriophage T7 produced by X rays, and concluded that all double strand breaks (DSB's) and no single strand breaks (SSB's) were lethal events. He used an analytical ultracentrifuge for the sucrose gradients and quantitated the DNA by UV absorption.

The use of sucrose gradients both alkaline (pH >12) and neutral in the last fourteen years has been extensive. It has led to a great increase in the knowledge of strand break production and repair, and the effects of alkali, on both DNA unwinding and also on the conversion of base damage to strand breaks above a certain ionic strength.

1.1.3. (a) Single Strand Breaks

In 1966 McGrath and Williams demonstrated that irradiation of the closely related resistant and sensitive E.coli B strains B/r and Bs-1, reduced the molecular weight of DNA sedimenting in alkaline sucrose compared to unirradiated bacterial DNA. Also they interpreted from their results that after post-irradiation incubation at 37°C, only the resistant strain was able to repair broken strands and increase the molecular weight back to the unirradiated control level. These observations led to extensive work by radiobiologists in an attempt to correlate strand breakage data with cell lethality.

Kaplan (1962, 1966) demonstrated that by incorporating halogenated pyrimidines such as 5-bromouracil (5 BU) into E.coli DNA, bacteria became sensitized two- to three-fold to radiation. These bacteria were less able to repair single strand breaks compared to the control, although using neutral sucrose they showed that neither
control cells nor cells with incorporated 5BU could repair double strand breaks. In 1970 Town et al. correlated the greater resistance of stationary phase cells compared to log phase cells of *E. coli* B/r, with a longer and more complete repair of single strand breaks. However, the log phase cells also degraded their DNA more rapidly and extensively. Although bacteria grown with glucose were 3-4 times more sensitive than those grown without glucose, cell death appeared to correlate more closely to a difference in DNA degradation than repair of single strand breaks.

The same authors (1971) made an important advance by discovering that a fast strand-break repair capacity of DNA polymerase I, controlled by the pol A* gene, occurred in buffer at room temperature. This explained why some authors (e.g. McGrath and Williams (1966) as well as themselves) obtained higher values than 60-75 ev required to produce a single-strand break. A similar fast buffer repair system was demonstrated in *Micrococcus radiodurans* by Alexander et al. (1970) with further repair requiring growth medium at 30°C. The buffer repair capacity eventually became known as Type II repair.

Further work on strand break repair in *E. coli* K12 strains (Town et al. 1972, Youngs and Smith 1973) demonstrated the existence of three types of repair. Type I repair which was ultrafast, acted preferentially on anoxic breaks, was independent of polymerase I and could be inhibited by physical or chemical pre-treatment of cells. The inhibition of this repair demonstrated that the initial yield of X-ray induced single strand breaks in vivo is largely independent of oxygen, but that the yield of breaks in anoxic cells is very rapidly modified by repair. Dean et al. (1969) recorded similar data. Using EDTA as a repair inhibitor at 0°C, they demonstrated an OER of 1.0 for strand break production in both *M. radiodurans* and a radiosensitive line of the L5178Y murine lymphoma cells. This corrected their earlier
report of an OER $> 1.0$ (Lett et al. 1967). In repair deficient strains of *E. coli* K12 Youngs and Smith (1973) revealed that pol A$_{1}$ (polymerase I deficient) was also deficient in type II (buffer) repair but not Type III (growth-medium dependent) repair. The exrA strains were not deficient in Type II repair but were deficient in type III repair. The double mutant pol A$_{1}$ exrA was deficient in both type II and type III repair and was more sensitive than either pol A$_{1}$ or exr A both in Air and N$_{2}$. Further considerable Type II repair could occur in pol A, if incubation in buffer was continued after irradiation. This repair was attributed to DNA polymerase III as it was absent in pol A dnaE strains. Town et al. (1973) considering the effect of oxygen on repairability of DNA strand breaks, demonstrated that both Type II and Type III repair was equally effective on aerobically or anoxically produced breaks. Type II repair could repair up to 90% of breaks but Type III apparently could only repair a further two breaks per genome before becoming saturated. Moreover the rec$^{+}$ gene controlling Type III repair was shown to affect the size of the oxygen enhancement ratio (OER). The absence of rec A$^{+}$ sensitizes anoxically irradiated cells to a greater extent than aerobically irradiated ones (OER 3.4$> 2.0$) and thus the rec A$^{+}$ gene must be involved with the repair of damage other than single strand breaks. Since the rec gene systems control the repair of UV induced DNA base damage, the rec A dependent recovery system could involve the repair of X-ray induced base damage. The rec B gene deficient mutant has a high OER (3.1) and the rec B gene product is therefore less involved in this type of repair.

**Anomalies of strand break analysis**

In 1976, Youngs and Smith became aware of a discrepancy in the estimation of the number of strand breaks produced, owing to the discovery that the apparent sedimentation rate of the DNA decreased
as the speed of centrifugation was increased above a certain level (Levin and Hutchinson 1973, Myers et al. 1972). DNA of large molecular weights had to be centrifuged at low speeds to avoid this effect. In addition, the non-random populations of DNA molecules obtained with only small amounts of DNA breakage invalidated the average molecular weight calculations. Hariharan and Hutchinson (1973) noted that because of this speed effect in neutral sucrose gradients, DNA of quite different sizes could sediment together in a gradient. This could readily hide repair of double strand breaks and lead to misleading conclusions. Youngs and Smith (1976) using centrifugation conditions of 21,000 for 4 hours for controls and up to 35,000 rpm for various times for irradiated cells repeated some of their earlier work. They found that they had underestimated the extent of DNA strand breakage by factors of 3.4 - 7.6, depending on the strain and conditions. The new data gave an initial yield of 32.4 breaks/genome/krad (9.0 eV/break) and the extent of type III repair was in fact double the previous estimation. The wild type bacterium had a frequency of 1.58 ssb/2.8 x 10^9 daltons after optimal repair, which is equal to about 3.6 breaks per genome, which does not correlate with single hit kinetics for cell lethality.

When the Smith group turned its attention to mammalian cells, a second defect in the alkaline sucrose gradient technique was exposed. Owing to the much higher molecular weight of mammalian cell genomes compared to bacterial cells (10^{12} vs 2 x 10^9) supralethal doses needed to be administered before the sensitivity of the technique could disclose the production and repair of single strand breaks (Roots and
Smith 1974). In addition, between doses of 5 to 15 Krad in air and 7.5 to 30 Krad in N₂ a varying OER for strand break induction was obtained, according to the conditions of irradiation, and was dependent upon radiation chemical events controlling fixation or restoration of DNA damage. Experiments performed at ice temperature led to the conclusion that if very fast repair occurred it was less than that observed for bacteria under similar conditions.

Palcic and Skarsgaard (1972) measured the effect of oxygen on the number of initial strand breaks in mammalian cells (mouse L60). They purposely avoided the anomalous sedimentation difficulty for unirradiated cells DNA. An OER of 2.9 was obtained for initial breaks and in a later paper (1972a) they showed that if irradiation was at 0°C, no rejoining or degradation took place up to 4.5 hours post irradiation for both aerobically and anoxically produced breaks.

Modig et al. (1974) demonstrated an OER of 2.3 for induction of strand breaks in X-irradiated Chinese hamster cells. Following a 30 minute post-irradiation incubation about 50% of the hypoxic breaks and 80% of oxic breaks were repaired. However the rejoining process was inhibited by 80% if post-irradiation incubation was performed under anoxia. They concluded that there was no correlation between cell recovery and rejoining of DNA breaks. However, several criticisms can be made on this work. Gradient lysis was performed at room temperature in light for 30 minutes, and irradiations were performed at 10°C. At these relatively high temperature conditions some repair could occur and light had been reported to cause strand breaks in DNA under high pH conditions (Elkind, 1970, Sedgwick and Bridges, 1972).

Another problem associated with the alkaline sucrose gradient technique was that a certain dose of irradiation such as 1 Krad was needed before a peak of DNA behaved according to sedimentation theories (Burgi and Hershey 1963). This peak was usually around 2 x 10⁸ daltons and
the preponderance of this peak even in bacteria (where doses are biologically relevant) was originally thought to be due to the existence of set length pieces of DNA interrupted by naturally occurring gaps, non-nucleotide linkers, or alkali-labile bonds (McGrath and Williams 1967). Elkind (1970) and Elkind and Chang-Liu (1972) showed that only after doses of 1440 rads in Chinese hamster ovary cells, did the main peak of $2.5 \times 10^8$ daltons become reduced in molecular weight by radiation induced strand breakage. Reformation of this smaller molecular weight material into the main peak took only 20 minutes at $37^\circ$C. In a later paper Shipley et al. (1971), by using BUdR incorporation, was able to find no close relationship between single-strand breaks and cell death. The BUdR in low concentration reduces the cells' capacity to sustain sublethal damage but at this concentration BUdR had negligible effect on the frequency and repair of single strand breaks.

Brewer and Nygaard (1972) have correlated the level of unrepaired DNA strand breaks with mitotic delay in Physarum polycephalum (slime mould). They felt that the biological response to radiation resulted from the fixation of damage which occurs if a damaged site becomes involved in replication before it can be repaired.

Durand and Olive (1979) could not correlate the enhanced survival of cells irradiated in spheroids as opposed to singly suspended cells, on a difference in capacity to repair strand breaks.

Skin fibroblasts cultured from patients suffering from the genetic disorder Ataxia telangiectasia (AT) are more radiosensitive than similar cells taken from normal subjects. However, the cells from the AT patients are as strand break repair proficient as the normal cells within the sensitivity of experimental techniques available (Lehmann 1977).
In conclusion, the majority of recent work does not support the proposal that unrepaired single-strand breaks are responsible for cell killing.

1.1.3b. Alkali-labile sites

Studies on single-strand breaks using the alkaline sucrose gradient technique also include the conversion by alkali at pH 12.0, of base damage to strand breaks. Bopp and Hagen (1970) demonstrated this effect by irradiating calf thymus DNA in dilute solution with 60Co \( \gamma \)-rays. Enzymes were used to characterize the terminal end groups following breakage in the absence of alkali treatment, and about 90\% of these breaks produced directly by radiation alone carried 5'-phosphate groups, and relatively few were 5'OH groups. The number of breaks and 5'phosphate end groups went up by a factor of 1.5 after 30 minutes treatment with strong ammonia solution (IN NH\(_4\) OH).

In earlier work Simon (1969) had shown that new terminal phosphate groups were formed above pH 7.0. Rhaese and Freese (1968) considered that this may be consequent either to the release of an oxidised terminal sugar from the phosphate or to the fact that new breaks are formed after alkaline treatment (pH 10.0).

In vitro work on phage by Kessler (1971) and Achey et al. (1971) obtained 40\% and 28\% more strand breaks after alkali analysis following oxic and anoxic radiation respectively. They considered this to be the result of indirect action since there was a reduction of alkali-labile breaks when irradiations were performed in the dry state (Kessler) or at -196°C (Achey et al.). This work was done with high doses of radiation. Paterson et al. (1973) were able to look at alkaline-labile strand break production in vivo using phage \( \lambda \) DNA introduced into minicells produced by an \( E.\) coli K12 mutant. The system also allowed an observation of strand break repair by the plasmids own enzymes. They obtained an OER of 3 to 4 for strand break production but
demonstrated that 35% of anoxic breaks were alkali-labile whilst only 20% of oxically produced breaks were labile. This contradicted the earlier in vitro studies of Kessler (1971) and Achey et al. (1971). Paterson found that virtually all of the direct chain breaks were repaired, but none of the alkali induced strand breaks. He suggested that these alkali-labile sites of damage were unsuitable for certain DNA repair enzymes operative in minicells.

In agreement with the work of Paterson, Lennartz (1973) also demonstrated that more alkali-labile sites were produced after anoxic radiation. They demonstrated that 27.8% of breaks produced under oxic conditions were from alkali-labile sites, and 49.8% under N\textsubscript{2}. The difference was explained by assuming that under anoxia more minor alterations in the DNA strand occur leading to strand breaks in alkali, whereas under oxic conditions more breaks are formed directly in the DNA backbone. Destruction of the furanose ring or the liberation of a nucleotide base may be considered such minor alterations.

Lucke-Huhle (1975) established that the major part if not all of the alkali-labile lesions were lethal for the double stranded phage ØX-RF-1-DNA, a finding which to her was not surprising since the toxic effect of alkali-labile sites produced by alkylating agents was well known. This system gave the figure of 22% as the proportion of strand breaks resulting from alkali-labile sites.

1.1.3c. The effect of oxygen on strand break production

The effect of oxygen, and the repair of strand breaks was studied by Johansen et al. (1974) using labelled phage DNA in infected host E.coli. This gave an in vivo phage analysis, and possibly allows an extra method for studying the repair deficiencies of E.coli mutants. They obtained an OER of between 4 and 5 for strand break production in the phage, but were able to show that the oxygen concentration needed to produce half the number of breaks induced in fully oxygenated
conditions (K value) was only 0.5 μM for phage in wild type and uvrA and recA mutants, compared with the much higher value of 8 μM for the host cell killing. This suggested that DNA lesions other than strand breaks were responsible for cell death in the presence of oxygen. This result was also found by Millar et al. (1980) in V79 Chinese hamster cells, using the rapid-lysis technique (see below 1.1.3d).

However, Chapman et al. (1974) demonstrated that the oxygen K value for cell killing and initial strand break production was identical in Chinese hamster cells. Their K value of 2.8 μM is intermediate to the two values quoted above. They obtained an OER of 2.9 for cell killing, and strand break production, and suggested that breaks in the presence of oxygen were qualitatively different from those obtained in the absence of oxygen. Chapman showed that 75% of oxic breaks were produced by indirect effect of the hydroxyl radical, and 25% more from direct breaks.

The advent of the rapid (within a very short period after irradiation) lysis techniques (1.1.3d below) allowed a more realistic time scale for the assessment of DNA strand break production and repair. Using the phage in vivo technique Johansen et al. (1975) demonstrated an OER of 4.0 for strand breaks within 100 msec post irradiation. The energy requirement per break was 12-16 eV and the same yields were obtained in phosphate buffer at 3°C and 22°C, and for growth medium at 37°C. The yields remained the same in the polA1 mutant. This data also questioned the ultrafast repair suggestion of Town et al. (1973). These authors suggested that Town's inhibitors were chemical or physical rather than biological, and would consequently be affecting differences in induction rather than repair.

1.1.3d. Rapid lysis techniques

These techniques essentially consist of a very rapid addition of the irradiated biological system to an ice-cold strong alkali-
detergent solution by hydraulic pressure.

Sapora et al. (1975), using a rapid lysis technique, were able to modify the ideas of Town, Smith and Kaplan (1970-1973) on bacteria. They showed that between 0 and 0.2 m secs post-irradiation, single strand breaks remained constant with an OER of 3.6. There was no sign of ultrafast repair. After 2 sec. there was both excision and strand break rejoining which was polymerase 1 dependent. They were unable to clarify the chemical events prior to 2 m secs.

Further work by Sapora et al. (1977) on E.coli B/r and Pol A− demonstrated that 1.3 radiation induced endonuclease sensitive sites were present for each initial strand break, and that oxygen was needed for this enzymatic action. Oxygen was dose modifying for strand breaks and endonuclease sensitive sites. Since the polymerase 1 dependent repair of strand breaks and the conversion of fixed damage to strand breaks by endonuclease activity occurred at the same time, the use of Pol A− strains allowed the evaluation of the breaks produced solely by endonuclease activity. Polymerase 1 activity in B/r repaired 90% of radiation induced breaks and 100% of endonuclease breaks, and left 0.7 and 4 ssb/single strand genome/Krad for B/r and Pol A− respectively.

The requirement of an oxic state for the proper functioning of some repair enzymes has been referred to already (Modig, 1974). Slower strand break rejoining in anoxia is also reported by Boye et al. (1974). Anoxia also inhibits Elkind or split dose repair in mammalian cells (Hall, 1972), chlamydomonas (Bryant, 1970) and the repair of single strand breaks in V79 Chinese hamster cells (Koch and Painter, 1975). Matsudaira et al. (1970) showed that Ehrlich ascites tumour cells could not repair strand breaks while held in anoxia, and correlated this to the reduction in ATP levels within the cells. Glucose could partially restore ATP levels and strand break repair. It has already been noted in the section on DNA degradation that the
exonuclease V responsible for degradation was ATP dependent. It would be interesting to determine if the limited repair of strand breaks in the rec A rec B mutant of *E. coli* was affected by anoxia. Millar et al. (1980) demonstrated that in V79 Chinese hamster cells the maximum yield of single strand breaks occurred at an oxygen concentration enhancing cell death by only 1.9. This cell line was unusual in having a biphasic relationship between $O_2$ concentration and cell death, with a plateau at an enhancement ratio of 1.9 over the range 1.5 to 7 $\mu$M $O_2$ (Millar et al. 1981). Below 1.5 $\mu$M a linear relationship between number of strand breaks and dose occurred.

Adams et al. (1976) (also using rapid mix techniques) have shown that the presence of oxygen is required for a much shorter time prior to irradiation for the maximal effect on single strand break production in mammalian cells than for the full OER on survival.

A general conclusion from these rapid mix studies is that there is no close correlation between the production of initial strand breaks and cell death.

In addition the role of oxygen is to enhance the fixed damage which is removed by endonuclease activity.

1.1.3e. **Double Strand-Breaks : production, repair and relevance to cell lethality**

Neutral sucrose gradients are traditionally used to measure double strand breaks and their repair. Repair has been demonstrated in some systems but not in others. Burrell et al. (1971) demonstrated the induction and repair of double strand breaks in *Micrococcus radiodurans*. The induction of double strand breaks by X-rays was found to be 520 eV/break and repair seemed efficient after sublethal doses up to 200 Krad in oxygen. In 1971 Wilkins showed that $\alpha$ rays were 2.4 times more efficient than X-rays at producing double strand breaks in *E. coli* B/r, with the respective yields being 0.19 and 0.08 double
strand breaks per 100 eV of dose absorbed in the DNA. Since the induction of single strand breaks was similar, he calculated that X-radiation produced 7.8 single strand breaks per double strand break, and α-radiation gave a ratio of 1.2:1. He calculated that the average number of double-strand breaks produced by the mean lethal dose of alpha and X-rays, 6.7 and 3.7 Krads respectively, was 0.8 and 3.3. He therefore concluded that a double-strand break would be the predominant lethal event after X-rays, and that some overkill would result from α rays since one α particle crossing the E.coli nucleus would produce about 3.2 double-strand breaks per chromosome.

Similar data was obtained by Bonura et al. (1975a). The E.coli K12 wild type JG 139 was irradiated with 50 kVp X-rays and 660 keV γ rays (137Cs) and the RBE effect on the production of DNA damage, repair, and cell killing was determined. Under aerobic conditions the 50 kVp soft X-rays were 1.47 times more lethal, and produced 1.93 times more double-strand breaks. Although the initial number of single strand breaks was identical, there were 1.57 x more single-strand breaks after full repair with soft X-rays. When a pol A1 strain was used there was still 1.33 times more unrepaired single-strand breaks with soft X-rays.

Work on E.coli (Krasin and Hutchinson 1977, Kirsch et al. 1976) demonstrated that double-strand break repair required a functioning recA+ gene and the presence of a duplicate genome. Their wild-type Wtab2497 repaired up to 3 to 4 double strand breaks per genome following 125I decay whereas no repair occurred in recA13 AB2487. A 1:1 correlation was found between cell killing and the number of post incubation residual double-strand breaks.

Ulmer et al. (1979) looked at the repair of double strand breaks in the DNA of E.coli K12 strains proficient and deficient in recombination repair. In the biologically significant dose range of
5 or 10 Krad of γ rays, the rec A+ wild type AB 2497 was able to repair double strand breaks whereas the deficient recA 13 AB2487 could not. The value of 2.7 initial double strand breaks/ genome/lethal event suggested that between one and two double-strand breaks were repairable in the wild type AB2497. These authors used such gentle lysis techniques that they could estimate the number of double strand breaks per lethal event without extrapolating from higher doses. Youngs and Smith (1976) reported that AB2497 was more radioresistant than their choice of strain (W3110) which might explain their inability to demonstrate repair of double strand breaks in this strain (Bonura et al. 1975b).

Krasin and Hutchinson (1977) were also able to imply from bacterial work that more than two or more genome equivalents were needed for double strand break repair (i.e. that repair depended on recombination).

Suhadi et al. (1972) investigated double strand break production and repair and its effect on survival in radiosensitive mutants of Micrococcus radiodurans. The initial number of double strand breaks were identical for the mutants and the wild type but the repair capacity of the sensitive mutants was limited and much slower. The authors associated the enhanced killing with delayed DNA repair, rather than no repair at all.

Experimental evidence from the investigation of strand breaks has led to the advancement of two theories of radiation action involving DNA as a primary target. Kellerer and Rossi (1971) have proposed that a double strand break produced by a single ionization event in DNA, is the lethal event. However, Chadwick and Leenhouts (1973) consider that an interaction between two separate events is needed to produce the lethal event: a dual target hypothesis. The rest of this section lists the data which supports one or other of these two hypotheses.
Munson et al. (1967) suggested that the proportion of double strand breaks (Type II damage) to single strand breaks (Type I damage) would increase with increasing LET. In addition Kellerer and Rossi (1971) considered that with increasing LET an increase in efficiency of cell killing should be evident, and that this would decline once a state of overkill was in operation. The deposition of energy over an increasingly large area may allow the breakage of both strands of DNA at once, and they considered that a double strand break caused by a single ionization event in the DNA would be the lethal event in a cell. Thus evidence was sought for a linear relationship between dose and double strand break production.

Alternatively Chadwick and Leenhouts (1973) proposed a theory of dual radiation action, such that with increasing LET two separately damaged but repairable targets could interact to form a lethal type of damage. To support this theory experimental evidence would be required to show a quadratic relationship between the number of double strand breaks produced and dose. Evidence has accumulated to support both ideas, and the followers of one theory are critical of data assessments of the other and vice-versa.

Corry and Cole (1973) obtained data for double strand breaks in Chinese hamster ovary cells. They speculated that since 1 rad $^{137}$Cs $\gamma$ rays appeared to give 0.15 - 0.5 double strand breaks per cell, then these must be repairable as 50-100 rad was not lethal. By incorporating chloroform and diethyl ether in their neutral sucrose gradients they were able to dissociate the slowly sedimenting very-radiosensitive complex reported by other workers (e.g. Elkind 1971) and look at production and repair of double strand breaks up to 50 Krads. Their results suggested full repair of double strand breaks up to 10 Krads but that doses above 50 Krad began to inactivate the repair system. Thus at 200 Krad no repair could be seen. This made them
critical of other work (Lehmann and Ormerod, 1970, Veatch and Okada 1969) which had shown lack of double strand break repair, using supralethal doses on mammalian cells and extrapolating the data back to the survival region of dose. Since they obtained a linear dose-response curve for double strand break production they concluded that double strand breaks were caused by a single hit event and not by interaction of two single strand breaks. Lehmann and Ormerod (1970) also obtained this linear relationship in L5178Y mouse cells.

Bonura et al. (1975b) demonstrated a linear increase of double strand breaks with dose in *E. coli* K12 JG139, an OER of 2.79, and a value of 532 eV per double strand break in air. This energy value was three times lower than that of Lennartz et al. (1973) and they considered that mechanical shearing of radiation induced single strand breaks could cause double strand breaks. This possibility has been demonstrated by Hayward (1974). Bonura et al. (1975b) also demonstrated that 1.3 to 1.4 double strand breaks per genome were produced at the $D_0$ dose and felt that double strand breaks could be lethal events. They estimated that only 5% of their double strand breaks could have been produced by the interaction of two single strand breaks.

Gillespie et al. (1975) and Dugle et al. (1976) considered that double strand breaks were responsible for death but the theory of dual radiation action by Chadwick and Leenhouts (1973) suggested that this hypothesis was untenable on microdosimetric grounds. This theory implied the requirement of the interaction of sublesions over distances two orders greater than the diameter of the double helix. However, using low speed gradients to overcome anomalous sedimentation Dugle et al. (loc. cit.) discovered that after repair at all doses, two unrepaired single strand breaks remained per double strand break in Chinese hamster cells. The number of double strand breaks was
also proportional to the square of the dose between 10 and 50 Krads; the expected correlation with the theory of two interacting single strand breaks.

Ritter et al. (1977) using Chinese hamster V79 cells, were able to observe strand break production and repair for a variety of LET values between 1 and 1953 keV μm⁻¹. When the efficiency for the induction of non-rejoining breaks was normalised to the value obtained for X-rays, a relative efficiency for break induction as a function of LET was obtained.

The peak response for this curve was similar to published data on the killing of some strains of bacteria and yeast; for induction of mutation in yeast, for killing mammalian cells in vitro and induction of chromosome aberrations in mammalian cells in vitro. Since a peaked response implies that two or more radiation target interactions are required to induce the effect they concluded that the non-rejoined breaks must be at least in part, double strand breaks. This conclusion, however, does partly assume that all double strand breaks need two hits which may not be the case for high LET radiation at least. Other possible double lesions could be closely associated single strand breaks, or a single strand break close to base damage or a cross-link on the complimentary strand. This paper produced much discussion and this was highlighted by Goodhead et al. (1978) in a letter to Nature, which had a reply from the original authors published at the same time (Ritter et al. 1978). Goodhead et al. felt that Ritter et al's comparison of high dose strand break repair data with low dose survival showing 'single hit' inactivation was not justified. They considered that at these high doses the sublethal interactions following multi-hit kinetics would be more closely associated with their data. Ritter's reply was that the initial slope, single hit mechanism of survival was
equally operative at all doses, and that since at high doses the percentage of non-rejoining breaks was constant with respect to dose, the linear relationship could be extrapolated back to low dose levels. They considered that multi-hit sublethal damage was a consequence of unrelated lesions.

Van der Schans (1978) irradiating several types of phage in vitro with X rays provided evidence that between 16 and 34 nucleotides were needed between opposite single strand breaks to prevent a double strand break, and suggested that the interaction was caused by the temporal local denaturation accompanying single strand breaks or in addition base damage which labilized the DNA helix. This data supports the dual radiation action theory.

Frankenberg-Schwager et al. (1980) using yeast, found that the linear relationship between DNA-double strand breaks and radiation dose (30 meV electrons) was converted to a quadratic function by cellular repair. It was also shown (by using split dose experiments) that double strand breaks remaining after 72 hours were unrepairable. This work gave evidence that only diploid yeast cells could repair double strand breaks, and that the recombinational repair processes involved required the presence of two homologous chromosomes.

Calculations by Frankenberg to explain the quadratic function suggested that two double strand breaks in the two homologous chromosomes could not be repaired when these breaks were closer than 10,000 base pairs. This data was gained in the survival curve dose range.

Another approach to the study of double strand breaks is the use of phage both in vitro and in vivo. Where irradiation is performed within the host bacteria, repair properties of the bacterium may be involved with the repair of phage. The points in favour of phage work in strand break studies is that they have a lower molecular weight DNA than bacteria and mammalian cells and their DNA is consequently more
shear resistant. Their use also overcomes the problem of anomalous sedimentation of high molecular weight DNA. Where the phage DNA exists as a circle, the breaks resulting in single or double strand breakage may be separated in neutral sucrose, and thus eliminate alkali induced breaks. Three forms may be produced. Species I is a covalently bound circular monomer containing several superhelical twists; species II is a circular DNA with one or more single strand breaks (a relaxed circle); and species III is a linear DNA molecule resulting from double strand breakage. These forms can be separated in neutral sucrose gradients and this allows the determination of single and double-strand breakage in the same gradient.

Boye (1980), Boye and Krisch (1980) measured double strand break production and repair in circular superinfecting Phage \( \lambda \) after irradiation in host cells. They demonstrated that \(^{125}\text{I}\) decays produced 0.39 single strand breaks: 1 double strand break whereas each \(^3\text{H}\) decay caused 0.2 single strand breaks and very few double strand breaks. In comparison 4 MeV electrons gave 42 single strand breaks per double strand break. No repair of double strand breaks was observed using the neutral sucrose gradient methods, although single strand breaks produced by \(^3\text{H}\) decay or 4 MeV electrons were repaired. No quadratic dose dependence of double strand break induction was found up to 500 Krad in oxygen and up to 1800 Krad in nitrogen. Since no difference in double strand breakage was observed between 60 and 80 Krad whether radiation was of 1 second duration or intermittently to allow repair, they concluded that the mechanism whereby two independently induced single strand breaks pair to form a double strand break was not significant in the biological dose range. Reviewing the single strand break data to date, Boye (1980) pointed out that results on double strand break repair in the chromosomal DNA of \( \text{E.coli} \) was conflicting, but the experiments showing
non repair of chromosomal double strand breaks involved relatively high radiation doses or were encumbered with sedimentation abnormalities. Only in vitro work appeared to give a linear-quadratic correlation between double strand breaks and dose, and he agreed with the Kellerer and Rossi (1971) theory that double strand break production followed single-hit kinetics. He was aware that the possibility remained that double strand breaks could be induced enzymatically by a slow repair process.

A different approach to DNA damage was used by Schafers and Kohnlein (1979), where they studied the loss in transfecting ability of B. subtilis phage Ø29' and SPPI. Biological inactivation occurred even though there were only 0.3 double strand breaks per genome. Biological activity was also lost to a measurable extent after irradiation doses which were less than 2 Krads, which is within the biological dose range. The results showed that other forms of DNA damage other than strand breaks could affect biological activity of DNA, although damaging events interfering with the uptake of DNA into the competent cell, or interfering with the obligatory primary recombination processes may not be relevant to cell lethality.

Thus although the pathway by which double strand breaks are produced remains in contention, there is still support for a role of double strand breaks in causing cell lethality. However, much of the evidence which purportedly favours double strand breaks as being the lethal event, derive from work where supralethal doses were given. In consequence some caution is needed in interpreting these results.

1.2. EFFECTS OF RADIATION ON MEMBRANES OR MEMBRANE CONSTITUENTS

Radiation effects on membranes are primarily a consequence of induced peroxidation of the unsaturated fatty acid constituents of the membranes. Also, unsaturated fatty acids undergo autoxidation at their
double bonds forming a free radical and losing a hydrogen atom H⁺.
The fatty acid free radical can then react with molecular oxygen to form a peroxide free radical, which in turn can react with another fatty acid to give a hydroperoxide and a fatty acid free radical. The process of radiation induced peroxidation is initiated by OH⁺ radicals produced from the radiolysis of water.

\[
\begin{align*}
RH \text{ (fatty acid)} & \rightarrow R^* + H^* \\
R^* + O_2 & \rightarrow RO_2^* \\
RO_2^* + RH & \rightarrow ROOH \text{ (hydroperoxide)} + R^*
\end{align*}
\]

\[
\begin{align*}
\text{RADIATION} & \\
H_2O & \rightarrow OH^* + e^- + H^+ \\
OH^* + RH & \rightarrow H_2O + R^* \\
OH^* + e^- & \rightarrow OH^- \\
OH^* + OH^- & \rightarrow H_2O_2
\end{align*}
\]

Hammer and Wills (1979) demonstrated that 2000 >10,000 Gy had little effect on saturated fatty acids but caused destruction of 98% of the highly unsaturated fatty acids (20:4, 20:5, 22:6) and 46% of the doubly unsaturated acids (18:2) in herring oil. This destruction was accompanied by an increase in lipid peroxide formation. Naturally occurring antioxidants such as glutathione and vitamin E will more readily give a hydrogen atom to the radiation induced free radicals from water than will a fatty acid molecule, and these substances therefore prevent the chain reactions associated with peroxidative processes.

Oxidative damage is dose rate dependent (Nakazawa and Nagatsu 1980) and a very sharp rise in the extent of oxidative damage occurs below 100 rad/min (Raleigh et al. 1977). In general, yields in chain reactions are inversely proportional to the square root of the dose-rate.
(Spinks and Woods 1976). Wills and Wilkinson (1967) observed the effects of radiation on lipid peroxide formation in subcellular fractions, and the quantity of peroxide formed was in the order microsomes > lysosomes > mitochondria > nuclei.

Several reports have demonstrated changes in membrane permeability both in cellular systems and model membranes. Watkins (1970) recorded the loss of acid phosphatase from isolated lysosomes and demonstrated an OER of 7-8 in this system. A delay of about 40 minutes occurred before any enzyme leakage and this compares favourably with the slow build up of peroxide recorded by Wills and Wilkinson (1967). Lysosomes exposed to autoxidising linoleic acid release enzymes in direct proportion to peroxidative damage (Tappel, 1973). Potassium release from irradiated erythrocytes has a low OER (~1.2, Nakken 1966) but peroxide formation in these membranes has not been associated with the permeability changes observed (Meyers and Bide, 1966, Sutherland and Pihl, 1968). Nakazawa and Nagatsuka (1980) have recorded a correlation between lipid peroxidation and loss of glucose from irradiated liposomes. Lipid peroxidation increases membrane lipid rigidity and negative surface charge, and membrane fluidity may be lowered by 20-30% following lipid peroxidation. The modification of the physical state of membrane lipids has been correlated with the loss of membrane functions, such as permeability (Dobretsov et al. 1977, Grzelinska et al. 1979, Vladimirov et al. 1980).

The effects of antioxidants on radiation induced lipid peroxidation has been extensively surveyed. Mice fed on a vitamin E deficient diet proved to be more radiosensitive than normal mice (Konings and Drijver, 1979) and X-irradiation in vivo shortened the lag period prior to rapid lipid peroxidation as measured in vitro. The length of the in vitro lag prior to peroxidation in various subcellular fractions was shown to be proportional to the amount of antioxidant present (Konings and
Oosterloo 1980). Vitamin E proved to be a more powerful protector than reduced glutathione (GSH) against oxidative damage in irradiated liver liposomes (Konings et al. 1979) and the unsaturated fatty acids arachidonic (20:4) and docosahexanoic (22:6) proved to be the most vulnerable. The effect of oxygen varied with dose rate in this liposomal system, the OER being 13 at 0.8 Gy/min and 5 at 8 Gy/min.

Konings and his colleagues have also demonstrated that the radiosensitivity of tumour cells grown in vivo or in vitro is decreased by the presence of vitamin E. (Konings and Trieling 1977, Fonck and Konings 1978). Vitamin E has also been shown to reduce erythrocyte fragility in vivo (Prince and Little 1973, Hoffer and Roy 1975). Although lipid peroxidation has definitely been correlated with subcellular and whole animal survival, the reduction in cell killing in the presence of antioxidants was not great.

1.3. EVIDENCE FOR TWO OR MULTIPLE TARGET INTERACTION

1.3.1. Effect of lipid peroxidation on other macromolecules

Tappel (1973) has recorded that proteins and enzymes undergo polymerization reactions, polypeptide chain scission, cross-linking and amino acid changes when exposed to oxidising lipid. He considered that malonaldehyde was possibly responsible for intra and inter-molecular protein cross-linking. When DNA was exposed to peroxidising arachidonic acid (20:4) structural changes occurred as measured by decreased melting point, decreased hyperchromicity, partial resistance to hydrolysis by DNase and by decreased template activity for RNA polymerase (Reiss and Tappel, 1974). DNA actually behaves as an antioxidant in the presence of oxidising liposomal suspensions (Pietronigro et al. 1976 and 1977) and has a synergistic antioxidant effect in combination with tocopherols in inhibiting the oxidation of methyl linoleate (Ikeda and Fukuzumi 1977).
Alper (1963, 1971 and 1974) has proposed that membrane and DNA may be radiation susceptible targets relatively sensitive and relatively insensitive to the presence of oxygen, respectively. It could be that indirect damage to DNA subsequent to DNA-membrane interaction, is the explanation for the enhancement of the radiation killing effects by oxygen (OER). In support of this theory some experimental evidence suggests that the peripheral region of the cell nucleus is the most sensitive part of the cell (Zerneno and Cole 1969; Datta et al. 1976) and this could be a site at which DNA and membrane are in close proximity. Further, Pietronigro (1977) has demonstrated that \emph{B. subtilis} transforming DNA lost its biological activity in a manner dependent on the magnitude of lipid autoxidation occurring, when incubated with oxidising liposomal suspensions. This last report was a great stimulus to the studies of altering the membrane fatty acid composition of cells and bacteria reported in this thesis.

1.3.2. \textbf{Membrane complexes}

(a) \textbf{Bacteria}

Alper postulated (1968, 1969, 1971) that the nucleic acid DNA could be defined as an 'N' type site of radiation action which was little affected by the presence of oxygen, and in addition there was an 'O' type site, (likely to be the cell membrane) damage at which was enhanced by the presence of oxygen.

Following the isolation by Godson and Sinsheimer (1967) of a membrane-complex containing most of the bacterial DNA from \emph{E.coli}, and the strong indications that DNA synthesis and initiation occurs at the bacterial cell membrane (Comings 1968, Ryter et al. 1968), Cramp et al. (1972, 1973 and 1975) used this system to observe any interaction between 'O' type (membrane) and N type (DNA) damage. Cramp's group showed (1972) that the effect of electron irradiation in depressing DNA
synthetic capabilities of the isolated complexes from E. coli B/r and
B5-1 had an OER of about 8.0, when complexes were isolated from the
irradiated cells. These results supported the Alper (loc cit) hypo-
thesis for dual target interaction. 20 Krads in air and 80 Krads in
N2 produced maximum depression of synthesis. However, low doses up to
50 Krad given directly to the isolated complex had no effect, and
higher doses stimulated DNA synthesis. Only at doses above 200 Krad
was an inhibition of DNA synthesis observed.

Further studies on this complex using radiosensitizers and
protective agents (1973) showed that cell survival and DNA-membrane
complex synthetic activity were not necessarily equally affected,
supporting the conclusion that other radiation sensitive sites occurred
within the cell, (Cramp and Walker 1973).

An evaluation of the complex isolation techniques used by
Okazaki (1970) and Stratling and Knippers (1971) led to Robertson et
al. (1978) questioning the role of the DNA-membrane attachment in
dNA synthesis. He demonstrated that the high OER of the radiation
induced reduction in DNA synthetic activity of membrane complexes was
not dependent on the attachment of DNA to membrane. Although this
questions the validity of using DNA synthesis as an endpoint of damage
to the complex, it does not necessarily refute its possible role in
dNA membrane complexes in the interaction of macromolecular damage.
The lack of an irradiation effect on the composition of the complex
agreed well with work by Myers and Johnson (1974). Continued work
with a modified complex isolation procedure (Robertson and Watkins,
1979) demonstrated that the onset of DNA synthesis coincided with the
dissociation from membrane of at least 70% of the genome DNA and all
of the nascent DNA in E. coli B/r and was concomitant with the action
of a nuclease on parental DNA. A radiation enhanced DNA synthetic
activity in the E. coli K12 strains (Watkins 1980) has prevented a
satisfactory comparison with the B strain results.

Following the discovery by Lark (1972) that the RNA attached to the DNA-RNA-membrane conglomerate of *E. coli* must be fully synthesized before new DNA-synthesis could begin, Cramp and Bryant (1974) tested the effects of rifampicin (an RNA polymerase inhibitor) on the survival of *E. coli* B/r and B$_{5-1}$ and the ability of their membrane complexes to synthesize new DNA after irradiation. The only effect of rifampicin was to greatly enhance the lethal effects of both electrons and neutrons on *E. coli* B/r under anoxic conditions. No effect was observed on killing of B$_{5-1}$. Only the DNA synthetic activity of the DNA membrane complex isolated from B/r irradiated with neutrons under anoxia was affected, and this exhibited a marked reduction in activity. The results suggest that part of the repair capabilities of B/r are dependent on the synthesis of an RNA molecule. The unexpected membrane-complex results implied that some reduction in RNA synthesis led to an enhancement of the effect of neutrons on B/r by increasing the proportion of type 'N' damage contributing to the reduction of DNA synthesis.

The discovery of structural roles of RNA have been made by several authors. Stonington and Pettijohn (1971) isolated the genome of *E. coli* in a protein-RNA-DNA complex. The DNA was in a highly folded conformation which unfolded in the presence of alkali or RNase. Although Worcel and Burgi (1972) suggested that the folded bacterial chromosome was stabilised by a single RNA core, Pettijohn and Hecht (1973) suggested that several RNA molecules would be required to both stabilize the folds in the DNA and segregate the DNA into domains of supercoiling which had been observed by both groups of workers.

Finally, Fralick and Lark (1973) presented data implicating the involvement of unsaturated fatty acids in initiating chromosome
replication in E.coli. However, Thilo and Vielmetter (1976) were not able to confirm this observation and criticised the use by Fralick and Lark, of an inhibitor which could also affect saturated fatty acid synthesis and general cell metabolism.

1.3.2. (b) Mammalian cells

Evidence of mammalian cell complexes which contain DNA, and undergo some kind of repair during post-irradiation incubation, was apparent from anomalous sedimentation properties of high molecular weight DNA. This has already been discussed in part under single-strand breaks, and the involvement of double-stranded DNA in alkaline conditions is referred to in the introduction of Chapter 2.

In 1972, Elkind and Chang-Lui studied the damage and repair, after X-rays, of a complex shown to be composed of lipid and DNA, and up to a dose of 194 rads they remained linked together. At about 388 rads the lipid and DNA began to disassociate. Following a dose as high as 722 rads they observed a time dependent reassociation repair of this lipid-DNA complex. These doses of 100 to 1500 rads used to resolve the complex, all within radiobiologically relevant cell survival levels, were not large enough to produce a detectable proportion of DNA breaks in this species during lysis. Since 10 to 20 times smaller doses were needed to damage the complex compared to the more rapidly sedimenting bulk DNA released from the complex, they deduced that the target size of the complex must be 2 to 4 \times 10^9 daltons.

Poppit and Fox (1977) have also demonstrated the occurrence, in P388F cells, of a nucleoprotein-like material, highly sensitive to X-rays, and partially reconstituted on post-irradiation incubation.

It is possible that repair of these complexes, which may be a reattachment of DNA to membrane, may mean the difference between survival and cell death in mammalian cells. Strand break determinations are
usually only possible at supralethal doses and may not be realistically related to cell death.

This type of DNA membrane-complex has been demonstrated in bacteria. Burrell et al. (1971) demonstrated that repair of a complex similar to that reported by Poppit and Fox (loc cit) which contained lipoprotein in *Micrococcus radiodurans*, was proportional to dose, and they speculated that this was evidence of repaired DNA re-attaching to membrane and that this prevented cell death.

The evidence considered in this section supports the hypothesis that there is interaction between macromolecules such as membrane, DNA and RNA in modifying initial radiation damage and in a co-operative attempt to recover from lethal events.
CHAPTER 2.

Strand break repair in *E. coli* B/r and B_{s-1} as detected by hydroxylapatite chromatography.
SUMMARY

In 1966, McGrath and Williams used alkaline sucrose gradients to demonstrate that the resistant \textit{E. coli} B strain B/r was apparently able to repair strand breaks to a greater extent than the more sensitive strain B$_{s-1}$. These authors introduced the concept that resistance to radiation was dependent on a proficiency of strand-break repair, and vice versa. However, these authors were not aware that DNA degradation had occurred during the repair time of their experiments, and that this had led to a misinterpretation of their data. I have repeated these experiments on \textit{E. coli} B/r and B$_{s-1}$, using the hydroxylapatite technique devised by Ahnstrom and Erixon (1974) to assess strand breaks, and also taken into account the occurrence of degradation. The results demonstrated that under all repair conditions the two bacterial strains were equally capable of repairing strand breaks. However, after complete strand-break repair following irradiation in growth medium, DNA degradation then occurred. At a dose of 10 Krad this degradation occurred earlier and to a greater extent in B$_{s-1}$ than B/r, and masked their equivalent strand-break repair capacities. It was also deduced that degradation probably occurred from sites other than strand breaks.

It was concluded that there was no correlation between strand break production and repair, and that some other site of damage causes the difference in radiation sensitivity between \textit{E. coli} B/r and B$_{s-1}$.
CHAPTER 2.

Progression from alkaline sucrose gradients to the hydroxylapatite technique:
A reassessment of DNA strand-break repair in E.coli B/r and B_{s-1}, using hydroxylapatite chromatography.

2.1. INTRODUCTION

As stated in Chapter 1, the traditional method for measuring DNA strand-break production and repair is the alkaline sucrose gradient technique first applied in radiobiology by McGrath and Williams (1966). These authors reduced mechanical shearing effects on DNA by performing lysis on top of the gradients. In this paper, there was an apparent demonstration that a radioresistant strain of E.coli (B/r) was more able to repair radiation induced strand breaks than the related sensitive strain, E.coli B_{s-1}. This observation has led to determined efforts using the alkaline sucrose gradient technique to demonstrate in many eukaryotic and prokaryotic cell lines, a correlation between proficient strand break repair and relative radioresistance. However, gradual refinements to the gradient technique occurred. An awareness of light induced strand breakage of DNA in alkali (Sedgwick and Bridges, 1972) and a better understanding of the behaviour of DNA in an alkaline environment has led to a partial rejection of this method and the emergence of a more versatile technique which avoids the problems associated with alkaline sucrose gradients. This technique, which involves the use of hydroxylapatite chromatography was first described by Ahnstrom and Edvardsson (1974). I have used this technique to demonstrate that the original conclusions drawn by McGrath and Williams (1966) were incorrect and that E.coli B/r and B_{s-1} are equally proficient at repairing strand breaks induced by radiation. The bulk of the data
has already been published (Ahnstrom, George and Cramp, 1978). Prior to presenting this data, however, I have outlined the major issues of controversy surrounding alkaline sucrose gradient measurements, which culminated in the development of the 'Ahnstrom hydroxylapatite technique'.

In theory, when alkaline treated DNA is centrifuged through an alkaline sucrose gradient it is considered to be single stranded and to sediment at a rate proportional to its molecular weight. Molecular weight, sedimentation coefficient, and distance travelled in a gradient may be related by the equations,

\[ \frac{S_2}{S_1} = \frac{M_2}{M_1} k \]

where \( S \) is sedimentation coefficient, \( M \) is molecular weight, and 1 and 2 denote two DNA's.

\[ \frac{D_2}{D_1} = \frac{M_2}{M_1} k \]

where \( D \) is the distance travelled in the gradient.

\( k \) is a constant in each case.

These formulae work very well for phage DNA, as discussed by Burgi and Hershey (1963). In principle, nature has provided in the phages a series of readily available DNA's of virtually precalibrated molecular weights. However, high molecular weight DNA above approximately \( 2 \times 10^8 \) daltons does not sediment according to theory. This DNA may be slowed down by viscous drag or may speed up as a consequence of molecular interactions dependent on concentration (Burgi and Hershey, 1963).

Elkind and Kamper (1970) experienced a very slow sedimentation of high molecular weight DNA from Chinese hamster cells and blamed this on an attachment to lipid. In contrast, McBurney et al. (1972) found that DNA released from unirradiated mouse L cells sedimented at about 500S, and was considered to be only loosely aggregated and possibly only partially single stranded. Only after irradiation, when the molecular weight had been reduced by strand breakage, did all these authors obtain
DNA which sedimented according to theory. McBurney and Whitmore (1972) were particularly concerned that the gradient technique yielded molecules of a much smaller dimension (about 2 to $5 \times 10^8$ daltons) in the hands of most researchers, than the size of DNA suggested from autoradiographs. McBurney et al. (1972) obtained a high molecular weight following centrifugation through a neutral sucrose layer before mouse L cells were lysed. However, the sedimentation patterns indicated that it was not single stranded. Davison (1966) showed that DNA unwinding in alkali at pH 12.2 slowed down rapidly above DNA molecular weights of $2 \times 10^7$ daltons. McBurney et al. (1972) concluded from this and from work done on DNA melting kinetics by Spatz and Crothers (1969) that their 500s ($= 5 \times 10^9$ - $10^{10}$ daltons) molecule would not be completely unwound after 16 hours at $4^\circ C$ (pH 12.0). This they proved by labelling one strand of their cell DNA with bromodeoxyuridine (BUdr) and inducing strand breaks uniquely to this strand by exposing the cells to fluorescent light. In alkali, the BU strands separated from the other strands (labelled with $^{14}C$ Tdr) which now appeared to have single stranded molecular weights in excess of $2 \times 10^9$ daltons.

In 1973 Ahnstrom and Erixon also demonstrated the incomplete unwinding of DNA in strong alkali. They lysed Chinese Hamster cells for 30 minutes at room temperature in 0.5M NaOH 0.1 MEDTA and then neutralised the reaction mixture by centrifugation through neutral buffered sucrose solution at pH 7.0. Incompletely separated strands were expected to renature in the neutral conditions (Davison, 1966) whereas completely separated single strands would renature at a much slower rate. This second renaturation was limited by using low DNA concentrations. Single and double stranded DNA arising from this procedure was then separated on hydroxylapatite columns according to the technique established by Britten and Kohne (1968). To prevent the renaturation of incompletely unwound DNA during chromatography the
samples were sonicated immediately after neutralisation.

Whereas most of the DNA from unirradiated cells exposed to this denaturation procedure eluted as double stranded material, irradiation up to 1 Krad produced an increase in single stranded material in proportion to dose, with 15-20% remaining double stranded at doses above this level. This latter material was considered to be partly alkali resistant, and partly a consequence of renaturation of repetitive DNA at 60°C occurring during the hydroxylapatite chromatography. Whatever the reason it was clear that complete denaturation into single-stranded DNA is very difficult. This paper also demonstrated that irradiated cells repaired DNA strand breaks extensively since after a post-irradiation time at 37°C in medium the hydroxylapatite chromatography subsequently showed only double stranded DNA. The results prompted these authors to devise a procedure of determination of DNA strand breaks which would avoid the difficulties encountered with strong alkali, and this is described by Ahnstrom and Edvardsson (1974).

This modification uses weak alkaline conditions for "unwinding" (0.02 to 0.03M NaOH) and assumes that single-strand breaks serve as untwisting points during separation. Since complete unwinding was still impractical because of the close packing of the DNA molecules, they proposed and proved that for a time-limited treatment, the DNA transformed to single-strand would be proportional to the number of strand breaks (single and double) present. However, to relate the percentage of single-stranded DNA formed by alkali treatment to the absolute number of strand breaks, a comparison would still be needed with identically treated cells subjected to velocity sedimentation. A linear relationship between dose and % single stranded DNA was observed between 50 and 2000 rads.

Unlike the work by Davison (1966) already mentioned with phage, higher molecular weight DNA is affected by intermolecular interactions
during strand separation and renaturation of DNA. Ahnstrom interpreted his results according to the following, as depicted in figure 2A. The untwisting of DNA starts from all strand breaks, and once a whole fragment between two breaks is unwound, it is released from the bulk DNA, resulting in the loss of two untwisting points. Neutralisation preserves the status quo of the unwinding process. Important observations noted in this paper were that the strand separation rate increased 2-3 fold by a rise in temperature from 4°C to 20°C, and the presence of 0.98 M NaCl increased the rate by a factor of ten. Attached cells gave poorer results than cells in suspension probably due to an effectively higher concentration of DNA released from a monolayer of cells. Finally these authors report that following a dose of 900 rad of X rays to Chinese Hamster cells, all strand breaks appeared repaired in 45 minutes at 37°C, whereas a substantial amount of breaks remained after 2 hours following a 4000 rad dose of neutrons although the initial number of breaks for both qualities of radiation were the same. Control cells already appeared to contain 700 to 2000 strand breaks per diploid genome, according to gradient calibration.

Rydberg (1975) comprehensively analysed the method of Ahnstrom and Erixon (1973). He was able to demonstrate that the amount of unwinding was proportional to the dose of 60Co X rays from 10 rads to 20 Krads using three different lysis procedures on Chinese Hamster fibroblasts. Experiments were completed at ice temperature such that repair of breaks was kept to a minimum Rydberg considered the rate limiting factor in DNA unwinding to be the friction of the rotating single-stranded portions. He assumed that the double-stranded segments did not rotate. The rate of unwinding also slowed down once the DNA between two breaks was fully unwound and caused the loss of two unwinding points.

McGrath and Williams (1966) overlooked one other major factor of importance. As described in Chapter 1, many irradiated bacteria degrade
Figure 2 A.

A schematic representation of the DNA unwinding technique for determining strand-breakage.
some of their DNA, and Yatvin et al. (1972) showed that when McGrath and Williams plotted gradient profiles as a percent of radioactivity recovered from the gradient rather than as a percent of the unirradiated bacterial DNA label, repair could be overestimated. Also, since DNA degradation is strain and dose dependent it would not be possible to correlate DNA breaks with cell death using a single dose of 20 Krads as attempted by McGrath and Williams (1966). Figure 2B (Cramp and Watkins, 1970) indicates that at 20 Krads B/r degrades nearly twice as much DNA as B_{S-1}. Yatvin et al. (1972) suggested that the lower molecular weight DNA following irradiation in B/r was the DNA degraded, leading to a false impression that all DNA was converted back to a higher molecular weight. Horan et al. (1972) obtained by selective mutation of B_{S-1}, a strain (PHHI) that did not degrade much of its DNA after irradiation at 20 Krads. This strain was more radioresistant than B/r and also repaired strand breaks to the same extent as B/r.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of bacteria

The bacteria were grown and labelled with $^{3}H$ thymidine in minimal salts medium in a similar way to Cramp and Watkins (1970). Cultures of bacteria were maintained on a 4% Blood Agar Base slope at 4°C and resloped every 3 to 4 months. Overnight cultures of 25ml of medium were prepared by adding 5ml of minimal salts medium, 2ml of a 10% glucose solution, and 20ml of distilled water to a 100ml conical flask. Each constituent was stored sterilised at room temperature in 100ml medical flats, and minimal medium pH 7.0 contained 27.5g KH$_2$PO$_4$, 57.5g Na$_2$ HPO$_4$, 3.19g COONa $\cdot$3H$_2$O, 0.625g Mg SO$_4$·7H$_2$O, 5.0g (NH$_4$)$_2$ SO$_4$ made up to 1 litre with glass distilled water.

The cultures were inoculated using a flame sterilised platinum wire loop which was scraped over the surface of a slope culture. Flasks were incubated overnight at 37°C on a rotary shaker. Next day, 1ml of
DNA degradation in *E. coli* B/r and B<sub>S-1</sub>, measured by loss of TCA-precipitable material following 90 minutes post-irradiation incubation at 37°C in minimal medium.
the overnight culture was added to 25ml of prewarmed medium (or 1ml to each 25ml multiple thereof) and after 30 minutes deoxyadenosine and $^3$H-thymidine were added to final concentrations of 320 ug/ml and 4 uCi/ml respectively. $^3$H-thymidine (TRK 418) was purchased from the Radiochemical Centre, Amersham. Growth was continued for 90 minutes and the bacteria harvested at 37°C at 2,700 rpm in an MSE MISTRAL 6L centrifuge. The bacteria were then resuspended and growth continued for 30 minutes in fresh 'cold chase' medium containing the deoxyadenosine and 100ug/ml of cold thymidine.

Irradiations were either performed in this cold chase medium at room temperature or the bacteria were recentrifuged at 37°C and resuspended in 0.2mM EDTA 0.39% NaCl for 15 minutes at ice temperature before washing by millipore membrane filtration and resuspending in ice cold 0.39% NaCl. Resuspending 25ml cultures in 2ml gave an approximate concentration of $10^{10}$ bacteria/ml.

2.2.2. Irradiation

Irradiation was at ice temperature, using an electron beam (7 MeV) from the MRC Linear Accelerator at a dose rate of 50 Krad/minute. Bacteria suspended in buffer were irradiated in specially made water jacketed vessels (Fig. 2C). To prevent strand break repair during the irradiations, iced water was pumped through the outer jacket by means of a peristaltic pump. Effluent water was still at 4-5°C. Otherwise, the vessels were essentially identical to those initially used by Cramp and Watkins (1970). For the later cold chase medium experiments, bacteria were irradiated in 0.25ml aliquots in polystyrene tubes in a perspex irradiation box containing melting ice, such that the sloping tubes were supported at their necks by the vertical side of the box and the bases were flush against the perspex front side facing the electron beam.
A cross-sectional diagram of the irradiation vessel illustrating the water jacket designed to regulate the temperature during irradiation.
2.2.3. Dosimetry

The dose received by the vessels was determined by the technique of ferrous ammonium sulphate dosimetry. 0.9804g of ferrous ammonium sulphate was added to 11.12ml of concentrated sulphuric acid (Analar) in a total volume of 500ml of distilled water. 30mg of NaCl was then added to this stock solution. Cleaned vessels, and sufficient glass conical sampling tubes, were left soaking overnight in this solution, rewashed with distilled water, and then refilled with 3.5ml of the iron solution. Irradiation of this solution produces the ferric ion which absorbs at an O.D. of 304 nm. The absorbed dose in rads is calculated from the equation

\[ 2.94 \times 10^4 \times (1 - 0.007t) \times \text{O.D. 304} \]

where \( t \) = the temperature in °C minus 20, at which the optical density is read. On this day the temperature was 25°C.

\[ = 2.94 \times 10^4 \times (1 - 0.007 \times 5) \times \text{OD 304} \]
\[ = 2.837 \times 10^4 \times \text{OD 304}. \]

Irradiations were performed at 20 Krads/minute and 5 Krad doses were given to 5 separate 3.5ml aliquots in each of 4 different vessels. The results and estimated absorbed doses are recorded in Table 2.1.

2.2.4. Post-irradiation Incubation

To allow strand break repair post-irradiation incubation was at 37°C for 30 minutes in the non-nutrient 0.39% saline when degradation was only 0-10%, or in minimal medium at 37°C when degradation in the lower dose-range was up to 50% after incubating for 60 minutes. For incubations in minimal medium, 0.25ml of double strength prewarmed minimal medium was added to 0.25ml of bacteria. In the case of the vessel irradiated cells, these were also dispensed into plastic tubes. Neither maintaining the temperature at 4°C or at 37°C under these conditions (Cramp and Bryant 1975), nor the treatment of EDTA changed the survival characteristics.
<table>
<thead>
<tr>
<th>Vessel</th>
<th>Dose</th>
<th>Divisions</th>
<th>O.D. 304 nm.</th>
<th>Estimated Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 Krad</td>
<td>100</td>
<td>.138</td>
<td>4.167 Krad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.153</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>103</td>
<td>.142</td>
<td>= 83.34%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99</td>
<td>.150</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>99</td>
<td>.133</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>.1432</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 Krad</td>
<td>99</td>
<td>.146</td>
<td>4.15 Krad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>.138</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>99</td>
<td>.138</td>
<td>= 83%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.143</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>.148</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>.1426</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5 Krads</td>
<td>101</td>
<td>.148</td>
<td>4.406 Krad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.147</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>.155</td>
<td>= 88.12%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>.154</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>.153</td>
<td></td>
</tr>
<tr>
<td>Av</td>
<td></td>
<td></td>
<td>.151</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5 Krads</td>
<td>100</td>
<td>.134</td>
<td>4.074 Krad</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.140</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>99</td>
<td>.137</td>
<td>= 81.48%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.143</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>101</td>
<td>.146</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>.140</td>
<td></td>
</tr>
</tbody>
</table>
from those obtained when bacteria were maintained at room temperature prior to growth on agar plates. Post-irradiation incubation of bacteria irradiated in cold chase medium was straightforward. Survival curves for \( B/r \) and \( B_{S-1} \) in cold chase medium were identical to survival reported by Cramp and Bryant (1975), in saline/phosphate buffer.

Following incubation, cells in medium were centrifuged at 4°C and washed once with 1ml 0.39% NaCl (at 4°C) prior to resuspension in 0.25ml 0.39% NaCl (or 0.98% where necessary) at 4°C. This washing was to remove the medium which buffers against the pH of the weak alkali. More bacteria already in 0.39% NaCl were cooled to 4°C in an ice bath.

2.2.5. **Alkaline lysis**

To determine the extent of DNA unwinding a modification of the technique described by Ahnstrom and Edvardsson (1974) was employed. Where repair was in saline, initial and final strand breakage was determined by adding 0.25ml of ice-cold 0.06M NaOH 1% Sarkosyl 0.39% NaCl to 0.25ml of the ice-cold bacterial suspension in 0.39% NaCl, in an ice-bath. Bacteria repaired in medium were either treated as above or were treated with 0.06M NaOH 1% Sarkosyl 0.98% NaCl. These bacteria had been resuspended in 0.98% NaCl after repair harvesting.

Lysis was allowed to proceed in the dark at 4°C, and the lysis solutions were then vigorously neutralised by squirting into each tube 1.2ml of ice-cold 0.2M NaH₂PO₄. Samples were then sonicated on an MSE sonicator for 10 seconds and 0.1ml of 2% sodium dodecyl sulphate added. Samples were then frozen at -20°C overnight.

2.2.6. **Hydroxylapatite Chromatography**

Samples were thawed slowly in cold water and eluted through 100mg of hydroxylapatite (Biorad HA grade) contained in a 1ml syringe equipped with a long 19 gauge needle and held in an electrically heated aluminium block at 58 - 60°C. Hydroxylapatite powder was held in place by a disc of porous polythene cut to size, and was washed first with 3ml of the
strongest elution buffer and then 3ml of the weakest elution buffer before loading. Up to 20 samples could be eluted at any one time as the block held 20 syringes.

It was important to keep the hydroxyapatite damp at all times, and particularly to elute double stranded DNA as soon as possible after the single stranded DNA. Allowing the hydroxyapatite to dry with sample still loaded led to anomalous results and some sticking of the DNA to the hydroxyapatite.

Either 1ml or all the sample was added to the preheated syringes as soon as possible after thawing. Eluates were collected in glass scintillation vials and the aluminium blocks were so constructed such that a 10 x 10 scintillation vial tray would fit snugly underneath it for convenience of sample collection.

Following elution of the sample the column was rinsed with 3ml of 0.012M potassium phosphate buffer (ph 6.8). The single stranded DNA was then separately eluted with 3ml of 0.125M buffer, and the double stranded DNA eluted with 0.25M phosphate buffer. Columns were used up to three times on the same day by cleansing between use with 3ml 0.5M phosphate buffer followed by 3ml of the 0.012M buffer.

To each eluate an equal volume of Instagel scintillant (Packard) was added, the vials capped and shaken, and the samples counted at 4°C on an LS250 Beckman Scintillation Counter. The wash through was also counted and usually yielded 10-15% of the loaded counts. These were considered to be labelled triphosphates from the triphosphate pool, since reloading onto a fresh column yielded almost a full recovery in the first wash.

The use of SDS in the samples was to restrict DNA protein binding and also to prevent this binding affecting the retention of the DNA by hydroxyapatite.
2.2.7. **DNA degradation**

DNA degradation following irradiation in growth medium without interruption of growth, has not been reported before. 1 ml samples of \(^3\)H Tdr labelled bacteria after various times of post-irradiation incubation were precipitated with 2 ml aliquots of ice-cold 10% Trichloroacetic acid in glass test tubes, and maintained at 4°C overnight. Precipitates were then filtered through glass fibre filters (Whatman G.F.C. 2.5cm) and washed with 0.39% NaCl. Filters were then counted in 5 ml of Instagel. Degradation was expressed as the percent of TCA precipitable DNA compared to control values. Degradation estimates from the loss in radioactivity eluting in single strand and double strand (SS and DS) form from hydroxylapatite columns were also made, and were in close agreement.

2.3. **RESULTS**

2.3.1. **Post-irradiation incubation in non-nutrient 0.39% NaCl.**

The value obtained for initial breaks, and the time course of their repair in *E. coli* B\(_{s-1}\) are illustrated by the typical results shown in Fig. 2 D. The extent of strand breakage is expressed by the function \(-\log ds/(ds + ss)\) where \(ds\) is the radioactivity in counts per minute eluted from the HA column by the 0.25M phosphate buffer, and is proportional to double-stranded DNA. SS is the equivalent c.p.m. eluted by 0.12M phosphate buffer and is proportional to single-stranded DNA. The value of this function increases with increasing dose as the amount of single-stranded DNA increases and double-stranded DNA decreases. The rate of repair was identical for B/r and repair was deemed completed at 30 minutes. Dose effect curves are therefore illustrated for initial damage at zero time and for residual damage after 30 minutes once repair is completed, for each set of irradiation conditions. Figs. 2E and 2F relate respectively, initial damage and damage remaining after repair reached a maximum, for both *E. coli* B/r and B\(_{s-1}\), and also for aerobic and
Graph illustrating the reduction in DNA unwinding with post-irradiation incubation time, after irradiation and incubation at 37°C in M/15 saline for E.coli B5-1. As the extent of unwinding increases with dose, the value of $-\log \frac{ds}{ds+ss}$ increases and, conversely, as repair of breaks occurs and the extent of unwinding decreases, this function decreases.
Dose-effect plots using the value of $-\log \frac{ds}{ds+ss}$ from hydroxylapatite gel separation from samples treated with weak alkali immediately after irradiation. Over the dose range used, linear plots are obtained.
Graph illustrating the amount of unwinding \(-\log\left(\frac{ds}{ds+ss}\right)\) obtained when the repair of strand breaks has reached a maximum. Irradiation and post-irradiation incubation was in M/15 saline (0.39\%, 0.066M).
anoxic gassing. Both strains of bacteria gave nearly identical responses, and as can be seen the oxygen enhancement ratios of the measured effects were between 6 and 8. This figure is much greater than for cell survival (OER = 2.0 for B₅₋₋ and 4 for B/r) and demonstrates that damage sustained in aerobic irradiation conditions may be slightly less easily repaired than anoxic damage, while the bacteria are held in non-nutrient suspension.

Under these post-irradiation conditions, DNA degradation was limited to a maximum of 10% after doses of 20 Krad in air or 45 Krad in nitrogen. A check on the amount of degradation could be achieved by expressing the amount of radioactivity in c.p.m. eluted as double-stranded and single-stranded DNA from an irradiated sample, as a percentage of the same c.p.m. obtained from a control sample.

2.3.2. Post-irradiation incubation in minimal-growth medium.

Following repair in minimal medium, lysis with 0.03M NaOH did not show up any residual strand-breakage over lysis times of 2.5 to 60 minutes. Only when the salt concentration was increased to 0.15M did evidence of unrepaired breaks appear. Dose-effect curves for 10 minute lysis times with 0.03M NaOH 0.5% Sarkosyl 0.15M NaCl are shown in figure 2G for initial breaks, and 2H for residual breaks. These results are the average of four experiments.

Identical results were obtained (fig. 2G) when the bacteria were irradiated at ice temperature in 0.15M NaCl or in minimal medium followed by immediate lysis (after centrifugation and washing, before resuspension in 0.15M NaCl). The lower dose scale of fig. 2G gives an indication of the sensitivity of the technique. If the bacteria were allowed to repair in warm minimal medium for 10 minutes, extensive repair was observed, even at high doses. Even after 60 minutes, when degradation was up to 50%, little change was recorded in the values of -log ds/(ds + ss). Thus the remaining DNA was
Dose-effect plots of $-\log \left( \frac{ds}{ds+ss} \right)$ obtained when repair is minimised by irradiating at ice-temperature, and lysing immediately afterwards. Lysis was in 0.15 M NaCl, 0.03 M NaOH, 0.5% sarkosyl. Note the low dose scale.
Dose-effect plots of \(- \log \left( \frac{ds}{ds+ss} \right)\) obtained when repair of strand breaks has occurred in minimal medium at 37°C for 10 minutes. Lysis was in 0.15 M NaCl, 0.03 M NaOH, 0.5% sarkosyl.
representative of the whole with regard to strand-break frequency and repair. Figures 2G and 2H demonstrate that the induction and repair of strand-break damage was nearly identical for B/r and Bs⁻⁻ with OER's of between 2.65 and 2.57 respectively following repair. Since the initial strand breakage OER's were 6.1 and 5.6 one can conclude that a greater percentage of strand breaks induced in the presence of oxygen are repaired than those induced under anoxia.

2.3.3. Results following irradiation in cold chase medium

Over the dose range 2-10 Krads in air, Bs⁻⁻ degraded more DNA than B/r and also began degradation at an earlier time. The time course of post irradiation degradation is shown in figure 2I after a dose of 10 Krads.

Where no time was allowed for strand break repair post radiation, the amount of strand breakage following 37°C irradiation was no greater than obtained with unirradiated bacteria. This implied that very rapid and complete repair had occurred during the irradiation time and before the bacteria were cooled to 4°C. For room temperature irradiations, full repair was completed within 5 minutes post irradiation incubation at 37°C, following a dose of 10 Krads. However, after 15 minutes Bs⁻⁻ began to degrade its DNA. This was revealed in the measure of strand breakage recorded by hydroxylapatite chromatography, and was evidently due to a specific reduction in the amount of double-stranded DNA eluting from the columns. This is demonstrated in figure 2J. Figure 2K expresses an apparent increase of DNA strand breakage during post irradiation incubation at 37°C over a period of 40 minutes. Under these circumstances there is a considerable difference in the amount of strand breakage between B/r and Bs⁻⁻ at low doses (Figure 2L). However as soon as the dose is high enough to cause degradation in B/r, an apparent strand breakage begins in this strain and increases with dose at the same rate as in Bs⁻⁻. Figure 2M displays the extent of
Figure 2.1

Time course for DNA degradation in *E. coli* B/r and B<sub>s-1</sub> following a dose of 100 Gy in minimal growth medium. Plot of TCA insoluble DNA against time of post-irradiation incubation in minimal growth medium (M.M.).
Figure 2 J.

Graph illustrating the correlation between DNA degradation and loss of double-stranded DNA.

![Graph](image)

Bacteria were irradiated in medium and allowed 60 minutes to repair at 37°C. Degradation seen as a loss in double-stranded (DS) cpm eluting from the hydroxylapatite column.

Crosses represent $B_{s-1}$. Circles represent $B/r$. 
Plot of log function of single-stranded DNA, which is proportional to strand breaks, against time of incubation in minimal growth medium.
Log function of single-stranded DNA obtained after 40 minutes incubation in minimal medium plotted against irradiation dose in Grays, showing a dose threshold before strand breaks occur in B/r.

TCA insoluble DNA plotted against dose following 40 minutes repair incubation at 37°C. No degradation occurs in B/r below doses of 60 Gy.
degradation which has occurred over this 40 minute post irradiation incubation at 37°C. When the alkali in the lysis procedure was omitted but the sonication step was retained, the bacterial suspensions in which degradation was at a maximum showed no detectable single-stranded DNA, confirming that degradation occurred on both strands simultaneously, possibly from residual double strand breaks.

2.4. DISCUSSION

The results are not easy to interpret as DNA degradation accompanies strand-break formation and repair. In non-nutrient conditions, a minimal amount of degradation occurs during the post-irradiation incubation period in which strand breaks are equally repaired by the two strains. Resuspension in minimal medium produces more degradation, up to 50% in one hour, and this is unequal between the bacteria. Nevertheless strand break repair, although not complete, appears to be equal in the remaining DNA. Presumably, even at 50% degradation, the residual DNA was representative of the whole with regard to strand break frequency and repair. Under any kind of post-irradiation incubation in minimal medium, the extent of unwinding, both for B/r and B_s-1, was very similar, with almost identical oxygen enhancement ratios (OER's) of between 2.4 and 2.8. However, a different picture emerged when irradiation was in growth medium. On this occasion the difference in timing and extent of degradation between the strains (earlier and to a greater extent in B_s-1) produced an apparent difference in the amount of strand-break repair. As mentioned in the results, in this situation the change in the values of -log DS/(DS + SS) appeared to be mainly a consequence of loss in the amount of radioactivity eluted as double-stranded DNA. The single-stranded fraction appeared to remain constant (figure 2J) and this suggests that the number of unwinding points is unaffected by degradation. This is difficult to comprehend as one would expect that the unwinding points producing
Diagrammatic representation of DNA degradation proceeding at the same time as strand break repair. The extent of unwinding in residual DNA should be independent of molecular size and indicate the relative number of breaks.
background unwinding in unirradiated controls would be randomly spaced throughout the DNA. Perhaps these single-stranded counts are so low compared to the initial double-stranded counts, that a small but real fluctuation in their level is obscure.

Before degradation has occurred, both bacteria have rapidly repaired all strand breaks down to the level of unirradiated bacteria. Thus there are fewer breaks under these conditions compared to when bacteria were irradiated in saline and incubated in medium. This probably means that degradation occurs in DNA that has been fully repaired. If one assumes that there is a complete repair as indicated by our technique, then degradation may occur from some marker other than a strand break. This could either be a different type of DNA lesion or a particular site on the bacterial genome not necessarily damaged itself by radiation. Alternatively the hydroxylapatite technique may not have been able to distinguish very low levels of strand breakage.

An attempt to prevent this DNA degradation using indane trione (Cramp and Watkins 1970) failed since the lowest concentration preventing degradation proved to be toxic. This would have allowed me to see if strand breaks reappeared during the period of time when degradation occurred.

Since omission of the alkali from the lysis procedure did not yield any single-stranded DNA, then degradation must degrade both strands simultaneously whether the lesion causing degradation extends over one or both DNA strands. Pollard et al. (1966) also demonstrated that no single-stranded DNA was exposed by degradation. In addition, since an equal percentage of DNA is unwound after conditions of minimal and maximal degradation for bacteria irradiated in saline and incubated in minimal medium, then degradation must remove DNA containing an equal proportion of unwinding points as the non-degraded
DNA. This implies that degradation continues past unrepaired single-strand breaks, and that single-strand breaks are considerably more numerous than sites for degradation.

Pollard and Fugate (1978) have independently published further work on the induction of inhibition of post-irradiation degradation, and its effect on strand break repair. They have also demonstrated that the repair process was more rapid than the degradation, and when degradation was inhibited, more repair was apparent. Cells that were lex−, which includes Bs−1, and AB 2476, could not be induced for inhibition of degradation. However, repair could clearly be seen after short times, and this repaired DNA was then degraded, suggesting that the signal for DNA degradation was not a strand break.

In 2.3.2. it was noted that breaks produced in aerobic conditions were repaired in growth medium to a greater extent than breaks produced under anoxia. Hesslewood (1978) also obtained this result using the hydroxylapatite technique in mammalian cells (L5178YR and S), and this is in agreement with the earlier publications of Modig et al. (1976) and Koch and Painter (1975). This observation suggests that more than one type of single-strand break is produced, and that less repairable breaks are produced only in, or to a greater extent in aerobic conditions of irradiation. Also, since the OER following break repair in saline is high (~8.0) and is low after repair in medium (~2.6), it follows that some breaks produced in aerobic conditions require nutrient or growth conditions for their repair.

Since the OER for strand breaks under any set of conditions was equal between B/r and Bs−1, and since the OER for cell lethality is higher in B/r (OER 4.0) than Bs−1 (OER 2.0) there is no correlation between strand breaks and cell lethality.
In conclusion, *E. coli* B/r and B$_{5-1}$ are equally proficient in strand-break repair, as measured by the hydroxylapatite technique, which suggests that other damage to DNA or damage elsewhere is responsible for their differences in radiosensitivity. The original work by McGrath and Williams (1966) using the same bacterial strains as used here, should no longer be interpreted as evidence that bacteria proficient in repairing DNA strand breaks are more resistant to irradiation. These authors omitted to account for DNA degradation. I have continued the work of this thesis, by considering the role that membrane fluidity might play in influencing bacterial and mammalian cell radiation sensitivity.
CHAPTER 3.

The influence of membrane fluidity on the induction and repair of DNA strand breaks in the unsaturated fatty acid auxotroph *E. coli* K 1060.
SUMMARY

E.coli K1060, an unsaturated fatty acid auxotroph, was used to study the effect of membrane fluidity on survival and DNA damage after exposure to electron radiation.

Oleic, elaidic, and other unsaturated fatty acids, were incorporated into the bacteria, and the temperatures of irradiation chosen in order to give the maximum difference in survival between the 'liquid crystal' and 'gel' states. Maximum sensitization was achieved by cooling the bacteria for 5 minutes at melting ice temperature before irradiation. No sensitization was observed if cooling was after irradiation. Under non-nutrient conditions bacteria grown on any of four unsaturated fatty acids tested, were able to repair DNA strand breaks to the same extent. When irradiation was in nutrient conditions, oleic and elaidic acid grown bacteria (the only ones tested) at all temperatures of irradiation, repaired DNA strand breaks equally well and to a greater extent than in non-nutrient suspension.
CHAPTER 3.
---------------

The Influence of Membrane Fluidity on Radiation Induced Changes in the DNA of E. coli K1060.

3.1. INTRODUCTION

The selection and isolation of unsaturated fatty acid auxotroph mutants of E. coli by Silbert and Vagelos (1967), has led to a greater understanding of the role that fatty acids play in membrane structure and function (Fox 1972). The fatty acid composition of E. coli membranes is almost entirely palmitic (16:0), palmitoleic (16:1, cis $\Delta^9$) and cis-vaccenic (18:1, cis-$\Delta^{11}$) (Fox 1972) but there is a low percentage of other fatty acids such as linoleic (18:2) and 16:2 (Yatvin et al. 1979). Unsaturated fatty acid auxotrophs grow in media containing a variety of unsaturated fatty acids including oleic (cis-$\Delta^9$ - 18:1) and elaidic (trans-$\Delta^9$ - 18:1) and their membranes can be altered quite readily. Esfahani et al. (1969) demonstrated that a 30-40% increase in the saturated/unsaturated fatty acid ratio occurred as the growth temperature was increased from 27°C to 42°C. This phenomenon, also demonstrated in wild type bacteria (Fox 1972), is thought to reflect a regulatory mechanism for maintaining the proper fluidity of the fatty acid side chains in the membrane lipids. The hydrocarbon side chains of these fatty acids undergo a transition from a more ordered (gel) to a less ordered (liquid crystal) state with increasing temperature, as revealed by a change in the X-ray diffraction pattern observed in the isolated membranes. Temperature transitions have also been shown to occur in the transport rate of glycosides in E. coli auxotrophs, and the transitional mid points vary according to the
unsaturated fatty acid supplied for growth (Wilson et al. 1970, Schairer and Overath 1969). Such transitions are marked by kinks in the Arrhenius plot of the log of the glycoside transport rate versus the inverse of the absolute temperature, and are interpreted as changes in membrane fluidity.

Yatvin (1976) was the first to demonstrate that the radiation sensitivity of *E. coli* could be dependent on membrane fluidity. When an auxotroph, *E. coli* K12 K1060, was grown exclusively on either oleic (cis 18:1) or linolenic (cis 18:3) unsaturated fatty acids, the bacteria had equal sensitivities at room temperature. However, the sensitivity of linolenic grown bacteria remained unaltered when the irradiation was at ice temperature (3-5°C) whilst the oleic grown bacteria were more sensitive at this lower temperature. Overath et al. (1970) have established the phase transition temperature of linolenate grown K12 auxotrophs as 4-6°C whereas the transition temperature of oleate grown auxotrophs was 13°C-15°C (Overath et al. loc cit., Wilson et al. 1970). Further work by Yatvin et al. (1979) reported the effects of a number of unsaturated fatty acids (18:3, 18:1, trans 18:1 and trans 16:1) and showed that survival after γ radiation above the transition temperature (Tt) was comparable for these fatty acid supplemented K1060s. On the other hand, below the transition temperatures (as reported by Schairer and Overath 1969, and Wilson and Fox 1971) the bacteria were sensitized to γ radiation. The authors concluded that membrane fluidity was important for cell survival. Although not reported by Yatvin and his colleagues, Redpath and Patterson (1978) demonstrated an increase in radiation sensitivity at room temperature when the same strain of bacteria was grown in fatty acids of increasing unsaturation. The radiation conditions used by Redpath and Patterson; room temperature irradiation,
oxygen and nitrogen gassing, and a dose rate of 1-2 Krad/minute were very comparable to Yatvin's (23°C, 1.8 Krad/minute). The increase in sensitivity recorded by Redpath was most marked in nitrogen (anoxic radiation conditions). This finding was considered to be dependent on lipid peroxidation rather than fluidity, and the subject of peroxidation has been detailed in Chapters 1 and 4.

Ghoulipour-Khalili (1978) failed to demonstrate temperature dependent radiation effects in several strains of *E.coli* K12 (N 1167, W3110 and HMT) and Yatvin (1979) believed that the presence of low levels of 16:2 and 18:2 unsaturated fatty acids in these strains (5-8%) were sufficient to prevent membrane gelling at ice temperature in wild-type strains.

Order-disorder thermal phase transitions of membrane phospholipids have been monitored using both X-ray diffraction and membrane fluorescent probes (Overath et al. 1975), and the transition is thought to derive from the ordering of the phospholipid hydrocarbon chains.

An additional phenomenon resulting from this thermal phase transition, has been termed lateral phase separation (Shechter et al. 1974, Letellier and Shechter 1976). Using freeze-fracture electron microscopy of *E.coli* fatty acid auxotroph membranes, these authors demonstrated lipid and protein segregation at temperatures below that of the phase transition. They concluded that at temperatures below the phase transition, lipids with only saturated fatty acids would tend to cluster into ordered domains from which the protein embedded in the lipid was excluded. Thus the proteins aggregated into regions of the membrane containing the lipids with two unsaturated and/or one saturated and one unsaturated fatty acid, which remained in a disordered state. Freeze-fracture photography of wild type *E.coli* K12 membranes, however, revealed no evidence for lateral lipid-protein phase separation.
Haest et al. (1974) showed lateral phase separation in *Streptococcus faecalis* membranes, but not in some *Bacillus* species or *Staphylococcus aureus*. Further research demonstrated that the presence of branched chain fatty acids prevented lateral phase separation, by incorporating *S. aureus* branched chain fatty acids into *Acholeplasma laidlawii B* membranes. The phase separation normally occurring in this organism at -5°C was lost.

Lee et al. (1974) proposed a further hypothesis to explain the anomalous thermal effects in some enzyme systems. Lee suggested a different form of clustering as an explanation for changes in the activity of membrane bound enzymes above the thermal transition temperature especially at around 25°C. This 'cluster', was considered to be a short-lived, more densely packed arrangement of molecules within an environment of freely dispersed molecules.

In Chapter 1, it was postulated that radiation cell killing might involve interaction between membrane and DNA. In addition, Yatvin et al. (1972) has demonstrated a lack of post-irradiation repair in membranes of the radiation sensitive *E.coli B*₅₋, and also a correlation between membrane fluidity and radiation sensitivity (Yatvin 1976). This chapter details experiments designed to determine whether or not there is any effect of membrane fluidity on the production and repair of DNA strand breaks in *E.coli K1060*. If the production and/or repair of DNA strand breaks is affected by fluidity changes, then it is possible that strand breaks are associated with cell killing. A negative result, however, although ruling out strand breaks involvement in sensitivity changes caused by fluidity changes, would not rule out a role for other possible DNA lesions.
3.2. MATERIALS AND METHODS

3.2.1. Growth and radioisotope labelling of K1060

**E. coli** K1060 were maintained on 4% Blood Agar Base plus 0.04% oleic acid, and 0.2% Brij 58. 6ml of the autoclaved mixture was added to a glass universal, and the agar allowed to set as a slope. Inoculations were carried out using a platinum wire loop sterilized by flaming.

Bacteria were grown in C/R Mineral salts medium (Anraku, 1967) supplemented with 0.5% glycerol, 0.3% casamino acids (Difco, vitamin-free) 0.2% 'Brij' 58 and 0.02% unsaturated fatty acid (UFA) (Overath, Schairer and Stoffel 1970). Fatty acids were obtained from Sigma Chemical Co., and kept as a 10% alcoholic solution at -20°C. The full ingredients are outlined below:

**Cohen Rickenberg Medium & Buffer**

pH to 7.3 with KOH

The following chemicals are made up to one litre with distilled water, to produce C/R buffer.

13.6g KH$_2$PO$_4$, 2.0g (NH$_4$)$_2$SO$_4$, 0.2g MgSO$_4$ 7H$_2$O, 0.0005g FeSO$_4$.7H$_2$O - approximates to one crystal, plus 1ml of a trace metal solution containing:-

- 480 mg Fe Cl$_3$.6H$_2$O
- 280 mg Mn Cl$_2$.4H$_2$O
- 270 mg CaCl$_2$
- 2000 mg ZnCl$_2$
- 290 mg H$_3$BO$_3$
- 130 mg CoSO$_4$

per litre

The following were also present in one litre of buffer to produce medium. 5g glycerol, 3g casamino acids (Difco - vitamin-free), amd 2g Brij 58.

Medium was sterilized in 100ml aliquots for 20 minutes at 15 psi in a bench gas autoclave.
Bacteria were grown overnight in 25ml C/R medium at 37°C (40°C for elaidic) with 50µl 10% UFA on a rotary shaker. One ml of the overnight culture (18 hours) was inoculated into 25ml of fresh preincubated C/R medium with the same UFA. Typical growth curves, which were not exponential are depicted in figures 3a and 3b. Where appropriate, after 30 min, deoxyadenosine and ³H-thymidine (Radiochemical Centre, Amersham, U.K.) were added to final concentrations of 320 µg/ml and 4 µCi/ml respectively. Growth was continued for one hour and the bacteria were harvested at 2,000 rpm for 10 minutes on an M.S.E. Mistral 6L centrifuge at growth temperatures. The cells were resuspended in cold chase medium containing deoxyadenosine and 100µg/ml of 'cold' thymidine for a further 30 minutes. Final harvesting (at 4°C, room temperature or growth temperature) concentrations were determined spectrophotometrically at 650 nm according to a standard cell count of 1.2 x 10⁸/ml giving an O.D. of 1.0 (Yatvin 1976). Bacteria were resuspended at 10⁶/ml in 0.15M NaCl or C/R buffer. For growth medium experiments bacteria were allowed to remain in cold chase medium.

3.2.2. Irradiation

Irradiations were carried out at ice temperature, room temperature, 37°C or 40°C using the same conditions and equipment as for B/r and B{s-1} in Chapter 2.

3.2.3. Strand break determination and DNA degradation

Strand break analysis and DNA degradation was assessed under the same conditions as Chapter 2, either immediately after irradiation or after post irradiation incubation at growth temperature.

3.2.4. Survival

Bacteria were diluted in C/R buffer and plated on nutrient agar plates. Plate medium comprised 11.5g nutrient agar, 1.0g Brij (0.2%) and 2 ml 10% oleic acid (0.04%) made up to 500 ml with distilled water. The Brij was dissolved before autoclaving, and the plates were hand poured.
Figure 3a

Growth curve for K1060 supplemented with oleic acid at 37°C. Since optical densities greater than 1.0 at 650 nm do not obey Beer-Lamberts Law, appropriate dilutions were made.
Growth curve for K1060 supplemented with elaidic acid at 40°C. Growth is very similar to that observed with oleic acid.
3.3. RESULTS

3.3.1. Harvesting and Cooling

Preliminary investigations did not produce consistent results with occasionally oleic acid bacteria, but often with elaidic acid bacteria. In an attempt to discover why elaidate bacteria often had poor (5%) or zero plating efficiencies, plates containing elaidic acid and incubated at 41°C were tried without any greater level of success. Yatvin (1976) and Yatvin et al. (1979) had not disclosed any difficulty associated with bacteria grown on this fatty acid. However, Esfahani et al. (1969) reported immediate loss in viability of another UFA auxotroph grown on elaidic acid, when placed below its growth temperature. Thilo and Vielmetter (1976) reported that elaidate grown K1062 were more liable to lysis. The time course (hours) of starvation effects reported by Henning et al. (1979) following UFA starvation seemed too slow to be relevant. The possibility of an effect of cold shock was then brought to light (Anraku 1967, Smeaton and Elliott 1967, Paton et al. 1978, Sato and Takahashi 1968, 1969 II & III, Haest et al. 1972). Various authors have reported that cold shocking can be lethal to both E.coli (Sato and Takahashi 1968, Haest et al. 1972) and Bacillus amyloliquefaciens (Paton et al. 1978) and that the release of small molecules from within the cell may cause the loss in viability. Elaidate bacteria harvested at growth temperature and resuspended in ice-cold 0.39% saline or C/R buffer, rapidly lost viability. This proved worse if washing was attempted. Consequently it was found that resuspension in saline or buffer at growth temperature in a volume of 10-20ml followed by slower cooling in an ice bath, prevented this viability loss; and so this procedure was adopted for all cooling experiments.
3.3.2. **Survival**

Owing to the length of irradiation times, Yatvin et al. (1979) did not analyse the timing protocol for producing survival changes induced by cooling. Using a 10X higher dose rate it was discovered that for oleic grown bacteria only a 5 minute pre-irradiation cooling was needed to produce the maximum change in survival. Post-irradiation cooling only, had no effect on survival and pre- plus post-irradiation cooling did not give an enhanced sensitization.

The survival curves for oleic, linoleic, and elaidic grown bacteria irradiated in non-nutrient saline or C/R buffer are shown in figures 3(c), 3(d) and 3(e) respectively.

Whilst the elaidate survival curve is comparable to that shown by Yatvin et al. (1979), the oleic survival curves are more sensitive. I believe that the oleic bacteria could undergo some repair during Yatvin's slower irradiation time whereas the elaidate bacteria could not. It could be that sublethal damage repair is displaying an effect due to membrane fluidity here. Indeed, Nolan et al. (1981) have demonstrated a dependence of sublethal damage repair on membrane fluidity in Chinese hamster (V79) cells. Scanty data by Yatvin (unpublished) indicates a similar increase in sensitivity with the higher dose rate.

Bacteria were also irradiated in full growth medium and diluted in C/R buffer before plating to determine survival. These bacteria were more resistant than those irradiated in non-nutrient conditions but the bacteria cooled to ice temperature before and during irradiation were still considerably more sensitive than those irradiated at room temperature (Figure 3(f)). The $D_0$ values from survival experiments are summarised in Table 3.1.

Although control samples maintained full viability at room
Survival curves for oleic acid grown E. coli K1060. Irradiation in air or oxygen-free-nitrogen gassing at ice temperature and room temperature. The bacteria were suspended in saline. Identical results were obtained if the bacteria were irradiated in C/R buffer. The error bars represent ± the standard error of the means.
Survival curves for linoleic acid grown K1060. Irradiation in air or oxygen-free-nitrogen gassing at ice temperature and room temperature. The bacteria were suspended in saline. Identical results were obtained if the bacteria were irradiated in C/R buffer.
Survival curves for elaidic acid grown E.coli K1060. Irradiation in air or oxygen-free-nitrogen gassing at ice temperature and room temperature. The bacteria were suspended in saline. Identical results were obtained if the bacteria were irradiated in C/R buffer.
Survival curves for oleic and elaidic acid grown E. coli K1060 irradiated in growth medium at room temperature and at ice temperature. Immediate dilution and plating.
<table>
<thead>
<tr>
<th>Temperature of irradiation</th>
<th>$D_0$ (grays)</th>
<th>Air irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oleic grown K1060</td>
<td>Elaidic grown K1060</td>
</tr>
<tr>
<td>Saline Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ice</td>
<td>53</td>
<td>17</td>
</tr>
<tr>
<td>Room Temperature</td>
<td>107</td>
<td>30</td>
</tr>
<tr>
<td>$40^\circ$C</td>
<td>350</td>
<td>175</td>
</tr>
</tbody>
</table>

These $D_0$'s are averages obtained from the results of four or more experiments.
temperature, during the course of the experiment the samples irradiated in fully supplemented growth medium steadily lost colony forming ability when held at this temperature. However, large differences in sensitivity between warm irradiated and ice irradiated bacteria were maintained (Fig. 3(g)).

This loss in viability post-irradiation came to light owing to a variation in survival. This variation had not occurred in buffer and so its presence was not investigated in non-nutrient conditions. The possibility of UFA peroxidation products in the growth medium having post radiation toxic effects was also not investigated.

If growth medium was added to oleate or elaidate grown bacteria irradiated in non-nutrient suspension, and incubated for 30-120 minutes at growth temperature before plating, survival of the bacteria remained unchanged from that obtained under normal non-nutrient conditions.

3.3.3. DNA degradation

DNA degradation in K1060 produced similar results to E. coli B/r and B_s-1. After irradiation in non-nutrient saline conditions, there was minimal degradation of DNA in oleic bacteria (Fig. 3(h)) and elaidate bacteria gave identical results. About 25% degradation occurred after a dose of 210 grays. Degradation following growth medium irradiations gave results reminiscent of B/r and B_s-1 data. Degradation was faster and more extensive in oleic grown K1060 which had been irradiated at 4°C. Figure 3(i) depicts this differential between 40°C and 4°C irradiated bacteria after a dose of 200 Gy.

3.3.4.1. Strand breaks in non-nutrient conditions

It was impossible to demonstrate a level of DNA unwinding for initial strand breaks at room temperature or 40°C owing to the very rapid repair of strand breaks at this temperature. When oleic acid grown K1060 were irradiated at either 4°C or 40°C followed by a 30 minute repair, incubation at 37°C, the yield of residual strand unwinding
Survival curves for oleic acid grown E.coli K1060 irradiated at ice and room temperature in growth medium. Samples were diluted and plated at 15 and 85 min after post-irradiation holding at room temperature.
Post-irradiation DNA degradation of oleic grown bacteria irradiated in non-nutrient saline or C/R buffer conditions. Post-irradiation incubation of 37°C for 30 min. Degradation for elaidic grown bacteria was to a similar extent. There was no degradation of DNA in unirradiated controls over the duration of the experiment.
Post-irradiation DNA degradation of oleic grown bacteria with time after irradiation (200 Gy) in fully supplemented growth medium. Degradation for elaidic grown bacteria was nearly identical after the same dose.
was the same (Fig. 3(j)). Results obtained with K1060 using the hydroxylapatite technique were more variable than those obtained with B/r and B<sub>s-1</sub>, however qualitatively identical results were achieved on a day to day basis. Consequently, Figures 3(j), 3(k), 3(l) and 3(m) illustrate the data from single experiments. Production and repair of strand breaks in non-nutrient conditions for elaidate grown K1060 appear in Fig. 3(k) and again show equal strand break repair for bacteria irradiated either at 4°C or 40°C.

3.3.4.2. Strand breaks in growth medium

Following irradiation, and post-irradiation incubation in growth medium, oleate and elaidate grown K1060 appeared to achieve less extensive repair after 4°C irradiation (Figures 3(l) and 3(m)). A revision of the DNA degradation data reported in 3.3, however, confirmed my belief that equivalent repair may well have occurred. Degradation of a more substantial amount of DNA (from double strand breaks?), ultimately yielded a decreased amount of double stranded DNA eluted from hydroxylapatite columns, producing an artifactual decline in break repair. The strand unwinding data derived for elaidate grown bacteria always resulted in higher levels of initial and residual DNA lesions, possibly implying an inherently elevated number of metabolic breaks in their DNA compared to oleate grown bacteria. Sato and Takahashi (1969 III) related cold-shocking in <i>E. coli</i> to a release of magnesium ions. They speculated that the major effect of this magnesium ion release was to inhibit DNA ligase activity, and demonstrated that more strand breaks were present in cold shocked cells. These authors also showed that <i>E. coli</i> B/r contained 13 times more DNA ligase activity than <i>E. coli</i> B<sub>s-1</sub> and that addition of magnesium acetate after cold-shocking, only stimulated recovery of B/r. Town et al. (1973) also observed that cold-shocking could reduce single strand break repair in <i>E. coli</i> K12.
Plot of $-\log \left( \frac{DS}{DS + SS} \right)$ (a function proportional to number of breaks - Ahnstrom, George and Cramp 1978) against dose showing extensive and equivalent repair in oleic grown bacteria irradiated at ice temperature and $40^\circ C$ in saline followed by a post-irradiation incubation time of 30 min at $37^\circ C$. 

Figure 3 j.
Plot of $-\log \frac{DS}{DS+SS}$ against dose showing extensive and equivalent repair in elaidic grown bacteria irradiated at ice temperature and $40^\circ C$ in saline followed by a post-irradiation incubation time of 30 min at $37^\circ C$. 
Plot of $-\log(DS/(DS+SS))$ against dose showing repair in oleic grown bacteria irradiated in growth medium at ice temperature and 40°C before and after repair.
Plot of $-\log\left(\frac{DS}{DS + SS}\right)$ against dose showing repair in elaidic grown bacteria irradiated in growth medium at ice temperature and $40^\circ\text{C}$ before and after repair.
The extent of unwinding in non-nutrient conditions was greater than double that achieved following growth media irradiation. Essentially similar data for DNA degradation and alkaline unwinding was obtained under anoxic conditions for oleic and elaidic acid grown bacteria. The emphasis in this work has been the investigation of radiation effects in fully aerated conditions where the maximum changes in sensitivity were observed.

Figures 3(n) and 3(o) demonstrate that equal repair of strand breaks resulting from 4°C and growth temperature irradiations were also obtained for linoleic and linolelaic grown bacteria.

3.4. DISCUSSION

As outlined in the introduction to this chapter, conclusions drawn from the literature support the interpretation that the more fluid the membrane at the time of irradiation, the more resistant is the bacterium to the lethal effects of radiation. Redpath and Patterson's (1978) analyses implicating peroxidation in K1060 membrane damage, and the work of Pietronigro et al. (1977) linking DNA damage with lipid peroxidation, is in full support of conclusions drawn by Cramp and Bryant (1975). To explain differences in sensitivity between E. coli B/r and B5-1 these authors envisaged that interaction must occur between two or more macromolecules. These views led to the work reported in this chapter to test for DNA strand breakage as a marker of DNA damage consequent on changes in membrane composition.

Oleic acid grown bacteria are less sensitive to irradiation than the elaidate grown bacteria. The sensitivities of both types, however, were increased by irradiation at ice temperatures. Holding bacteria at ice temperatures for just a few minutes prior to and throughout the irradiation, was all that was necessary to achieve maximum sensitization, whereas post-irradiation cooling had no effect.
Figure 3 n.

Dose-effect curve of strand-breakage in linoleate grown K1060 irradiated in saline at ice-temperature or 37°C, after post-irradiation incubation at 37°C for 30 minutes.
Dose-effect curve of strand breakage in oleate or linoleleaidate grown K1060 irradiated in saline at ice temperature or 37°C, before and after repair at 37°C for 30 minutes.
However, ice temperature irradiations clearly did not lead to a higher level of residual breaks. Although elaidic acid bacteria were more sensitive than oleic grown bacteria, and albeit yielded a generally greater amount of strand unwinding in alkali, because of the amount of scatter on the data it is not possible to accurately show increased sensitivity with increased DNA lesions. The higher level of breaks revealed in unirradiated elaidate bacteria especially in non-nutrient conditions supports this caution. The further complication of a differential in DNA degradation in nutrient conditions between $4^\circ C$ and $40^\circ C$ irradiated bacteria was unfortunate. In work reported in Chapter 2 this kind of dilemma was overcome because degradation did not commence until strand break repair maxima had already been achieved. With K1060, however, DNA degradation began immediately post-irradiation and prevented a true analysis of strand break repair. One is forced to conclude from this work that although changes to membranes cause dramatic changes in surviving numbers, there is neither a correlative change in residual strand breaks, nor sufficient loss in total DNA to justify considering DNA degradation as a measure of relative sensitivity. This pair of bacteria were adequately suitable for a study on the temperature effect on survival, which was the main aim of the project. More rewarding data from research designed to investigate the peroxidation issue, may have been achieved from a comparison of oleic and arachidonic acid grown bacteria. Indeed, Redpath and Patterson (1978) have shown that K1060 grown in arachidonic acid have a marked increase in sensitivity over oleic grown bacteria, at room temperature.

Exhaustive studies on quantitative changes in post radiation DNA damage such as strand breakage and degradation have not shown close relationships between these parameters of the effect of radiation,
and cell killing. There is a need for the development of techniques to determine more subtle or specific changes in DNA both immediately and at later times after irradiation than those investigated here. Damage leading to differences in sensitivity may only come to light during or following post-irradiation replication, that is after plating of the bacteria to observe colony formation. The possibility of survival being dependent on the total disruption of the cell due to membrane fragility during handling should not be overlooked.

Sensitivity changes dependent on a temperature change only at the time of irradiation needs further consideration. The results suggest that this increase in sensitivity at ice temperature is dependent upon the deposition of energy in and around the membrane. It is possible that in the 'gel' state lesions are imposed within the membrane that are less repairable, or that because of the juxtaposition of other macromolecules more or different lesions arise. In support of the first suggestion, it is possible that in the gelled membrane, free radical diffusion is greatly restricted leading to a greater level of radical interaction (radical-radical recombination). Thus a much greater level of hydrogen peroxide could be produced giving an enhanced peroxidation of unsaturated fatty acids. The greater production of by-products following the restriction of radiation induced radicals has been recorded in ice-lattices (aqueous-glasses) (I. Wilson, personal communication). Unpublished studies by Yatvin give some support to the second suggestion. He investigated the amount of DNA remaining attached to a membrane complex isolated from K1060 on alkaline sucrose gradients (Yatvin, personal communication). When this bacterium was grown on a variety of individual fatty acids, a greater proportion of the DNA remained attached to the membrane complex when the cells were cooled immediately before lysis. However,
post-irradiation release of DNA from this complex was greater in bacteria irradiated and lysed below their membrane transition temperatures (i.e. at 4°C).

With regards to work reported in this chapter, a surprising result was the equivalent survival of bacteria irradiated at 40°C in non-nutrient and nutrient conditions, whereas at lower temperatures a greater radiation cell-kill occurred in non-nutrient conditions.

A temperature dependent membrane leakage effect would be difficult to reconcile with the fact that pre-cooled bacteria irradiated at a higher temperature produce the higher temperature survival response. However, it is possible that osmotic shock in combination with temperature changes causes the difference in sensitivity between buffer and medium. Only the medium contains glycerol, and the Brij 58 in the medium, being a detergent, could well alter the fluidity of the membrane and decrease the phase transition effects. Preliminary investigations on non-protein bound SH levels in cold-shocked E. coli K1060 did not demonstrate any loss due to leakage at growth temperature or 4°C, during an hour's suspension in C/R buffer.

A more likely explanation may be that there is an alteration of the intracellular physiological environment to a more sensitized condition which is enhanced by pre-irradiation maintenance in non-nutrient conditions. It is difficult to proffer a more specific hypothesis.

One final observation on the data presented in this chapter and by Yatvin et al. (1979) has been considered in more depth by Alper et al. (1981). In non-nutrient conditions only the anoxic survival curves are shouldered. The only effect of pre-irradiation cooling on the anoxic survival response is the loss of this shoulder. This is another rare example of oxygen being non dose-modifying.
Only the presence of oxygen has led to a true radiobiological change in sensitivity, and that is defined by a change in the final slope $D_0$. I have recently demonstrated in a single experiment that 30 minutes pregassing in nitrogen at room temperature does not induce a shoulder on the oxic survival curve but gives identical results to those already presented here. This rules out the induction of a shoulder by a conditioning process in a nitrogen atmosphere.

The major part of this chapter has been published, (George, Cramp and Yatvin, 1980).

My concluding remarks are that I have found no evidence to correlate an increased level of residual strand breaks with the increased level of cell killing occurring where bacteria are irradiated below the membrane phase transition temperature instead of above it. In growth conditions, where one would expect repair to be maximal, strand-break repair is obscured by concurrent degradation.
CHAPTER 4.

The influence of changes in the unsaturated fatty acid composition of membranes on the radiosensitivity of the human lymphoid cell line, LDV.
SUMMARY

The work described in this chapter was an investigation into the effect of the alteration of the membrane fatty acid composition on radiation sensitivity, of a mammalian cell line grown in suspension. The survival of cells grown in delipidated medium plus either linoleic acid or oleic acid at 12.5 μg/ml, was compared to the survival of control cells grown in normal foetal calf serum. Equal sensitivity between these cell types was obtained under fully oxygenated or fully anoxic conditions or in the presence of 1% oxygen; at room temperature or at ice temperature. That is, although incorporation of exogenously supplied fatty acids resulted in substantial increases in their contribution to the fatty acid composition of unfractionated membrane phospholipids; radiation sensitivity was unaltered. It was noted, however, that the nuclear membrane phospholipid contained a considerably lower percentage of unsaturated fatty acids and was less prone to substantial alteration following growth in exogenously supplied fatty acids. If the hypothesis that the nuclear membrane is important as a radiation sensitive site is valid, then the lack of change in cell sensitivity in these experiments is not surprising. It was also observed that increased incorporation of the exogenously supplied singly and doubly unsaturated fatty acids into membrane phospholipid led to a decline in the contribution of polyunsaturated fatty acids. This homeostatic effect could prevent a major overall alteration in membrane properties.
CHAPTER 4.
-------------

Effect of irradiation on the survival of LDV human lymphoid cells, and the consequence of alteration of the fatty acid composition of their membranes.

4.1. INTRODUCTION

4.1.1. The alteration of mammalian cell membrane fatty acid composition.

Following the successful attempt to alter radiation sensitivity of E. coli K1060, by changing the unsaturated fatty acid composition of its membranes, it was decided to attempt similar experiments with mammalian cells in cell culture. This area of research is relatively new but has already received wide attention. Changes in membrane composition have been brought about in whole animals by adjusting the dietary intake of fatty acids and analysing spleen cells (Harms-Ringdahl, 1980) and tumour cells (Yatvin et al. 1980); mouse leukaemia cells L1210 (Burns et al. 1979); and Ehrlich ascites cells (Solomonson et al. 1975). Membrane changes in cell culture using exogenous fatty acids have also been successfully achieved in mouse LM cells using chemically defined, serum free media (Williams et al. 1974; Doi et al. 1978); delipidated serum media for CHO cells (Rintoul et al. 1978); dialysed serum media for human skin fibroblasts (Spector et al. 1979) and simply the addition of exogenous fatty acids to normal 10% foetal calf serum media for lymphocytes and Baby Hamster Kidney (BHK) cells (Hoover et al. 1980; Klausner et al. 1980). Hosick (1979) has used liposomes containing free oleic acid to modify mouse mammary epithelial cells in culture. The isolation of an unsaturated fatty acid auxotroph of CHO cells by Chang and Vagelos (1976) gave another potential way for the modification of membrane fatty acid composition.
Although the addition of non-esterified fatty acid has been reported as being toxic to mammalian cells (Moskowitz 1967; Geier, 1967) the addition of oleic (18:1) or linoleic (18:2) unsaturated fatty acids has been reported to give normal growth rates (Spector et al. 1979; Chang and Vagelos, 1976; Doi et al. 1978). Addition of arachidonic acid slowed growth rate in human skin fibroblasts (Spector et al. 1979) but not in mouse LM cells (Doi et al. 1978). Addition of exogenous fatty acids does cause a build up of triglycerides in the cell, and Spector et al. (1979) demonstrated an increase in 18:2 triglyceride from 4 to 44% as a percentage of total lipid, when linoleic acid was supplied.

Owing to the great variability in membrane fatty acid composition between different cell lines and different animal tissues, the outcome of membrane manipulations are difficult to predict; however the cells do attempt to maintain some sort of equilibrium between saturated and unsaturated fatty acids. For example, addition of linoleic acid via the diet or through the medium actually causes a decrease in oleic acid as compensation, but whether elongation products of linoleic acid become incorporated into phospholipids depends on the cell type. Spector et al. (1979) recorded an increase in 18:2 (3.7% → 25.4%), 20:2 (0.5% → 6%) but a decrease in 20:4 (12% → 5.2%) for human skin fibroblasts. Burns (1979) revealed increases in the phospholipid content of 18:2, 20:2, 20:5, 22:4 and 22:6, but no change in 20:4 in L1210 murine leukaemia cells grown ascitically.

Harms-Ringdahl (1980) however has demonstrated rises in spleen phospholipids 18:2 and 20:4, when mice were fed a high essential fatty acid diet. Thus the commonest elongation product of linoleic acid (18:2), arachidonic acid (20:4), has responded in all three possible ways (decrease, increase and unaltered) following the administration of high linoleic acid diets. Animal cells are normally grown in media containing serum and consequently derive almost all their lipids from the serum.
Endogenous synthesis is strongly inhibited by outside sources of fatty acids (Bailey, 1967). Evidence for the alteration of the fatty acid composition of confluent cells is contradictory. Whereas Spector et al. (1979) demonstrated an alteration of human fibroblast cells within 24 hours, Lynch (1976) could not demonstrate changes in mouse L fibroblast cells.

Since the nuclear membrane may be more important in affecting cell radiation sensitivity, it was my intention to compare the alterations of the nuclear membrane lipids in comparison to the cell as a whole. Such studies have not yet been reported following attempts to alter the membrane fatty acid composition of cells, but several papers have been produced comparing the nucleus and/or nuclear membrane fatty acid composition with other cell membranes in mammalian liver in normally fed animals. The nuclear membrane lipid composition has proved to be very similar to other cell membranes but compared to the microsomal membranes does have one or two distinct features. The nuclear membrane contains a higher proportion of saturated fatty acids in phosphatidylcholine and phosphatidylethanolamine (which comprise about 80% of most cell phospholipids) in pig liver (Stadler and Kleinig, 1971) and bovine liver (Kennan et al. 1972). The presence of four times more esterified cholesterol in the nuclear membrane compared to the microsomal membrane has also been reported for the rat and pig liver (Kleinig, 1970).

Although one would expect the nuclear membrane to contain virtually all the lipid present in the nucleus, Khandwala and Kasper (1971) have demonstrated that the body of the nucleus contained 30% more arachidonic acid (20:4) and 100% more docosahexatrienoic acid than the nuclear membrane of rat liver. Schlager and Ohanian (1980) also demonstrated a high saturated fatty acid content of nuclear membranes from Line-10 guinea pig hepatoma cells, but the actual fatty acid composition is markedly different from other cell lines reported in the literature.
4.1.2. **Effects of membrane fatty acid composition on radiation sensitivity**

Several papers on the effect of essential fatty acid deficiency on radiation sensitivity have been reviewed by Holman (1971). Becker et al. (1950) demonstrated that symptoms common to X-ray injury and EFA starvation were intensified in the irradiated fat-deficient animals, particularly with respect to gut damage. Cheng et al. (1952 and 1953) found that the linoleic acid content of cotton-seed oil protected EFA deficient rats against multiple sublethal doses of X-rays. Linolenate offered only slight protection but acted synergistically with linoleate (Subbaram et al. 1964). Recently, however, Harms-Ringdahl (1980) has presented evidence suggesting that EFA deficiency may reduce sensitivity of mice to whole body irradiation of 6 Gy, although a higher dose of 12 Gy appeared to show no difference between mice fed essential fatty acid deficient or proficient diets.

Changes in X-ray sensitivity dependent on membrane fluidity changes in mammalian cells has been indicated both in vivo and in vitro. Yatvin et al. (1980) demonstrated an increased lifetime of tumour infected mice given whole body irradiations following injection of the membrane fluidising anaesthetic, procaine. That is, the procaine sensitised the malignant cells to radiation. Yau (1979), however, has demonstrated that procaine protected the response of oxygenated murine 3T3 or L5178Y cells in vitro although he does not state the temperature of irradiation.

4.1.3. **Membrane alterations and membrane fluidity**

Several main types of membrane composition alterations can influence membrane fluidity (Shinitzky and Inbar, 1976). The most prominent of these is the molar ratio of cholesterol to phospholipids. Increased levels of cholesterol in membranes lead to decreases in microviscosity (i.e. increase in fluidity).
Other characteristics are the degree of unsaturation of the phospholipid acyl chains, the relative amount of sphingomyelin in the system, and the presence of neutral lipids like triglycerides. The effect of the degree of unsaturation of the phospholipid acyl chain is to increase the partial specific volume of the molecules, due to the kinking of the chains (Chapman and Wallach, 1968). Secondly, sphingomyelin is highly saturated and its presence tends to reduce fluidity. This tends to be counteracted by the presence of lecithin which is highly unsaturated. Thirdly, the net fluidising effect of double bonds is far from being proportional to their number per molecule. Thus, replacement of stearic (18:0) with oleic (18:1) markedly increases the fluidity, but further replacement of oleic acid with linoleic acid (18:2) has only a small effect (Shinitzky and Henkart, 1979). Longer acyl chains reduce the differences between saturated and unsaturated fatty acids.

Membrane bound proteins may also influence membrane fluidity, and the dynamic characteristics of the biological membrane lipid layer in model liposome systems is not the same, or only partially similar, as that within the cell when they are made from protein free lipid extracts (Shinitzky and Barenholz 1978). Proteins act in a qualitatively similar way to cholesterol. They increase microviscosity and decrease the flow activation energy especially in membranes with low cholesterol to phospholipid ratios such as the mitochondria. It is thought that membrane proteins become more exposed to the aqueous surroundings after increases in the microviscosity and vice-versa (Borochov and Shinitzky, 1976; Shinitzky and Inbar, 1976).

4.1.3.i. Measurements of membrane fluidity. The fluidity of membranes has been assessed by a variety of techniques such as electron spin resonance (King and Spector, 1978), nuclear magnetic resonance, and 1 See Addendum for explanation of Activation Energy.
lipophilic fluorescent probes such as 1, 6-diphenyl-1,3,5-hexatriene (Johnson and Robinson, 1979). Controversy still surrounds the interpretation of data obtained with fluorescent probes, both on theoretical grounds (Hare et al. 1979; Hildenbrand and Nicolau, 1979; and Shinitzky and Barenholz, 1978) and on the localization of the probe (Collard and de Wildt, 1978; Johnson and Nicolau, 1977).

Membrane microviscosity is a measurement representing the average taken for this non-homogeneous organelle. The degree of rotational mobility of fluorescent probes which are embedded within biological membranes or lipid bilayers is an indication of fluidity (Shinitzky and Inbar, 1974). The greater the rotational mobility of the probe the more fluid its environment is thought to be. The degree of depolarization of the fluorescent emission of a probe in a particular lipid environment depends on the rotational mobility and the excited state lifetime of the probe. In general, given no change in the fluorescent lifetime, the greater the polarization the less mobile the probe, and therefore the less fluid the membrane. However, it is never certain that the probe microenvironment is representative of the whole membrane.

Diphenylhexatriene has proved to be the most popular fluorescent probe and was chosen for the study in this instance of membrane fluidity in LDV cells. It has excellent spectral properties and partitions equally well between gel and liquid crystalline phases (Herring et al. 1980). The probe has been used in many investigations on lipid vesicles, isolated plasma membranes and membranes of intact normal and tumour cells (Collard and de Wildt, 1978).
Collard and de Wildt (loc. cit) have demonstrated the dangers of interpreting data obtained on whole cells in terms of microviscosity of plasma membranes. They demonstrated that all lipid materials of the cell, including cytoplasmic lipid droplets were labelled with DPH. Johnson and Nicolau (1977) using human tonsil lymphocytes, concluded that the probe distribution corresponded to that of the phospholipids, except that the fluorescent intensity of the probe in the plasma membrane was enhanced by about 15% relative to its chemical distribution.

4.1.3.ii. Effect of membrane fatty acid alterations on membrane fluidity. Herring et al. (1980) could detect no change in plasma membrane fluidity of Dictostelium discoideum measured at 20°C following a substantial increase in the incorporation of polyunsaturated fatty acids in the membrane. In addition, phosphatidyl choline isolated from cells grown on both polyunsaturated fatty acid supplemented and unsupplemented medium exhibited similar mobilities of an incorporated spin probe when dispersed in aqueous solution.

However, Berlin et al. (1980) using DPH as a probe, demonstrated that the relative fluidities at 37°C of rabbit platelet membrane isolates correlated with the polyunsaturated fatty acid contents of the membrane phospholipids following dietary changes. Unsaturation was important but not the only determinant of membrane fluidity. They felt that the rigidity induced influences of the membrane proteins might also contribute.

Using an ESR technique, King and Spector (1978) also obtained changes in plasma membrane fluidity following short term exposure of intact Ehrlich Ascites cells to media containing various fatty acids during short term stationary cultures. In contrast, Stubbs et al. (1980) using diphenylhexatriene in lymphocytes, failed to find a change in
fluidity correlated with large changes in the unsaturated fatty acid composition of the plasma membrane phospholipids. Changes in fluidity were correlated with the appearance of lipid droplets in the cytoplasm of the cells. The fatty acid pattern of the neutral lipids extracted from the purified plasma membrane showed very little incorporation of the exogenous fatty acid; cells being incubated for 20 hours at 37°C. The triglycerides of the lipid droplets and the plasma membrane were therefore very distinct. Lipid droplets were not found in the cytoplasm when cells supplemented with saturated fatty acids were observed, and there was no change in membrane fluidity.

4.1.4. Other effects of fatty acid alterations

Hoover et al. (1980) have reported several functional and cellular changes after fatty acid alterations and include altered membrane enzyme function; morphology; lectin agglutination; cell adhesion; and cellular differentiation. To this list may be added changes in the degree of exposure of surface proteins; lateral mobility of receptors; responses to mitogenic stimuli; endocytosis; amino acid transport; and cholesterol esterification (Spector et al. 1979).

4.2. MATERIALS AND METHODS

4.2.1. Maintenance of LDV cells

4.2.1.i. Storage and growth of LDV cells Cells were stored in RPMI 1640 medium plus 10% dimethyl sulphoxide in liquid nitrogen at about 3 x 10^6 cells/ml in 2ml presterilized screw cap vials (Sterilin). To start a cell culture, a new vial was quickly thawed until a film of liquid was visible between the inside of the vial and the remainder of the cell suspension. The vial was flamed, the cap removed, the neck reflamed and the contents dropped into 15ml of fresh medium in a plastic centrifuge tube. To maintain sterility all opening operations were carried out in a laminar flow hood. The cells were centrifuged at 800 rpm
for 3 mins in a bench top centrifuge, the supernatant discarded, and
the cells resuspended in the residue by light tapping of the tube.
10ml of fresh medium was added, and the cells examined before incubating
at an appropriate dilution (see 4.2.1.iv).

The growth medium was RPMI 1640 (Gibco Biocult). 1 packet for
10 litres of medium was made up in 1 litre of sterile dd. H₂O, stirred
with a magnetic stirrer for about one hour, and then allowed to settle.
This 10X strength was sterilized by vacuum filtration through a 47mm
0.22μ ASWP millipore filter. The actual cell medium was prepared in
sterile 500ml serum bottles according to the table below, and stored at
-20°C while not in use.

| 400 ml | double distilled water, autoclaved. |
| 50 ml  | 10X RPMI sterile                   |
| 50 ml  | 10X NaHCO₃ (20g/l - filter sterilized) |
| 82 ml  | Foetal calf serum (Gibco Biocult)   |
| 0.8 ml | 10³ x antibiotic (Penstrep)         |

When preparing for use the medium was neutralized to pH 7.4
according to the RPMI phenol red indicator (straw coloured) with 1N
sterile HCl. Antibiotic was stored at -20°C in 20ml aliquots at 10³ x
the required concentration in plastic universals. Two vials each of
penicillin (100 units/ml) and streptomycin (100 μg/ml) (both Glaxo
Laboratories) were dissolved in 20ml sterile distilled water.

4.2.1.ii. Serum delipidation 14g liposorb (Artery U.S.A.)
plus a magnetic stirrer were autoclaved in a litre flask. 500ml of
foetal calf serum was added and stirred for 3 hours at 37°C. The mixture
was then decanted, centrifuged in 25ml capped M.S.E. polyallomer tubes in
the Mistral 6L MSE centrifuge for 10 minutes at 2,000 rpm, and the super-
natant decanted again into universals. The serum was centrifuged again
for 20 minutes at 2,000 rpm, and the supernatant decanted into a sterile
serum bottle for storage at -20°C.

G.L.C. analysis revealed that about 15-18% of the oleic acid and
Palmitic acids were left after this procedure.

4.2.1.iii. **Freezing of LDV cells** 300ml of cells were grown to about $2 \times 10^5$/ml and dispersed into plastic universals. The cells were harvested at $37^\circ$C at 800 rpm for 3 minutes, the supernatant discarded, and the cell pellet resuspended gently. 1 ml of fresh medium was added to each universal and all universals combined. Each universal was rinsed with 5 ml of fresh medium which was added to the original 15 ml. These cells were placed on ice along with labelled sterile freezing vials and allowed to cool. 10% v/v of the preservative dimethyl sulphoxide was then added to the suspension.

Each freezing vial was then filled using a sterile pasteur and the lids screwed on tightly. The cell volumes were adjusted to ensure that all air was removed by an overflow of surplus cells. All vials were then placed in the neck of a liquid $N_2$ bank for 3-4 hours before finally fixing to a 'straw' and suspending in liquid nitrogen until required. After 24 hours one vial was thawed to check viability.

4.2.1.iv. **LDV growth characteristics** LDV human lymphoid cells were grown on RPMI 1640 medium substituted with 10% foetal calf serum or 10% delipidated foetal calf serum. Normal cells grew with a division time of about 12 hours at $37^\circ$C. Cells were grown in 10ml amounts in 2oz glass medical flats lying almost horizontally, except where larger volumes of cells were required.

Throughout the experiments an approximation of the dilution of the original calf serum present in normal RPMI has been indicated by a fraction ($\frac{1}{x}$) such that any lipids or other components not removed in the delipidation procedure will be additional to this ($\frac{1}{x}$) factor.

**Control cells** (no fatty acids added to growth medium)

Control cells and other cells will grow up from very low concentrations, but were normally passaged from between $5 \times 10^3$/ml and $10^4$/ml. Control cells grew happily to $8 \times 10^5$/ml but took 24 hours to attain confluency at $10^6$/ml. The cells usually clump quite extensively
and undisturbed cultures needed to be agitated by pipetting into single cells for accurate haemocytometer counting. Confluent cells die off very rapidly at this concentration (10^6/ml). Fig. 4A shows a growth curve for control cells.

**Delipidated cells (cells grown using delipidated medium)**

Cells grown solely on unsubstituted 10% delipidated foetal calf serum grew normally to confluency at \( \frac{1}{50} \), but following a second passage at between \( \frac{1}{500} \) and \( \frac{1}{5000} \) cells divided more slowly for 1-1.5 divisions and then began to die off. At the higher values of \( \frac{1}{x} \) cells became singletons, were very rounded, and also very much enlarged (see Fig. 4B).

**Fatty acid substituted cells**

Oleic acid, linoleic acid, or occasionally both unsaturated fatty acids, were added as 1% solutions in absolute ethanol, which is equivalent to 10mg/ml. 12.5 \( \mu \)l of this stock was added to 10ml of delipidated RPMI to give a final concentration of 12.5 ug/ml or multiples thereof (Rintoul et al. 1978).

(a) Oleic acid Cells substituted with 12.5 \( \mu \)g/ml oleic acid grow reasonably well up to \( \frac{1}{2500} \) but on one exceptional occasion attained 5 \( \times \) 10^4/ml at \( \frac{1}{800,000} \) after 6 passages. Cells divide normally up to between \( \frac{1}{100} \) and \( \frac{1}{2500} \) depending on whether the first passage is with or without oleic acid. Without oleic acid in the first passage cells grew with about 20 hour doubling times to 2 \( \times \) 10^5/ml or 2.7 \( \times \) 10^4/ml depending on the \( \frac{1}{x} \) value of \( \frac{1}{210} \) or \( \frac{1}{1000} \) respectively. Again, stressed cells tended to become singletons and also round up and enlarge. Very stressed cells maintained after growth had ceased, often lysed without agitation. Irradiations were usually performed on cells grown between \( \frac{1}{20} \) and \( \frac{1}{250} \). Early results demonstrated that doubling the amount of oleic acid and hence the alcohol did not upset the growth rates but 4 times the amount prevented growth. Up to 50\( \mu \)l of ethanol added to control cultures had
The graphs show growth curves starting from several different cell densities.
GROWTH OF SECOND PASSAGE CELLS IN DELIPIDATED MEDIUM WITHOUT FATTY ACID SUPPLEMENTS DILUTED FROM A FIRST PASSAGE OF CELLS GROWN TO $2.7 \times 10^5$ /ML AT 1/50 FCS
no effect on their growth.

(b) **Linoleic acid** Cells were generally less able to grow on linoleic acid and were possibly more fragile than oleic grown cells. Growth on 12.5μg/ml linoleic acid was at the normal rate to \( \frac{1}{200} \) but cells divided only once every 16.5 hours at \( \frac{1}{1900} \) with both 25μg and 37.5μg/ml. 12.5μg/ml at this dilution could not support cell division. Cells at 25 and 37.5μg/ml could be maintained alive up to a 5th passage at \( \frac{1}{19,000} \).

Cells grown with a combination of 12.5 μg/ml or 25 μg/ml of each fatty acid appeared to grow more slowly and stopped growing during the 4th passage at \( \frac{1}{450} \). Fig. 4C gives an indication of the relative growth of cells passaged at \( \frac{1}{1000} \) with oleic or linoleic acid.

**Identical passaging**

When continuous passaging at the same \( \frac{1}{x} \) value was performed, cells appeared to grow quite happily with 12.5μg/ml of either oleic or linoleic acid for at least six passages at the two values tested \( \left( \frac{1}{20} \text{ & } \frac{1}{100} \right) \) and also 20+ at \( \frac{1}{250} \).

**Control Cells grown in full medium plus fatty acids**

Control cells grown in full medium and further supplied with 12.5 μg/ml of oleic or linoleic acid grew at the normal rate for at least 4 weeks or about 14 passages.

**4.2.2. Lipid Analysis**

**4.2.2.1. Cell preparation procedure** For whole cell analysis, whole cell membrane phospholipid and nuclear membrane phospholipid analyses, \( 3 \times 10^6, 10^7 \), and 2-4 x \( 10^7 \) cells were required, respectively.

Cells were harvested at 1,000 rpm (300g) for 5 mins using a bench centrifuge (B & T bench autocentrifuge) and resuspended and washed 2 x with physiological saline. The pellet was allowed to drain dry in a ground glass conical centrifuge tube (10 ml).
GROWTH OF CELLS AT \( \frac{1}{1000} \) FCS AFTER ONE PASSAGE IN DELIPIDATED MEDIUM WITHOUT FATTY ACIDS TO \( 1.2 \times 10^5/\text{ML} \) (\( \frac{1}{100} \) FCS)
Samples were grown according to Tables 4A and 4B. Where required, nuclei were extracted according to the following technique.

To the washed cell pellets resuspended in their own residue, 20 ml of ice cold nuclear detergent was added and the suspension left on ice for 10 mins. The nuclear detergent (sterile) consisted of 0.25M sucrose, 3.3 mM CaCl₂ and 0.25% non-ident P40 non-ionic detergent. Samples were then checked for nuclei and where necessary a quick "whirlimix" preceded centrifugation for 5 mins at 1000 rpm. Alternatively, if nuclear isolation was incomplete, harvesting was followed by a further 5 mins in a fresh 20 ml of nuclear detergent. However, this appeared to lead to loss of fatty acid grown nuclei by lysis, and in later experiments this stage was omitted. The nuclear pellet was then washed twice with 20 ml of ice cold sterile phosphate buffered saline (PBS), resuspended in 5 ml PBS and pelleted into a ground glass conical centrifuge tube (10 ml) in the MSE Chilspin centrifuge at 3,000 rpm. The supernatant was then discarded and the pellet allowed to drip dry by inverting the centrifuge tube and standing it on tissue paper.

4.2.2.i. Folch extraction of cells A modification of the Folch Extraction technique (Folch et al. 1957) was used to extract the total lipid extract. All solvents had been redistilled.

1. Added 2 ml CHCl₃ : MeOH (2:1) to the pellet and vortexed vigorously.
2. The sample was then gassed with N₂, sealed with parafilm, and left overnight in the cold room.
3. The extract was then centrifuged for 5 minutes at 900g in the MSE Chilspin.
4. The supernatant was decanted and saved, while the pellet was re-extracted with a further 2 ml CHCl₃ : MeOH (2:1) and the two supernatants combined.
### TABLE 4A

**FATTY ACID ANALYSES**

Details of cell growth (continued in Table 4B)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>FCS</th>
<th>GROWTH</th>
<th>FINAL CONCENTRATION /ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1. Whole Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td></td>
<td></td>
<td>6.7 x 10^5</td>
</tr>
<tr>
<td>Control 2</td>
<td></td>
<td></td>
<td>3 x 10^5</td>
</tr>
<tr>
<td>Control 3</td>
<td></td>
<td></td>
<td>7.6 x 10^5</td>
</tr>
<tr>
<td>Oleic 1% 1/10</td>
<td>2 days growth from 1.6 x 10^4/ml at 1/10</td>
<td>2 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Oleic 1% 1/510</td>
<td>Passage of above: 0.2/10 4 or 5 days growth from 4 x 10^3</td>
<td>1.1 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 1% 1/10</td>
<td>2 days growth from 1.5 x 10^4/ml</td>
<td>3.2 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 2% 1/10</td>
<td>2 days growth from 1.5 x 10^4/ml</td>
<td>3 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 3% 1/10</td>
<td>2 days growth from 1.5 x 10^4/ml</td>
<td>2.6 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Expt. 2. Whole Cell Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td></td>
<td></td>
<td>5.6 x 10^5</td>
</tr>
<tr>
<td>Control 2</td>
<td></td>
<td></td>
<td>5.5 x 10^5</td>
</tr>
<tr>
<td>Linoleic 1% 1/30</td>
<td>3 days growth (over weekend) from 5 x 10^3/ml</td>
<td>1.8 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 2% 1/30</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>2.2 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 3% 1/30</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>7 x 10^4</td>
<td></td>
</tr>
<tr>
<td>Oleic 1% 1/30</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>2.4 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Oleic &amp; Linoleic 1% 1/30</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>3 x 10^5</td>
<td></td>
</tr>
<tr>
<td>O + L 1% 1/30</td>
<td>&quot; &quot; &quot; &quot;</td>
<td>(double alcohol) 1.4 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Expt. 3. Nuclear Membranes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>150 ml static</td>
<td>2.2 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Control 2</td>
<td>Large volume</td>
<td>7 x 10^4</td>
<td></td>
</tr>
<tr>
<td>Control 5</td>
<td>Large volume. Twin of 2</td>
<td>4 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Control 6</td>
<td>200 ml</td>
<td>1.5 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Oleic 3 1% 1/14</td>
<td>45 ml controls + 270ml DLRPMI (1/7) grown</td>
<td>10^5</td>
<td></td>
</tr>
<tr>
<td>Oleic 4 1% 1/14</td>
<td>for 3 days (to 4 x 10^4/ml) and then diluted to give twins for one day at 1/14</td>
<td>6 x 10^4</td>
<td></td>
</tr>
<tr>
<td>Oleic 7 1% 1/20</td>
<td>2 days 1/5 50ml @ 1.75 x 10^5/ml diluted 4x and grown for 1 day</td>
<td>1.5 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 8 1/10 2%</td>
<td>2 days 1/5 50ml @ 9 x 10^4</td>
<td>7 x 10^4</td>
<td></td>
</tr>
<tr>
<td>Linoleic 9 1/15 1%</td>
<td>diluted x3 150ml 1/15 for 1 day</td>
<td>1.6 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Linoleic 10 1/10 1%</td>
<td>2 days 1/5 150ml @ 1.25 x 10^5</td>
<td>1.15 x 10^5</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>1/FCS</td>
<td>Growth</td>
<td>Final Concentration per ml.</td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>--------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>5 Oleic 1%</td>
<td>1/10</td>
<td>2 days growth from $2.2 \times 10^4$/ml</td>
<td>$3.4 \times 10^5$/ml</td>
</tr>
<tr>
<td>6 Linoleic 1%</td>
<td>1/10</td>
<td>2 days growth from $3 \times 10^4$/ml</td>
<td>$4 \times 10^5$/ml</td>
</tr>
<tr>
<td>7 Control + Oleic 1%</td>
<td></td>
<td>6th passage</td>
<td></td>
</tr>
<tr>
<td>8 Control + Linoleic 1%</td>
<td></td>
<td>6th passage</td>
<td></td>
</tr>
<tr>
<td>9 Oleic 1%</td>
<td>1/10</td>
<td>7th passage. nuclei</td>
<td></td>
</tr>
<tr>
<td>10 Linoleic 1%</td>
<td>1/10</td>
<td>7th passage. nuclei</td>
<td></td>
</tr>
<tr>
<td>12 Oleic 1%</td>
<td>1/20</td>
<td>25ml 8th passage to $3.5 \times 10^5$/ml</td>
<td>$4 \times 10^5$/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dilute 4 x for 1 day. 100ml nuclei</td>
<td></td>
</tr>
<tr>
<td>13 &amp; 14 Linoleic 1%</td>
<td>1/20</td>
<td>25ml 8th passage to $1.3 \times 10^5$/ml</td>
<td>$4.4 \times 10^5$/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dilute 2 x for 1 day to $3.4 \times 10^5$/ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50ml) add 75 ml for 1 day. Divide into 2.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Used 14 for nuclei).</td>
<td></td>
</tr>
</tbody>
</table>
5. 0.8ml of 0.1M KCl was then added to this supernatant, and the sample was vortexed, thoroughly gassed with N₂, and left to stand to allow the phases to separate.

6. The aqueous top phase was then removed and discarded to leave the total lipid extract, which was stored under N₂ in the cold room until the next procedure.

Serum extracts. To obtain the total lipid extract of foetal calf serum samples, 400 µl of test serum was added to 0.6ml 0.85% NaCl in a ground glass conical centrifuge tube and 4ml CHCl₃ : MeOH (2:1) was added. The sample was then gassed with N₂, vortexed thoroughly and sealed with parafilm and stored in the cold room overnight.

4.2.2.iii. Phospholipid / Neutral lipid separation

1. Silicic acid was activated by overnight heating at 120°C in an oven. Activated silicic acid (Bio-Sil HA minus 325 mesh, Bio-Rad) was stored in a dessicator.

2. Chloroform was added to slurry the silicic acid.

3. 5ml constant width glass pipettes were used as silicic acid columns. These were plugged with glass wool and rinsed with chloroform.

4. Sufficient slurry was added to make a 3ml column volume. Columns producing air locks were discarded.

5. The column was washed thoroughly with chloroform until translucent, and prevented from drying out until used, by occasional flushing with further chloroform.

6. Lipid extracts in CHCl₃ : MeOH (2:1) were dried down and redissolved in 1ml chloroform. Water was omitted at all costs since the functioning of the columns was destroyed by its presence. The extract was then added to the column using a finn pipette and allowed to filter through.
7. The neutral lipids plus free fatty acids were extracted first using 3 separate elutions of 5ml of chloroform.

8. The glycolipid fraction could then be removed by eluting with 5ml of 4% methanol in chloroform.

9. The phospholipid fraction was eluted with 6 x 5ml aliquots of methanol.

10. All samples were dried down, using a rotor evaporator where necessary and stored under N₂ in a small volume of hexane in parafilm sealed ground-glass stoppered centrifuge tubes. Storage was at -20°C.

4.2.2.iv. Transesterification This procedure was performed in pyrex, screw capped, teflon lined, bacteriological culture tubes.

To the dried lipid extract was added 1 ml of Boron trifluoride in methanol (14g BF₃ per 100 ml CH₃OH). This compound was pipetted using a safety pipette in a fume cupboard. The sample was flushed with nitrogen and heated in a water bath at 100°C for 10 minutes. After cooling, 2ml of hexane and 1ml of distilled water were added, the sample vortexed vigorously, and allowed to stand to separate the phases. The hexane phase was transferred to a ground glass stoppered conical centrifuge tube and dried down under a N₂ flow in a fume cupboard. The dried extract was stored at -20°C in a small amount of hexane, under N₂, until ready for gas liquid chromatography.

4.2.2.v. Gas liquid chromatography The machine used for G.L.C. was a Pye Series 204 chromatograph equipped with a temperature programmer and a Pye Unicam DP88 integrator. The peaks were recorded on a chart recorder.

0.5 - 2μl samples were analysed using a one meter column packed with 5% EGSSX 100 on chromosorb W (Varian associates) as the liquid phase, and the carrier gas was argon flowing at 20ml/min. The samples were run isothermally at 170°C.
All recordings were calibrated using various fatty acid standards purchased from Sigma Chemical Co. Ltd.

4.2.3. Membrane Fluidity: Cell Preparation

A fluorescence polarization technique using the membrane fluorescent probe 1,6-diphenyl-1,3,5,-hexatriene (DPH) was used to study the effect of membrane fatty acid alteration and temperature on the membrane fluidity.

Preservative-free tetrahydrofuran (THF) and diphenylhexatriene were purchased from Aldrich Chemical Company. Diphenylhexatriene was dissolved in hot acetone filtered through Whatman No. 1 filter paper and cooled to crystallize the DPH. A $4 \times 10^{-3}$M solution of DPH in THF was then prepared and stored at room temperature under N$_2$ in a darkened glass bottle (molecular weight DPH = 232.3, therefore dissolved 9.29 mg in 10 ml THF).

0.1 ml of DPH stock solution was added, using a finely drawn out Pasteur pipette, to 50 ml of rapidly stirred phosphate buffered solution so that the DPH was efficiently dispersed.

Cells were prepared by incubating in this solution at 37°C for 30 minutes at a concentration of about $10^7$ cells/20 ml solution. The suspension was then centrifuged to remove the DPH solution and the cell pellet was resuspended at about $3 \times 10^6$ cells/ml in phosphate buffered saline. These cells were then kept on ice until ready for polarization measurements, the Elscint microviscosimeter being at a site several miles away (C.R.C. Northwick Park Hospital, MRC Immunology Unit, courtesy of Dr. J. Farrant).

4.2.4. Cell Survival

4.2.4.i. Agar plates Survival was assessed by growing cells on 0.35% agar noble plates. These were prepared according to the following procedure sufficient for 80 plates.

1.4 g of Agar noble was added to 256 ml of sterile double
distilled or deionized distilled water. This was autoclaved for 20 minutes at 15 lb/sq. in. and cooled to about 50°C (hand hot). To this was added:-

- 32 ml 10 X RPMI
- 32 ml 10 X NaHCO₃
- 80 ml Foetal calf serum
- 0.4ml 10³ X antibiotic

and the mixture adjusted to pH 7.4 with IN HCl. 5ml of agar was added to each plate (Sterilin bacteriological grade) using a 25ml plugged glass pipette (sterilized overnight at 125°C in a dry oven) and the plates allowed to set. Experimental plates were usually prepared the previous day and left overnight at room temperature.

Cells were appropriately diluted such that approximately 100 viable cells were dropped onto each plate in a 0.1ml aliquot and the plates were incubated in a 5% CO₂ 100% humidity incubator for 9 to 10 days before the resultant colonies were counted.

A simple preliminary experiment to determine the optimum agar strength was conducted according to the following. 100ml samples of plating medium were prepared with the following strengths of agar 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, and 0.6%. 4 plates of each agar strength were inoculated with 100ul of the following numbers of cells, 50, 100, 150, 200, 300 and the results are illustrated in Table 4C. It was considered that agar strength had no effect on cell survival over this range of concentrations and 0.35% agar plates were used successfully for all survival experiments.

4.2.4.11. Feeder cells A feeder cell experiment was performed which involved diluting a known number of viable cells into a volume of irradiation killed cells (X ray dose of 4 Krads). The results depicted in Table 4D demonstrated that feeder cells were not necessary and were therefore not routinely used. Approximately 100 viable cells were added to each plate.
<table>
<thead>
<tr>
<th>Agar Strength</th>
<th>Cell Number</th>
<th>Plating Efficiency</th>
<th>Agar Strength</th>
<th>Cell Number</th>
<th>Plating Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25%</td>
<td>50</td>
<td>88</td>
<td>0.45%</td>
<td>50</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>Too wet</td>
<td></td>
<td>100</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>Too wet</td>
<td></td>
<td>150</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>88</td>
<td></td>
<td>200</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>Too wet</td>
<td></td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>88</td>
<td></td>
<td>AV</td>
<td>80</td>
</tr>
<tr>
<td>0.3%</td>
<td>50</td>
<td>50</td>
<td>0.5%</td>
<td>50</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>54.5</td>
<td></td>
<td>100</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>62.7</td>
<td></td>
<td>150</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>90</td>
<td></td>
<td>200</td>
<td>84.5</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>-</td>
<td></td>
<td>300</td>
<td>65.3</td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>64.3</td>
<td></td>
<td>AV</td>
<td>79.4</td>
</tr>
<tr>
<td>0.35%</td>
<td>50</td>
<td>52</td>
<td>0.55%</td>
<td>50</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>68</td>
<td></td>
<td>100</td>
<td>77.5</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>78.7</td>
<td></td>
<td>150</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>70</td>
<td></td>
<td>200</td>
<td>76.5</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>75</td>
<td></td>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>AV</td>
<td>70.5</td>
<td></td>
<td>AV</td>
<td>69</td>
</tr>
<tr>
<td>0.4%</td>
<td>25</td>
<td>55</td>
<td>0.6%</td>
<td>50</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>42</td>
<td></td>
<td>100</td>
<td>74.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>50</td>
<td></td>
<td>150</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>52.67</td>
<td></td>
<td>200</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>62</td>
<td></td>
<td>300</td>
<td>60.2</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td></td>
<td></td>
<td>AV</td>
<td>70.1</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>59.5</td>
<td></td>
<td>AV</td>
<td>53.5</td>
</tr>
</tbody>
</table>
TABLE 4 D

FEEDER CELL EXPERIMENT

Approximately 100 viable cells were mixed with various sterilized feeder cell numbers and plated on 0.35% agar to determine the optimum feeder cell number.

<table>
<thead>
<tr>
<th>No. of Feeder Cells/ml</th>
<th>Plating Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero</td>
<td>103.5</td>
</tr>
<tr>
<td>Zero</td>
<td>126 (50µl)</td>
</tr>
<tr>
<td>Zero</td>
<td>105 (150µl)</td>
</tr>
<tr>
<td>9.5 x 10^2</td>
<td>84.8</td>
</tr>
<tr>
<td>2.8 x 10^3</td>
<td>84</td>
</tr>
<tr>
<td>9.2 x 10^3</td>
<td>122</td>
</tr>
<tr>
<td>2.8 x 10^4</td>
<td>114.2</td>
</tr>
<tr>
<td>9.3 x 10^4</td>
<td>132.7</td>
</tr>
<tr>
<td>1.82 x 10^5</td>
<td>50.8</td>
</tr>
</tbody>
</table>
4.2.5. **Irradiation of cells**

4.2.5.1. **Electrons**  Irradiation of cells was with an electron beam from the MRC Linear Accelerator (7 MeV) at 2 Krad per minute in air or oxygen free nitrogen gassing at 2 litres/minute. The system was pre-gassed and followed by another 30 minutes gassing prior to irradiation. The irradiation temperatures (4, 22, 37°C) were maintained by using water-jacketed vessels through which cooled or heated water was circulated. The vessel was identical to that used for E.coli K1060 except the glass scinter bubbler was replaced by an insert allowing a passage of gas across the surface of the liquid at 2 litres per minute. A small glass magnetic stirrer was used to maintain a continual mixing of the contents. A diagram of the vessel is shown in fig. 4D and a photograph in Photo 1.

Cells were normally irradiated without harvesting in 2.5ml of their growth medium (± fatty acid supplements). Irradiations performed in phosphate buffered saline (pH 7.4) gave identical results. Cell death due to mechanical damage was assessed by continuous sampling of unirradiated batches of cells.

Results obtained with this system indicated the possible presence of residual oxygen. Gassing was calibrated according to the scheme below and hypoxia was confirmed. Consequently a different irradiation technique using X rays was adopted.

4.2.5.1i. **X rays**  5ml of cells were irradiated in 40ml roller bottles at 22°C or 4°C according to the technique originally described by Cullen and Walker (1980a). Bottles were rotated at 100 rpm and flushed with N₂ at 1 litre/minute. Rotation causes the cells to be carried over the upper surface of the inside of the bottle and efficient degassing occurs without bubbles. Seven minutes N₂ gassing at 22°C was required to obtain equivalent partial pressures of oxygen in the nitrogen and the liquid (Cullen and Walker, loc. cit) and where irradiations were to be at 4°C, the cooling occurred after the state of anoxia was
Vessel used for electron irradiations.

Details as for Fig. 2 C except as indicated.

Figure 4 D.

Rotating magnet

Rotating stirrer

2 ml. of LDV cells

Air / N₂ / 10% O₂ at 2 litres/minute
achieved. Irradiations were commenced after the seven minutes at 22°C or after a further 5 minutes at 4°C using the MRC 250 kVp X-ray set with a dose rate of 94 rad/min. Samples were taken using 1ml disposable syringes with stainless steel wide gauge needles. To prevent oxygen reintroduction to the vessel when sampling, the needle with plunger fully depressed was inserted about halfway along the bottle tube. The plunger was withdrawn to about the 0.1ml mark and the gas slowly expelled. This process was repeated twice before the needle was pushed right into the bottle cavity when again 0.1ml of gas was drawn into the syringe before the needle was inserted into the cell suspension.

Again, a measure of cell loss due to the gassing procedure was monitored. Because electron survival data indicated a possible K value change with fatty acid grown cells, survival after 1% oxygen gassing was also measured. Cylinders containing 1% oxygen were used from British Oxygen Company, and the oxygen content checked with the Hersch cell.

4.2.5.iii. Gassing vessels used for electron radiation

The rate of removal of oxygen from 2ml (or more) of medium was assessed using a platinum/silver electrode hermetically sealed by wax into the vacant side arm of the vessel.

The electrode was connected to an Oxford chart recorder and the loss of electrode current was monitored (a simplified circuit diagram is shown in Fig. 4E). The current flowing through the platinum electrode is directly proportional to the partial pressure of oxygen in the medium, and when the current stabilises, equilibrium of oxygen in the medium and overhead gas is reached. A sample recording is shown (Fig. 4F). If gassing is linear, a straight line is obtained by plotting the log of the electrode current versus gassing time.

(A measure of the increase or decrease in volume of this liquid due to condensation or evaporation resulting from 4°C or 37°C gassing,
A simplified circuit diagram of the oxygen electrode used for monitoring the removal of oxygen from the vessel used for electron irradiation.
Figure 4. Chart recording showing the rate of loss in conductivity to the rate of oxygen removal from the irradiation vessel, recorded by the oxygen electrode, which is proportional.
was also made).

From this plot one can also easily deduce the time needed for the electrode current to fall to half the original value which indicates that the oxygen concentration has also halved. Considering that air is 210,000 ppm oxygen, it takes approximately 15 half-times to achieve anoxia (≈ 6 ppm). Thus half-times of more than 2 minutes will lead to irradiations where oxygen is still present at radiobiological concentrations if a pregassing time of only 30 mins were used.

Table 4E comprised half-times and predictions of the amount of oxygen left in the gas phase after 30 minutes. It can be seen that in cases where the volume exceeds 2 ml, or the vessel is held at 4°C or even room temperature on occasions, incomplete anoxia is attained after 30 minutes gassing.

2 ml of medium degassed at 37°C is reasonably anoxic in all of the seven vessels tested.

A Hersch cell was used to measure the level of oxygen present in the cylinder gas both before and after passage through the irradiation vessel. The inflow oxygen concentration was 10 ppm and the outflow was 16 ppm. At the gas flow rate used (2 litres N₂/minute) these values were unaffected by the use of either copper tubing or rubber pressure tubing. It was also noted that sampling did not introduce air if the sampling capillary was already full of medium.

The amount of fluid lost and gained due to evaporation or condensation in the vessel is illustrated in Table 4F. During irradiation the humidifying bottle was immersed in water at the same temperature as the irradiation vessel to minimise these volume changes.

4.2.6. Estimation of non-protein bound sulphydryl levels

The levels of non-protein bound sulphydryl present in the three cell types was determined according to the colorimetric method of Ellman (1959).
## TABLE 4 E

<table>
<thead>
<tr>
<th>Vessel Number</th>
<th>Vessel Volume</th>
<th>Half-Time (minutes)</th>
<th>Temperature °C</th>
<th>ppm after 30 mins on top of inflow</th>
<th>Temperature ppm  after 30 mins on top of inflow</th>
<th>15 x half time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1.7</td>
<td>37</td>
<td>1</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2.4</td>
<td>37</td>
<td>36</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2.2</td>
<td>37</td>
<td>14</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1.9</td>
<td>37</td>
<td>5</td>
<td>28.5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2.1</td>
<td>37</td>
<td>10</td>
<td>31.5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2.0</td>
<td>37</td>
<td>6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2.3</td>
<td>37</td>
<td>25</td>
<td>34.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>5</td>
<td>20</td>
<td>3,000</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2.25</td>
<td>20</td>
<td>20</td>
<td>33.75</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>12.5</td>
<td>2</td>
<td>40,000</td>
<td>187.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1,000</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1.7</td>
<td>37</td>
<td>1</td>
<td>25.5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>37</td>
<td>1,000</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>3.8</td>
<td>37</td>
<td>885</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>Temperature °C</td>
<td>Volume</td>
<td>Gas and Flow Rate</td>
<td>Stirring</td>
<td>Volume Change</td>
<td>% Changes</td>
</tr>
<tr>
<td>--------</td>
<td>----------------</td>
<td>--------</td>
<td>-------------------</td>
<td>----------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>1</td>
<td>37</td>
<td>2ml</td>
<td>41 O₂/min</td>
<td>+</td>
<td>-.18ml</td>
<td>-5%</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>2ml</td>
<td>21 N₂/min</td>
<td>-</td>
<td>-.3ml</td>
<td>-15%</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>4ml</td>
<td>21 N₂/min</td>
<td>+</td>
<td>-.4ml</td>
<td>-10%</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>4ml</td>
<td>21 O₂/min</td>
<td>+</td>
<td>-.3ml</td>
<td>-7.5%</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>2ml</td>
<td>21 O₂/min</td>
<td>+</td>
<td>-.3ml</td>
<td>-15%</td>
</tr>
<tr>
<td>6</td>
<td>37</td>
<td>2ml</td>
<td>21 N₂/min</td>
<td>+</td>
<td>-.2ml</td>
<td>-10%</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>2ml</td>
<td>21 O₂/min</td>
<td>+</td>
<td>-.3ml</td>
<td>+15%</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>2ml</td>
<td>21 O₂/min</td>
<td>+</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

Sample 8 had the gas bubbler in ice, the rest were at room temperature. Volume changes were measured over a period of 30 minutes. Experiments performed under conditions of sample 8 led to extra water of condensation entering the irradiation vessel.

**VOLUME CHANGES OCCURRING WITHIN THE IRRADIATION VESSEL**

**DUE TO THE HIGH GAS FLOW RATE OF 2 LITRES PER MINUTE.**
4.3. RESULTS

4.3.1. Membrane fatty acid alterations

Tables 4A and 4B describe the way in which cells were grown before their lipids were extracted and analysed by gas liquid chromatography. Table 4G summarises the average results. The fatty acid methyl esters of greater retention time than arachidonic acid methyl ester, were assessed only in the fourth experiment (Tables 4B and 4K). This was due to a late awareness of their existence in all samples.

The following fatty acid methyl ester standards were run in order to identify the sample peaks. Myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3), arachidonic (20:4), behenic (20:0), erucic (20:1), lignoceric (24:0) and docosahexatrienoic (22:6). The identifications of the other fatty acids were based tentatively on the relative retention times (RRT) and also by comparison with values given in the literature. The relative retention times are expressed as a ratio: Time taken for unknown peak/Time taken for stearic acid methyl ester. The RRT's obtained are listed in Tables 4K and 4L. The proposed identities of 12:0, 14:1, 15:0, 15:1, 16:1 and 17:0 are reasonably certain, but the other fatty acid identifications must remain tentative.

4.3.1.i. Whole cells. Tables 4G and 4H include data obtained from whole cell extracts, and it may be readily confirmed that the fatty acids added to the growth medium are clearly taken up in some form or another. The major increases above control cell values expressed as molar percentages of total fatty acids are:-

<table>
<thead>
<tr>
<th>Added Fatty Acid</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Oleic (12.5 µg/ml medium)</td>
<td>24.6 → 34.8</td>
</tr>
<tr>
<td>1% Linoleic (12.5 µg/ml medium)</td>
<td>2.8 → 22.6</td>
</tr>
<tr>
<td>2% &quot; (25 µg/ml medium)</td>
<td>2.8 → 27.9</td>
</tr>
<tr>
<td>3% &quot; (37.5 µg/ml medium)</td>
<td>2.8 → 37.6</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td>2.9</td>
</tr>
<tr>
<td>W.C. Phospholipid</td>
<td>4.2</td>
</tr>
<tr>
<td>Whole Nucleus</td>
<td>4.7</td>
</tr>
<tr>
<td>W.N. Phospholipid</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>Oleic</strong></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td>2.4</td>
</tr>
<tr>
<td>W.C. Phospholipid</td>
<td>4.0</td>
</tr>
<tr>
<td>Whole Nucleus</td>
<td>1.6</td>
</tr>
<tr>
<td>W.N.P.</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>6.5</td>
</tr>
<tr>
<td>2.</td>
<td>5.6</td>
</tr>
<tr>
<td><strong>Linoleic</strong></td>
<td></td>
</tr>
<tr>
<td>Whole Cell</td>
<td>1.6</td>
</tr>
<tr>
<td>W.C. Phospholipid</td>
<td>2.0</td>
</tr>
<tr>
<td>Whole Nucleus</td>
<td>1.6</td>
</tr>
<tr>
<td>W.N.P.</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>7.0</td>
</tr>
<tr>
<td>2.</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>14:0</td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
</tr>
<tr>
<td>Oleic 1/10</td>
<td>1.9</td>
</tr>
<tr>
<td>(1%) 1/10</td>
<td>2.9</td>
</tr>
<tr>
<td>Average</td>
<td>2.4</td>
</tr>
<tr>
<td>Oleic + Linoleic (1% + 1%)</td>
<td></td>
</tr>
<tr>
<td>Linoleic 1/10</td>
<td>1.4</td>
</tr>
<tr>
<td>1% 1/30</td>
<td>1.8</td>
</tr>
<tr>
<td>Average</td>
<td>1.6</td>
</tr>
<tr>
<td>Linoleic 2%</td>
<td>1.2</td>
</tr>
<tr>
<td>Linoleic 3%</td>
<td>1.4</td>
</tr>
</tbody>
</table>

**Unsaturation Index**

Experiment 1: Molar Percentages

1.4

152.
Whole cell membrane lipids

Table 4G and 4I shows how the increased uptake noted above, is distributed between the major lipid classes, namely: neutral lipids, glycolipids, and phospholipids.

Neutral lipids (including free fatty acids, diglycerides and triglycerides). This fraction is normally 5-15% of the total cell lipid. There is a large increase in the neutral lipid and free fatty acid component of the cells grown in fatty acid supplemented media. The oleic acid (18:1) contribution has risen from 21.6% to 47.5% when supplied at a concentration of 12.5 µg/ml, and the linoleic acid contribution has risen from 2.9% to 46.7% when supplied at 25µg/ml. It is highly likely that this increase is an increase in the triglyceride pool, but no quantitative measurements were made to test this.

Glycolipids. The glycolipid fraction is usually about 5% or less of the total cell lipid, and may well be confined to the plasma membrane. This fraction appears to contain a much higher proportion of saturated fatty acids. The changes noted above control cell glycolipid values in the proportion of oleic and linoleic fatty acids, when these are supplied in the medium were

<table>
<thead>
<tr>
<th>Fatty Acid Supplement</th>
<th>Molar Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1 (12.5µg/ml)</td>
<td>19.1 → 24.7</td>
</tr>
<tr>
<td>18:2 (25µg/ml)</td>
<td>4.0 → 9.7</td>
</tr>
<tr>
<td>18:2 (37.5µg/ml)</td>
<td>4.0 → 10.1</td>
</tr>
</tbody>
</table>

Phospholipids (normally about 85% of total cell lipid)

The exogenously added fatty acids are certainly incorporated into membrane phospholipid. This could be either biochemical incorporation of the free fatty acid into macromolecules or just a passive infiltration of membrane lipids by fatty acids bound to protein. Fatty acids become bound to serum proteins, which facilitates their entry through the plasma membrane. The increases recorded over control levels were:-
TABLE 4 I
WHOLE CELL EXTRACT FATTY ACID ANALYSIS

Experiment 2: Molar Percentages

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fatty Acids</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:1</th>
<th>20:2</th>
<th>20:3</th>
<th>20:4</th>
<th>U.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>All 1/10 FCS</td>
<td>14:0</td>
<td>16:0</td>
<td>16:1</td>
<td>18:0</td>
<td>18:1</td>
<td>18:2</td>
<td>20:1</td>
<td>20:2</td>
<td>20:3</td>
<td>20:4</td>
<td>U.I.</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.7</td>
<td>31.1</td>
<td>5.4</td>
<td>23.5</td>
<td>21.6</td>
<td>2.9</td>
<td>10.9</td>
<td>1.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>37.5</td>
<td>34.9</td>
<td>19.1</td>
<td>4.0</td>
<td>4.5</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>4.1</td>
<td>31.1</td>
<td>4.5</td>
<td>20.4</td>
<td>23.9</td>
<td>4.5</td>
<td>t</td>
<td>t</td>
<td>3.0</td>
<td>8.6</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>4.7</td>
<td>17.0</td>
<td>7.5</td>
<td>10.7</td>
<td>47.5</td>
<td>2.1</td>
<td>2.4</td>
<td>2.8</td>
<td>3.6</td>
<td>1.7</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>1% G</td>
<td>42</td>
<td>26.9</td>
<td>24.7</td>
<td>2.5</td>
<td>3.9</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2.6</td>
<td>25.1</td>
<td>5.7</td>
<td>15.8</td>
<td>40.5</td>
<td>1.1</td>
<td>t</td>
<td>1.7</td>
<td>2.4</td>
<td>5.2</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>Linoleic</td>
<td>2.3</td>
<td>12.8</td>
<td>1.6</td>
<td>9.7</td>
<td>8.1</td>
<td>46.7</td>
<td>-</td>
<td>3.0</td>
<td>12.7</td>
<td>3.2</td>
<td>6.45</td>
<td></td>
</tr>
<tr>
<td>2% G</td>
<td>46.6</td>
<td>26.7</td>
<td>13.5</td>
<td>9.7</td>
<td>3.6</td>
<td>0.65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>21.2</td>
<td>1.9</td>
<td>19.0</td>
<td>9.1</td>
<td>31.0</td>
<td>-</td>
<td>4.6</td>
<td>7.3</td>
<td>4.3</td>
<td>2.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic F</td>
<td>3% G</td>
<td>38.8</td>
<td>31.3</td>
<td>17.9</td>
<td>10.1</td>
<td>2.0</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>2.2</td>
<td>23.6</td>
<td>3.4</td>
<td>16.6</td>
<td>8.5</td>
<td>31.4</td>
<td>-</td>
<td>6.0</td>
<td>4.1</td>
<td>4.1</td>
<td>2.72</td>
<td></td>
</tr>
<tr>
<td>Oleic + Linoleic F</td>
<td>3.1</td>
<td>22.0</td>
<td>3.0</td>
<td>13.1</td>
<td>25.5</td>
<td>15.2</td>
<td>13.2</td>
<td>4.8</td>
<td>3.08</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: F Neutral Lipids + Triglycerides + Free Fatty Acids
G Glycolipids
P Phospholipids
Fatty Acid Supplement     Molar Percentage
18:1 (12.5μg/ml)           23.9 → 40.5
18:2 (25μg/ml)             4.5 → 31
18:2 (37.5μg/ml)           4.5 → 31.4

It was apparent that the maximum membrane fatty acid alterations were produced by adding 25μg/ml of fatty acid to the delipitated medium.

4.3.1.ii. Nuclei  As outlined in Chapter 1, the nuclear membrane has been postulated as a possible target of radiation sensitivity, and may undergo damage which could affect the DNA within the nucleus. Thus changes to the nuclear membrane following addition of unsaturated fatty acids to the growth medium, may be more important than other membrane changes. As can be seen from Tables 4G and 4J, the exogenously supplied fatty acids certainly enter the nucleus. In summary for whole nuclei, increases were:-

Fatty Acid Supplement     Molar Percentage
18:1 (12.5μg/ml)           18.6 → 57.8
18:2 (12.5μg/ml)           1.7 → 48.4

However, results of two experiments on isolated nuclear membrane phospholipid extracts, devoid of nucleoplasm and the pool of free fatty acid plus glycerides, were in conflict. (Tables 4J and 4K).

In the first experiment, where cells were grown for two days with exogenous fatty acid, the nuclear membrane appeared to undergo little change. This growth period had produced the large changes in total cell membrane phospholipid described in the preceding section. Unfortunately, this experiment was done without simultaneously measuring the bulk cell phospholipids for a direct comparison. Nevertheless, a second experiment with appropriate controls, did reveal nuclear membrane changes after a longer period of growth (7 passages, about 15 days) in exogenously supplied fatty acids. (Expt. 3 Table 4J and Expt. 4 Table 4K respectively).

The increase in molar percent, over control cell values are recorded below, and this includes all data plus the average result:-
## TABLE 4 J

### FATTY ACID ANALYSIS

<table>
<thead>
<tr>
<th>Expt. 3. Molar Percentages</th>
<th>Nuclei only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty Acid</td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>14:0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>1N</td>
<td>1.8</td>
</tr>
<tr>
<td>1G</td>
<td>4.9</td>
</tr>
<tr>
<td>2N</td>
<td>2.2</td>
</tr>
<tr>
<td>2G</td>
<td>3.6</td>
</tr>
<tr>
<td>2P</td>
<td>7.4</td>
</tr>
<tr>
<td>6AN</td>
<td>7.5</td>
</tr>
<tr>
<td>6AG</td>
<td>7.7</td>
</tr>
<tr>
<td>6AP</td>
<td>4.6</td>
</tr>
<tr>
<td>6B</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
<td>Oleic</td>
<td></td>
</tr>
<tr>
<td>4N</td>
<td>2.8</td>
</tr>
<tr>
<td>4G</td>
<td>6.4</td>
</tr>
<tr>
<td>4P</td>
<td>9.0</td>
</tr>
<tr>
<td>7N</td>
<td>3.4</td>
</tr>
<tr>
<td>7G</td>
<td>2.0</td>
</tr>
<tr>
<td>7P</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
</tr>
<tr>
<td>Linoleic</td>
<td></td>
</tr>
<tr>
<td>9N</td>
<td>1.6</td>
</tr>
<tr>
<td>9G</td>
<td>5.7</td>
</tr>
<tr>
<td>9P</td>
<td>3.9</td>
</tr>
<tr>
<td>1ON</td>
<td>2.3</td>
</tr>
<tr>
<td>1OG</td>
<td>3.9</td>
</tr>
<tr>
<td>1OP</td>
<td>9.4</td>
</tr>
<tr>
<td>8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Abbreviations as for Table 4 I. No letter following sample number indicates that sample is a whole nucleus.
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>RRT</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Average Neutral Lipid</td>
<td>Lipid</td>
<td>Lipid</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td>Oleic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>all samples)</td>
<td>Lipid</td>
<td>Lipid</td>
<td>Nuclei</td>
<td>Nuclei</td>
<td>18:2</td>
<td>18:2</td>
<td>18:2</td>
<td>18:2</td>
<td>18:2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:0</td>
<td>0.148</td>
<td>3.20</td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:1</td>
<td>0.214</td>
<td>3.90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>0.287</td>
<td>8.27</td>
<td>7.28</td>
<td>3.68</td>
<td>4.64</td>
<td>5.32</td>
<td>8.84</td>
<td>1.37</td>
<td>2.01</td>
<td>1.62</td>
<td>48.18</td>
<td>7.31</td>
</tr>
<tr>
<td>14:1</td>
<td>0.322</td>
<td>0.71</td>
<td>0.41</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15:0</td>
<td>0.361</td>
<td>2.40</td>
<td>3.03</td>
<td>0.99</td>
<td>2.04</td>
<td>3.46</td>
<td>1.60</td>
<td>0.95</td>
<td>0.82</td>
<td>0.79</td>
<td>0.6</td>
<td>2.41</td>
</tr>
<tr>
<td>15:1</td>
<td>0.390</td>
<td>3.46</td>
<td>4.06</td>
<td>1.75</td>
<td>1.96</td>
<td>3.68</td>
<td>1.2</td>
<td>0.79</td>
<td>1.52</td>
<td>0.6</td>
<td>2.41</td>
<td>1.06</td>
</tr>
<tr>
<td>15:2</td>
<td>0.444</td>
<td>2.41</td>
<td>0.60</td>
<td>1.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>0.522</td>
<td>18.70</td>
<td>30.29</td>
<td>24.64</td>
<td>16.78</td>
<td>19.17</td>
<td>29.87</td>
<td>23.6</td>
<td>26.0</td>
<td>20.83</td>
<td>15.33</td>
<td>26.85</td>
</tr>
<tr>
<td>16:1</td>
<td>0.594</td>
<td>6.40</td>
<td>3.47</td>
<td>3.57</td>
<td>4.98</td>
<td>4.72</td>
<td>5.82</td>
<td>5.0</td>
<td>0.79</td>
<td>1.71</td>
<td>3.9</td>
<td>4.07</td>
</tr>
<tr>
<td>16:2</td>
<td>0.631</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17:0</td>
<td>0.722</td>
<td>2.35</td>
<td>1.33</td>
<td>0.23</td>
<td>0.96</td>
<td>0.92</td>
<td>0.88</td>
<td>0.9</td>
<td>0.34</td>
<td>1.65</td>
<td>1.77</td>
<td>0.94</td>
</tr>
<tr>
<td>17:1</td>
<td>0.809</td>
<td>0.09</td>
<td>0.41</td>
<td>1.13</td>
<td>0.52</td>
<td>0.67</td>
<td>0.5</td>
<td>0.59</td>
<td></td>
<td></td>
<td>0.50</td>
<td>1.12</td>
</tr>
<tr>
<td>18:0</td>
<td>1.0</td>
<td>24.96</td>
<td>21.58</td>
<td>17.10</td>
<td>12.45</td>
<td>12.41</td>
<td>18.05</td>
<td>20.9</td>
<td>15.26</td>
<td>17.47</td>
<td>6.14</td>
<td>18.42</td>
</tr>
<tr>
<td>18:1</td>
<td>1.126</td>
<td>19.87</td>
<td>15.40</td>
<td>23.13</td>
<td>38.16</td>
<td>32.58</td>
<td>24.64</td>
<td>28</td>
<td>13.88</td>
<td>16.53</td>
<td>7.18</td>
<td>22.03</td>
</tr>
<tr>
<td>18:2</td>
<td>1.408</td>
<td>0.98</td>
<td>2.68</td>
<td>0.9</td>
<td>1.15</td>
<td>0.11</td>
<td>2.38</td>
<td>3.0</td>
<td>20.87</td>
<td>18.54</td>
<td>2.40</td>
<td>2.15</td>
</tr>
<tr>
<td>20:1</td>
<td>2.199</td>
<td>1.12</td>
<td>0.72</td>
<td>1.9</td>
<td>0.65</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>20:2</td>
<td>2.768</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.73</td>
<td>3.26</td>
<td></td>
<td></td>
<td></td>
<td>3.82</td>
</tr>
<tr>
<td>20:3</td>
<td>3.150</td>
<td>0.46</td>
<td>0.05</td>
<td>1.71</td>
<td>0.79</td>
<td>0.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.17</td>
<td>2.5</td>
</tr>
<tr>
<td>20:4</td>
<td>3.520</td>
<td>0.55</td>
<td>7.68</td>
<td>4.27</td>
<td>6.78</td>
<td>2.51</td>
<td>3.41</td>
<td>3.88</td>
<td>7.44</td>
<td>4.90</td>
<td></td>
<td>6.23</td>
</tr>
<tr>
<td>22:0</td>
<td>3.900</td>
<td>0.98</td>
<td>2.87</td>
<td>0.38</td>
<td>0.28</td>
<td></td>
<td>7.59</td>
<td></td>
<td>1.88</td>
<td>5.76</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>22:5</td>
<td>4.781</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.66</td>
<td>6.32</td>
</tr>
<tr>
<td>22:6</td>
<td>6.77</td>
<td>0.63</td>
<td>2.38</td>
<td>1.84</td>
<td>1.69</td>
<td></td>
<td>1.73</td>
<td>0.64</td>
<td></td>
<td></td>
<td>2.29</td>
<td></td>
</tr>
<tr>
<td>22:5</td>
<td>9.300</td>
<td>1.52</td>
<td>0.24</td>
<td>7.04</td>
<td>2.46</td>
<td>4.89</td>
<td></td>
<td>2.61</td>
<td>3.35</td>
<td></td>
<td>2.44</td>
<td></td>
</tr>
<tr>
<td>22:6</td>
<td>10.400</td>
<td>0.64</td>
<td>4.20</td>
<td>2.80</td>
<td>2.25</td>
<td></td>
<td>2.52</td>
<td>2.13</td>
<td>3.23</td>
<td>2.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>4.6</td>
<td>5.3</td>
<td>0.63</td>
<td>0.14</td>
<td>1.6</td>
<td>1.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>U:1</td>
<td>1.21</td>
<td>0.49</td>
<td>2.93</td>
<td>3.00</td>
<td>2.80</td>
<td>0.84</td>
<td>2.107</td>
<td>2.87</td>
<td>3.13</td>
<td>0.88</td>
<td>1.04</td>
<td>3.66</td>
</tr>
<tr>
<td>Saturated F.A.%</td>
<td>50.86</td>
<td>66.38</td>
<td>47.05</td>
<td>36.87</td>
<td>41.28</td>
<td>59.24</td>
<td>47.7</td>
<td>44.43</td>
<td>42.36</td>
<td>73.91</td>
<td>59.91</td>
<td>39.51</td>
</tr>
<tr>
<td>Unsaturated F.A.%</td>
<td>49.14</td>
<td>33.67</td>
<td>52.95</td>
<td>63.13</td>
<td>58.72</td>
<td>40.76</td>
<td>52.3</td>
<td>55.57</td>
<td>57.64</td>
<td>26.09</td>
<td>40.09</td>
<td>60.49</td>
</tr>
</tbody>
</table>
This data implies that oleic acid does eventually become incorporated into phospholipid, but linoleic does not. However, the data obtained from the second experiment were from weak traces (insufficient experimental material on the G.L.C. column) and the composition of the other acids were not consistent (e.g. myristic acid was 48% in sample 10). More experimental data would be required to establish this point.

Another possible conclusion would be that although the nuclear membrane analyses of experiment one demonstrated no change, the anomalous results of experiment two could be due to nuclear contamination with other cell membrane fractions.

One certain conclusion, however, is that the nuclear membrane phospholipids contain higher proportions of saturated fatty acids, have lower unsaturation indices, and are therefore less fluid, than the bulk cell phospholipids.

The unsaturation index is defined as:

$$U.I. = \frac{(16:1 \times 1) + (18:1 \times 1) + (18:2 \times 2) + (20:4 \times 4) + (22.6 \times 6) \text{ etc.}}{(16:0) + (18:0) + (20:0) \text{ etc.}}$$

i.e. the sum of the molar percentages of the unsaturated fatty acids, including a weighting for the number of double bonds; divided by the sum of the molar percentages of the saturated fatty acids.

4.3.1.iii. Evidence of homeostasis. In order to maintain a balance between saturated and unsaturated fatty acids, and hence maintain correct membrane structure and function, the cells have undergone changes
in the relative amounts of other fatty acids.

**Oleic acid**  Incorporation of oleic acid led to a decrease in the molar percentages of 16:0 (31.1% → 25%), 18:0 (20.4% → 15.8%) and 20:4 (8.6% → 5.2%) in the bulk phospholipids.

The direct elongation product of 18:1, 20:1 (Eicosaenoic acid) which hopefully has been correctly identified, is only 3% in control cells and may have actually decreased in oleic fed cells. Where a slight increase in 18:1 has been recorded in the nuclear phospholipid, a compensatory increase in 18:0 has apparently occurred, with a small decrease in 16:0. Where an increase in 18:1 has occurred in the nuclear phospholipid 16:0 has decreased significantly along with 20:4.

**Linoleic acid**  In the bulk phospholipids, an increased incorporation of 18:2 has led to a major decrease in 18:1 (25.8% → 16.8%) and a small decrease in 16:0 (30.4% → 26.0%). There would also appear to be a genuine increase in the direct elongation product 20:2 (Eicosadienoic acid) from 0.2% to 4.4%, however, no such change was apparent in the nuclear membrane. Contrary to expectations, arachidonic acid composition fell rather than increased following linoleic acid feeding.

**Higher polyunsaturated fatty acids**  22:4, 22:5, 22:6

Cells grown in culture derive almost all their lipids from the serum (Bailey, 1967; Bailey and Dunbar, 1973). The fatty acid analysis of several batches of sera are shown in Table 4L, and serum composition is well documented (e.g. Spector et al, 1979). The data, by comparison, aids the identification of the fatty acids present in the cell tables, as one then knows which fatty acids could or should be present.

Significant amounts of these fatty acids were recorded in control cell bulk phospholipid (2.4, 7.0, and 4.2 molar percent of the total fatty acids). These decreased in cells grown in fatty acid supplemented medium. Although possibly absent in the nuclear membrane of fatty acid fed cells, no control cell nuclear extracts were analysed for polyunsaturated fatty acids. (Table 4K).
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>RRT</th>
<th>SERA U902001D</th>
<th>SERA L906401D</th>
<th>24/6/80</th>
<th>L906401D delipidated</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.2321</td>
<td>t (trace)</td>
<td>t</td>
<td>4.28 (too high)</td>
<td>-</td>
</tr>
<tr>
<td>14:0</td>
<td>0.3058</td>
<td>5.09</td>
<td>3.79</td>
<td>8.15</td>
<td>5.21</td>
</tr>
<tr>
<td>14:1</td>
<td>0.3817</td>
<td>t</td>
<td>2.70</td>
<td>)</td>
<td>4.27</td>
</tr>
<tr>
<td>15:0</td>
<td>0.4107</td>
<td>2.10</td>
<td>3.61</td>
<td>3.66</td>
<td>)</td>
</tr>
<tr>
<td>15:1</td>
<td>0.4531</td>
<td>0.79</td>
<td>t</td>
<td>0.94</td>
<td>0.63</td>
</tr>
<tr>
<td>16:0</td>
<td>0.5379</td>
<td>19.6</td>
<td>20.68</td>
<td>18.56</td>
<td>14.18</td>
</tr>
<tr>
<td>16:1</td>
<td>0.6250</td>
<td>5.91</td>
<td>6.02</td>
<td>5.94</td>
<td>2.53</td>
</tr>
<tr>
<td>17:0</td>
<td>0.7388</td>
<td>1.45</td>
<td>1.55</td>
<td>1.87</td>
<td>1.64</td>
</tr>
<tr>
<td>17:1</td>
<td>0.8415</td>
<td>1.63</td>
<td>1.73</td>
<td>1.77</td>
<td>1.78</td>
</tr>
<tr>
<td>18:0</td>
<td>1</td>
<td>11.55</td>
<td>12.98</td>
<td>12.75</td>
<td>9.41</td>
</tr>
<tr>
<td>18:1</td>
<td>1.1272</td>
<td>24.28</td>
<td>24.68</td>
<td>20.00</td>
<td>19.73</td>
</tr>
<tr>
<td>18:2</td>
<td>1.4129</td>
<td>4.56</td>
<td>3.98</td>
<td>3.88</td>
<td>2.38</td>
</tr>
<tr>
<td>?</td>
<td>1.5826</td>
<td>t</td>
<td>0.77</td>
<td>1.64</td>
<td>t</td>
</tr>
<tr>
<td>?</td>
<td>1.8973</td>
<td>0.67</td>
<td>0.54</td>
<td>0.27</td>
<td>0.25</td>
</tr>
<tr>
<td>20:1</td>
<td>2.1406</td>
<td>0.23</td>
<td>t</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>20:2</td>
<td>2.3549</td>
<td>0.32</td>
<td>0.38</td>
<td>0.84</td>
<td>0.47</td>
</tr>
<tr>
<td>?</td>
<td>2.7679</td>
<td>0.03</td>
<td>t</td>
<td>0.03</td>
<td>t</td>
</tr>
<tr>
<td>20:3</td>
<td>3.0826</td>
<td>1.03</td>
<td>1.25</td>
<td>0.54</td>
<td>2.07</td>
</tr>
<tr>
<td>20:4</td>
<td>3.4821</td>
<td>5.12</td>
<td>4.79</td>
<td>4.39</td>
<td>8.88</td>
</tr>
<tr>
<td>22:0</td>
<td>3.8259</td>
<td>t</td>
<td>0.04</td>
<td>2.20</td>
<td>3.0</td>
</tr>
<tr>
<td>22:5</td>
<td>4.7813</td>
<td>1.67</td>
<td>1.66</td>
<td>1.92</td>
<td>5.09</td>
</tr>
<tr>
<td>22:6</td>
<td>8.8393</td>
<td>4.36</td>
<td>3.90</td>
<td>1.32</td>
<td>6.11</td>
</tr>
<tr>
<td>?</td>
<td>10.2098</td>
<td>4.41</td>
<td>3.62</td>
<td>2.23</td>
<td>7.26</td>
</tr>
<tr>
<td>?</td>
<td>11.8978</td>
<td>4.26</td>
<td>3.87</td>
<td>(missed)</td>
<td>t</td>
</tr>
<tr>
<td>?</td>
<td>13.35</td>
<td>0.95</td>
<td>t</td>
<td>&quot;</td>
<td>5.01</td>
</tr>
</tbody>
</table>
Unsaturation indices

Membrane fluidity changes are implicated by changes in the unsaturation indices of membranes (increases indicate more fluidity).

Increases in the indices for whole cells and whole nuclei for cells grown in fatty acid supplemented media almost certainly result from high triglyceride pools of the fed fatty acids.

The bulk phospholipid values also increase, however, implying increased membrane fluidity in the unsaturated fatty acid grown cells. Linoleic supplemented cells undergo the greatest changes, the unsaturation indices being 1.45, 1.90, and 2.28 for control, 18:1 and 18:2 cells respectively. Dividing the control value into the others, an increase in fluidity is indicated by the values 1.31 for oleic and 1.57 for linoleic fed cells.

For the nuclear membrane, however, which is already less fluid than the bulk cell membranes, the changes are less clear. Values are 0.730 (control) 0.511 and 0.818 (oleic) and 0.754 and 0.964 (for linoleic). These can be converted in a similar way to .70 and 1.12 for oleic fed cells and 1.03 and 1.32 for linoleic fed cells. If a change is present it is certainly less marked. As one would expect, linoleic fed cells give the highest changes.

However, these unsaturation indices do not include the polyunsaturated fatty acids. These have been accounted for in Table 4K. Although the difference between the nuclear membrane and the bulk cell phospholipid is maintained, the difference between the different cell types are virtually lost when they are taken into consideration, except for the linoleic sample number 13.

Increased incorporation of the 18:1 and 18:2 acids has led to a decrease in the higher polyunsaturated fatty acids leading to a stabilization of the number of double bonds present. The data obtained with the fluorescent probe diphenylhexatriene (DPH) (see section 4.3.2)
showing apparent fluidity changes may therefore be solely due to the excess neutral lipids.

4.3.2. **Membrane Fluidity**

Figures 4G and 4H demonstrate the results of polarisation analyses using the fluorescent probe diphenylhexatriene. P values are plotted against optical density to elucidate any variation in P caused by light scattering effects dependent on cell or particle density. Johnson and Robinson (1979) obtained little effect on this parameter caused by cell density but a considerable effect caused by membrane fragment density. These authors extrapolated their data to zero density before making comparisons between data. Figure 4G displaying the results of two experiments (marked 1 & 2) on whole control and whole oleic cells, reveals a change in P value for the same cell type between experiments. To counteract this, Yatvin and coworkers (unpublished data) subtract control bacteria values from fatty acid grown bacteria values at the same optical density before analysing the data. In any event the results conclusively show that oleic grown whole cells appear to be more fluid than cells grown in the absence of oleic acid. Figure 4H showing data from a third experiment, similarly show that linoleic grown cells are also more fluid than control cells. The difference is about 0.06 between control and oleic cells at 37°C and 0.07 to 0.08 for linoleic grown cells which may indicate a slightly higher fluidity level of linoleic grown cells over oleic grown cells. The results also demonstrate that cell fluidity increases when the temperature rises from 4°C through room temperature to 37°C.

In response to literature criticism by Collard and Wildt (1978) on probe data using whole cells, a crude membrane extract was also studied and the results are also displayed in Figure 4H. The results from this single experiment still shows fluidity changes due to temperature and membrane alteration although the increase in fluidity caused by oleic
The graph shows fluorescence polarisation measurements carried out on whole control (C) or oleic (O) cells in two different experiments (denoted 1 & 2).
Details as for Fig. 4 G except M refers to membrane preparations of prelabelled cells, which were sonicated and centrifuged at to pellet the membrane fraction.
acid incorporation is less pronounced. The difference is approximately 0.03 to 0.04 with this data.

4.3.3. Irradiation

Survival of controls - electrons. Figures 4I 1 - 8 demonstrate a colony survival assay of various cells gassed under various temperatures. Fig. 4I 7 also gives survival as seen down a microscope for comparison. The results indicate an approximate 15-20% loss in colony forming ability over the half-hour period during which irradiations are performed. This was thought to be due to mechanical damage. Although experimental handling of the linoleic cells appeared to indicate a greater fragility, this observation has not been confirmed here. I do not feel that this variability change due to fragility significantly affects conclusions drawn from the survival curves to which no correction has been made.

Electron irradiation. Figures 4J 1 - 3 depict survival curves for control, oleic, and linoleic acid grown cells respectively. Nitrogen data displaying breaks in survival lines were assumed to be incompletely anoxic at the commencement of irradiation and were excluded. The data comprises the averages of up to 6, 10, and 4 experiments for control, oleic and linoleic grown cells, respectively. There was no difference in survival between 4°C and 37°C irradiated cells and the data for these temperatures were therefore combined. Fig. 4K shows the separated data for oleic acid grown cells.

A summary of OER's and D_0's appear in table 4M and Fig. 4L combines the six survival curves. The data suggests that linoleic cells irradiated in air are more sensitive by a factor of 1.2.

The nitrogen data also suggests that fatty acid grown cells were more sensitive than control cells, and this had originally been more exaggerated in preliminary experiments. However, since the OER's for these survival lines in these early experiments were less than 3.0 it
Cells were gassed with Nitrogen at 2 litres per minute and samples were diluted and plated immediately.

Loss of viability of unirradiated cells due to the gassing and stirring procedure used for electron irradiation.
Cells were gassed with \( N_2 \) at 2 litres per minute and samples were diluted and plated immediately.
Crosses represent cell counts using a microscope
Survival was assayed as for 1-6
Survival of LDV control cells following electron irradiation at 2 Krad/min under aerated or anoxic gassing conditions. The data points are averages of experiments done at both 40°C and 37°C. Error bars represent ± the standard error of the means. Gassing, at 2 litres/min, was commenced at least 30 minutes prior to irradiation.
Survival of LDV cells grown on oleic supplemented medium and irradiated at 2 Krad/min under aerated or anoxic gassing conditions. The data points are averages of experiments done at both 4°C and 37°C. Error bars represent ± the standard error of the means. Gassing at 2 litres/minute was commenced at least 20 minutes prior to irradiation.
Survival of LDV cells grown on linoleic supplemented medium and irradiated at 2 Krad/min under aerated or anoxic gassing conditions. The data points are averages of experiments done at both 4°C and 37°C. Error bars represent \( \pm \) the standard error of the means. Gassing at 2 litres/minute was commenced at least 30 minutes prior to irradiation.
The graph shows survival data of oleic grown cells irradiated in air or anoxia, and at 4°C or 37°C. Experimental details were as for figure 4 J.
### TABLE 4 M

O.E.R. and $D_0$ values for LDV cells irradiated with electrons.

<table>
<thead>
<tr>
<th>Fatty acid supplement</th>
<th>O.E.R.</th>
<th>$D_0$</th>
<th>AIR</th>
<th>$N_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (None)</td>
<td>2.74</td>
<td>1.2</td>
<td>3.36 Gy</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2.38</td>
<td>1.2</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>2.74</td>
<td>1.0</td>
<td>2.72</td>
<td></td>
</tr>
</tbody>
</table>
The survival curves summarise the data obtained for electron irradiations.
was considered that either fatty acid grown cells were more sensitive under anoxic conditions, or they were more sensitive in the presence of very low levels of oxygen. That is, the three cell types were differentially sensitive at low levels of $O_2$. To test for this the experiments were repeated using the more refined irradiation procedures devised by Cullen & Walker (1980).

**X-ray survival**

Figure 4M demonstrates that little or no loss in unirradiated cell viability occurs during the time course of an experiment.

The result of an experiment illustrated in Fig. 4N at room temperature (22°C) produced an OER of 3.06. It was concluded that the fatty acid cells were no more sensitive than control cells under anoxic conditions and that the earlier electron irradiated cells were not fully anoxic. Although no attempt to differentiate the lower survival of linoleic grown cells irradiated in air was made, the linoleic acid data lies below that of the other two cell types.

To test for the possibility of K value differences, irradiations were performed at one concentration of $O_2$ (1%). Preliminary experiments suggested that the K value was close to this concentration. The results, including error bars for the standard errors of the means, are shown in Figs. 4 O, P and Q. The slopes of these lines are identical and I concluded that the different cell types were likely to have identical values for the oxygen effect K value although this is not strictly proven.

Variations on a day-to-day basis often occurred, but any possible differences in sensitivity between the cell types were not significantly large to distinguish from differences caused by age of culture. The average results therefore comprise data from $1/20 \rightarrow 1/350$ FCS. Cullen and Walker (1980) have shown that the radiobiological oxygen constant K varies with the proliferative activity of the cells. Cullen et al. (1980)
Survival of unirradiated cells subjected to the gassing and rolling conditions used for X-ray irradiations.
Survival of LDV cells (control, oleic grown, and linoleic grown) following X-irradiation at 94 rad/min. Cells were rolled and pregassed at room temperature for 7 minutes prior to irradiation at room temperature.
Figure 4.0.

X-ray survival line of control cells irradiated in 1% oxygen. The error bars denote the standard error of the means of about 12 experiments. Dose rate 94 rad/min.
Figure 4. P.

X-ray survival line of oleic grown cells irradiated in 1% oxygen. The error bars denote the standard error of the means of about 12 experiments. Dose rate 94 rad/min.
X-ray survival line of linoleic grown cells irradiated in 1% oxygen. The error bars denote the standard error of the means of about 12 experiments.
also demonstrated that this variation was dependent on a decline in the non-protein sulphhydryl content with increasing cell density. The average cell density of cells used in the survival experiments were: control, $2.75 \pm 0.30 \times 10^5$/ml; oleic $3.65 \pm 0.28 \times 10^5$/ml; linoleic $2.46 \pm 0.22 \times 10^5$/ml. However, this is an unreliable criterion on which to judge the average results presented, related to cell non-protein bound SH content.

In case any radical scavengers present in the medium prevented expression of peroxidative damage, test experiments were performed in phosphate buffered saline. This procedure, however, produced identical results to those obtained using medium. Cells irradiated at $4^\circ$C, by partial-immersion of the roller bottles in a melting ice bath, also caused no change in radiation cell survival. The increase in dose caused by bulk scatter from the water surface was allowed for. (J. Lunec - personal communication).

4.3.4. Non-protein bound SH

The non-protein bound SH levels were plotted against cell growth density in Figure 4R. Although the SH levels decline with increasing density there appeared to be no change due to the alteration of the membrane fatty acid composition.
Figure 4. Variation of NPSH levels with cell density in LDV cells.

LDV cells grown in RPMI medium
- Control
- Oleic
- Linoleic
4.4. DISCUSSION

Procedures outlined in this chapter have successfully led to the alteration of the LDV cell membranes and membrane phospholipids, but it remains to be seen whether excess linoleic acid is taken up by the nuclear membrane. The fact that the nuclear membrane phospholipid contains a higher percentage of saturated fatty acids compared to the total cell phospholipid is in agreement with the observations of Stadler and Kleinig (1971) for pig liver, Kennan et al. (1972) for bovine liver, and Schla ger and Ohanian (1980) for guinea pig hepatoma cells. It would also explain why, because of a lower degree of unsaturation, the nuclei were less susceptible to peroxide formation than other subcellular membrane fractions (Wills and Wilkinson, 1967).

The lack of effect on radiation sensitivity, following membrane alterations is in contrast to the E.coli K1060 data described in Chapter 3. One explanation for this may be the mammalian cells' capacity for reducing the number of double bonds present by reducing the amount of the polyunsaturated fatty acids like arachidonic (20:4) and docosahexanoic (22:6) when fed 18:1 and 18:2 acids. An alternative explanation could be that the levels of natural antioxidants in the bacteria vary with fatty acid supplementation or more simply bacteria contain lower levels of antioxidant capacity compared to mammalian cells which is overcome following membrane alteration. The fact that the E.coli K1060 are more sensitive in buffer may indicate a leakage of antioxidant in the pre-irradiation holding stages. However further experimentation did not show this to be true in K1060.

I have shown (Fig. 4R) that no change in non-protein bound SH levels occur subsequent to changes in LDV membrane fatty acid composition. The results do suggest, however, that LDV cells grown with excess linoleic acid may be more sensitive than control cells in
using liposomes, have demonstrated that alteration of membrane fatty acid compositions can influence fluorescent probe rotational relaxation time (and hence membrane fluidity) once the phospholipids are within about 10°C of the oleic phospholipid liquid-crystalline to solid phase transition. They point out that this is unlikely to occur under physiological conditions.

The indications of greater radiosensitivity of rats fed essential fatty acid deficient diets (Cheng et al. 1952 and 1953) and the contra-indications reported by Harms-Ringdahl (1980) of essential fatty acid deficiency reducing mouse radiosensitivity following whole body irradiation, are difficult to interpret. It may be that whole animal radiosensitivity changes are more dependent on physiological changes independent of theoretically possible peroxidative damage caused to the cell membranes. Since essential fatty acids are also precursors of prostaglandins (Holman, 1971) the consequences may be dependent on hormonal changes. Another factor which was not controlled in the animal experiments was relative humidity. It has been shown (Holman, 1971) that low humidity will greatly enhance the expression of symptoms of essential fatty acid deficiency.

The survival data for LDV cells did not reveal any change in radiosensitivity following electron irradiations at 4°C or 37°C at 3 Krad/min. Similarly, no change in radiosensitivity occurred following X-ray irradiation at a dose rate of 94 rad/min at 4°C and 22°C. Gholipour-Khalili (1978) did not show any increase in sensitivity of a number of E. coli wild type strains when the temperature was lowered from room to ice-bath temperatures. Only the unsaturated fatty acid auxotroph E. coli K1060 proved to be more sensitive below a membrane phase transition temperature (Yatvin et al. 1979). Schechter et al. (1974) have shown by freeze-fracture electron microscopy in a similar mutant that, as the temperature is lowered below a phase transition
air. A yet lower X-ray dose-rate might amplify the effect. Raleigh et al. (1977) have demonstrated a sharp rise in oxidative damage below 100 rads/min which has also been seen for erythrocyte plasma membrane by Konings (1981).

From the full fatty acid analyses of Fig. 4K, one might conclude that the change in membrane fluidity following membrane changes as detected by diphenylhexatriene, are in contradiction of the unsaturation indices. Stubbs et al. (1980) in extensive studies, correlated a change in membrane fluidity with the appearance of lipid droplets in the cytoplasm of the lymphocyte cell supplied with exogenous fatty acids. They were able to show that the fatty acid pattern of the neutral lipids extracted from the purified plasma membrane showed very little incorporation. However, their incubation times were only 20 hours, compared to the period of at least 2 days growth and 4-5 divisions in the LDV cells. Thus my data may be compared more favourably with the alteration of fluidity of rabbit platelet membrane isolates following dietary changes demonstrated by Berlin et al. (1980).

Harder conclusions cannot be drawn from either my data or that of Berlin (loc cit) since neither author has actually isolated neutral lipid extracts from purified membrane extracts. My analyses were performed on whole nuclei still probably containing a large nucleoplasmic pool of exogenously supplied fatty acid triglycerides.

As reported in the introduction to this chapter, there is some evidence in membrane fluidity data which supports the fact that linoleic acid (18:2) incorporation will have very little greater effect on membrane fluidity than incorporation of oleic acid (18:1) (Shinitzky and Henkart, 1979).

Johnson et al. (unpublished data, quoted in Stubbs et al. 1980)
temperature the lipids and membrane bound proteins aggregate into separate domains. This has not been shown to occur in wild type E. coli bacteria, as their transition temperature is probably below 4°C. However, phase separations of solid and fluid lipids have been observed in freeze-fracture electron micrographs of phospholipids cooled from temperatures below enzyme activation energy disorder-order transitions (Luna and McConnel, 1977). In addition electron micrographs of rat liver mitochondrial membranes showed large smooth areas of gel phase lipid with protein particles aggregated into small areas of fluid phase lipids (Hackenbrock et al. 1976).

Increases in the activation energies of membrane bound enzymes when measured at low temperatures has been taken as supportive evidence that some proteins may phase separate with the more fluid lipids at these low temperatures (<15°C) (McMurchie and Raison, 1979). These authors demonstrated an increase in the activation energies of succinate oxidase from the mitochondria from sheep liver and kidney, when cooling from 40°C to 0°C. Therefore it seems unreasonable to propose differences between LDV cells and E. coli K1060 on a phase separation argument to explain why the bacteria have altered radiation sensitivity and the LDV cells do not. Activation energy changes indicate that membrane lipid fluidity has a direct influence on the conformation of the active site of some membrane-associated enzymes. Since the lipids exert their influence by interacting at hydrophobic regions of the enzymes distinct from the active site, they can be considered allosteric effectors of membrane associated enzymes.

However, phase transitions inferred from kinetic data are only indirectly related to lipid fluidity. Fluorescence polarization studies, which are specifically confined to the hydrocarbon region of the lipid layer, have produced reports of only two actual lipid phase transitions to date (Shinitzky and Henkart, 1979). Both of these studies
demonstrated a broad transition around 25°C.

**Addendum**

Since the completion of this Chapter I have done several experiments to further test for an increased sensitivity of LDV cells grown in linoleic acid and irradiated in air. No such increase in sensitivity was obtained. However, see the addendum to Chapter 5.

**Flow Activation Energy** $E$ (Shinitzky and Henkart 1979)

The flow activation energy ($E$) can be derived from the following equation by determining the change in microviscosity ($n^-$) with temperature.

$$n^- = \frac{E}{RT}$$

Where $n^-$ = membrane microviscosity

$T$ = temperature

$A$, and $e$ are constants. $R$ is the universal gas constant.

The equation holds for a given system. Adding proteins to the system alters the system, and therefore alters the flow activation energy. Phase changes also alter the flow activation energy.
CHAPTER 5.

Conclusions and General Discussion.
Chapter 5:

Conclusions and General Discussion.

5.1. INTRODUCTION

It was my intention in this thesis to consider more than one aspect of radiobiology. Initially, the effects of radiation on DNA structure and function were considered as either direct evidence for events responsible for cell lethality (e.g. strand breaks) or as indicators that the cell killing event occurred in DNA (e.g. DNA synthesis and DNA degradation). Having disclosed, for a variety of reasons, that these phenomena were not entirely satisfactory reflections of the characteristics of cell killing, particularly with regard to the influence of oxygen present at the time of irradiation, an alternative site of radiation damage leading to cell death was postulated to be in membrane. A resume of the literature presented ideas that membrane damage may result in cell death directly or that interaction between membrane and DNA, one or both of which may be damaged, might result in the lethal lesion(s).

In Chapter 2, the techniques and conclusions of a classical paper (McGrath and Williams, 1966) implicating the importance of strand-break repair in bestowing relative radiation resistance, was critically refuted. These authors had ignored the loss of TCA precipitable DNA from their sucrose gradients, which was an indication of the occurrence of DNA degradation. Their conclusion that the radiation resistant bacterial strain _E. coli_ B/r was able to repair strand breaks more efficiently than the sensitive strain Bs-1, could be easily discredited by taking into account the phenomenon of DNA degradation. I have demonstrated that these strains are equally strand-break repair proficient and have taken this as evidence to support the existence of
an alternative lesion responsible for radiation cell killing.

In \textit{E. coli} K1060, Yatvin (1976) and Yatvin et al. (1979) presented evidence that membrane fluidity could influence the radiation sensitivity of bacteria. Bacteria irradiated below the temperature whereby the cellular membrane structure was changed from a liquid-crystal to a gel condition, were more sensitive to radiation. In the same organism, Redpath and Patterson (1978) had also shown that the number of double bonds present in the unsaturated fatty acids incorporated into membrane could also influence survival at a particular temperature. An increased number of double bonds enhanced the killing effect of radiation. These authors considered that post-irradiation lipid peroxidation was responsible for the changes in bacterial sensitivity. Knowing that Pietronigro (1977) had indicated that lipid peroxidation could affect the biological integrity of DNA \textit{in vitro}, I have tested whether the alteration of the fatty acid composition of the membranes of \textit{E. coli} K1060 could alter the amount of initial or residual DNA strand-breakage. This work was reported in Chapter 3, and indicated that membrane fluidity changes do not affect the levels of strand breakage. The experiments reported do not discount a role for lipid peroxidation in strand-break kinetics as the fatty acids investigated in detail, oleic and elaidic both containing only one double bond, could be oxidised to the same extent.

A more comprehensive investigation of the role of membrane fluidity and lipid peroxidation was attempted in Chapter 4. This reports investigations on the effect of irradiation temperature and the effect of increasing the content of unsaturated fatty acids containing one (oleic) or two (linoleic) double-bonds on the radiosensitivity of the LDV mammalian cell line. However, since manipulation of the unsaturated fatty acid composition produced no change in radiation
cell sensitivity, the production and repair of strand breaks was not investigated.

Thus a brief conclusion to the work detailed in this thesis is that -

1. *E. coli* B/r and Bs-1 are equally strand-break repair proficient.
2. Membrane fluidity affects the survival characteristics of the *E. coli* unsaturated fatty acid auxotroph K1060, but induces no concomitant change in DNA strand-break kinetics.
3. That manipulation of the fatty acid composition of the LDV mammalian cell line produces no change in radiation survival characteristics, even though one would expect that increases in the unsaturated fatty acid content of the membrane would increase the probability of lipid peroxidation and that the conditions of irradiation would instigate changes in membrane fluidity.

I propose to complete this thesis by discussing the results more fully; by considering future work along these lines; and by reassessing the hypotheses behind the experimentation and conclusions drawn, in the light of more recent publications.

5.2. Repair of DNA Strand Breaks

Experiments reported in Chapter 2 clearly demonstrate that the conclusions drawn by McGrath and Williams (1966) were erroneous due to DNA degradation. Strand-break repair within an hour post-irradiation was shown in this thesis work to be equal between *E. coli* B/r and Bs-1 within the limits of the hydroxylapatite technique. Pollard and Fugate (1978) in concurrent experiments, came to identical conclusions using the traditional alkaline sucrose gradient technique. The question arises as to whether the difference in radiation sensitivity is dependent on other DNA lesions, lesions in other macromolecules which may or may
not act in conjunction with DNA lesions, or from undetectable residual levels of strand breaks undergoing repair at later times than those reported in this thesis.

To date, no differences have been reported concerning other DNA lesions in B/r and Bs-1. There is the possibility that degradation also masks a difference in the production and/or repair of base damage in the alkaline sucrose gradient technique. The hydroxylapatite technique uses pH values which do not convert base damage to strand breaks. Alkaline-sucrose gradients employ stronger alkali which converts base damage to strand breaks. Attempts to demonstrate unwinding with higher levels of alkali would require very short lysis periods, but could be tried. Paterson et al. (1979) described evidence that base damage had a low O.E.R. or an O.E.R. of less than one in skin fibroblasts, and are therefore not oxygen sensitive sites of damage. However, Lafleur et al. (1981) demonstrated that O-ray induced alkali-labile sites in ØX174-RF-DNA were not identical with apurinic and/or apyrimidinic sites. These authors suggested that a damaged deoxyribose molecule which had lost its base, in an otherwise intact sugar-phosphate backbone, could be the alkali-labile site. Considering the differences between oxic and anoxic strand-breaks, Edgren et al. (1981) have published further evidence that they are qualitatively different in human fibroblast cell lines. In addition, the repair capacity of glutathione was active only in the repair of oxically induced single-strand breaks.

Yatvin et al. (1972) clearly indicated that Bs-1 appeared less proficient at some form of membrane repair compared to B/r and has also shown differences in the phospholipid composition between these two strains post-irradiation (Jacobson and Yatvin 1976). These authors noted a lag period of 30-45 minutes before any changes were manifested, which is similar to the lag period noted in changes in the DNA synthetic
capacity of DNA membrane complexes in these strains (Cramp et al. 1972), and also to the onset of lipid peroxidation in isolated lysosomes (Watkins, 1970) and other subcellular organelles (Wills and Wilkinson, 1967).

It is possible that damage envisaged as an interaction between DNA and membrane is the detachment of DNA from membrane due to strand-breakage. Abe et al. (1977) has demonstrated that DNA is released from E.coli membrane complexes by single-strand endonucleases, and that approximately twenty DNA-membrane attachment sites exist per cell. Not all of these sites were susceptible to single-strand endonuclease. There is a possibility that damage to DNA could result in DNA release. Dardalhon-Samsonoff and Averbeck (1980) showed that wild type M. radiodurans was twice as efficient at restoring DNA attachment to membrane compared to a radiosensitive mutant U.V.17, following irradiation. This reattachment correlated with survival and DNA degradation but always preceded the onset of DNA synthesis after X-irradiation. They believed that the DNA-membrane complex protected irradiated DNA from further breakdown and allowed further repair to occur.

Another consideration is whether a differential occurs in residual strand break repair occurring at later times than the 30 minutes recorded in my data. Although the work involved mammalian cells, Ahnstrom and Edvardsson (1979) have demonstrated a fast, intermediate and slow repair of strand breaks. These authors speculated that fast repair involved single-strand breaks, while intermediate repair involved double strand breaks with a structural support. They also believed that slow repair, which took 15 hours, concerned those double-strand breaks which occurred between the nucleosomal bodies in the 'linker' DNA and required a three body collision to form a
repair complex. This form of repair is not applicable to bacteria, but the principle, nevertheless, could be relevant. (Such an explanation would have been considered for differences in mammalian cell sensitivity following membrane alteration, had such a result been obtained in Chapter 4). Thus, experiments looking at longer repair times may improve our knowledge on the lethal lesion(s) in bacteria. I would use the more sensitive method suggested by Rydberg (1980), who improved the hydroxyapatite technique by treating simultaneously, unirradiated cells labelled with one radionuclide and irradiated cells labelled with a different radionuclide. However, one would still have to observe DNA remaining post degradation.

Whatever the lethal lesion, differences between cell lines may not come to light until the cell enters division. This event is generally believed to be where the relevant lesions manifest their lethality following irradiation. Any lesion leading to a disproportionate sharing of DNA between daughter cells, or to loss of genetic material is likely to be lethal.

If DNA membrane attachment is important in ensuring equal sharing of DNA in daughter cells then a lethal lesion could be not one double-stranded break but two, since two would be needed between attachment points to separate a section of DNA completely from membrane, resulting in a loss of DNA in one of the daughter cells.

A final consideration on this topic would be that DNA degradation, although unrelated in extent to cell death, may be initiated at the lesion inflicting lethality other than residual breaks, or particular sites on the DNA. That such lesions are in small numbers is indicated by the results and discussions in Chapter 2.

5.3. Membrane Damage

5.3.1. E.coli K1060. There is no doubt that K1060 is more
radiosensitive when irradiated below its membrane transition temperature, and that elaidate grown K1060 are more sensitive than oleate grown K1060. Chelack et al. (1975) have also demonstrated that Acholeplasma laidlawii is more sensitive to radiation below 12°C in air; the membrane phase transition temperature according to spin label studies. However, the opposite effect was obtained in nitrogen.

Reasons proposed to explain the temperature effect include membrane leakage, and incurrence of different amounts of lethal damage owing to a difference in macromolecular interaction at the time of irradiation. Yatvin et al. (1979) considered that the important change was the lateral phase separations (segregations) of lipid and protein domains as reported by Haest et al. (1974) and Shechter et al. (1974). Yatvin et al. (loc. cit) believed that these separations only occurred in K1060 at the temperatures of increased radiosensitivity, and not at all in the wild type above 0°C. They also felt that lateral phase separation was not recorded in the wild type E. coli strains owing to a level of 16:2 and 18:2 fatty acids capable of maintaining membrane homogeneity.

Reports on lateral phase separation of lipids, and the resultant segregation of lipid and protein domains are complicated, and can lead to confusion. Letellier et al. (1977) proved by chemical analysis that phase separations involved a separation of the lipids containing two saturated fatty acid chains, from the remaining lipids which contained two unsaturated fatty acid chains or one saturated and one unsaturated fatty acid chain. The membrane particles seen in freeze-fracture electron microscopy proved to be proteins. Due to the closer packing of the two saturated fatty acid chains (the phase transition), the proteins present in these fully saturated lipids are squeezed out into the still fluid mixed unsaturated lipids. Thus proteins undergo a lateral
phase separation or aggregation, becoming more closely packed in these more fluid areas. This is illustrated in Figure 5A. The bacterium used was the unsaturated fatty acid auxotroph K 1059 grown with linolenic acid. 

However, an occurrence of lipid protein segregation is not sufficient to explain the increase in radiosensitivity in K 1060 irradiated below the phase transition temperature, since it has been reported in K 1060 where sensitization does not occur, and also in several wild type strains. Although Kleeman and McConnell (1974) did not detect any aggregation in the plasma membranes of wild type E. coli K12 1100 thiamine between 3.5 and 42°C, Haest et al. (1974) obtained some aggregation of proteins at 20°C in E. coli K12 wild type with a complete separation at 5°C. A kink in the Arrhenius plot at 12°C occurred in the membrane bound NADP oxidase activity. He did not separate the outer and inner bacterial membranes. Work done by Janoff et al. (1979) would also suggest that lateral phase separations occur below 8°C in E. coli W 1485 F- (wild type) outer membranes. In addition Letellier and Shechter (1976) demonstrated phase separations at 0°C for K 1059 cytoplasmic membranes grown with linolenic acid, although no kink in the Arrhenius plot of proline transport occurred in the whole cell membranes between 0°C and 45°C (Shechter et al. 1974), whereas Yatvin (1976) reports no change in radiation sensitivity in linolenate K1060 between 0°C and 22°C. Furtado et al. (1979) also obtained lateral phase separations in the blue green alga Anacystis nidulans.

Yatvin (1979) has proposed that small percentages of fluid membrane will probably maintain the function of processes essential to repair or survival, and demonstrated that only 6% of linolenic acid in palmitelaidate grown cells will prevent any sensitivity increase at 4°C. However, his proposal that levels of 16:2 and 18:2 fatty acids
Shecter et al. (1974) found that freeze-fracture electron micrographs of oleate grown *E. coli* K1059 cytoplasmic membrane vesicles frozen from 45°C had fracture surfaces covered with densely packed particles. However, when frozen from 0°C the fracture surfaces displayed a mixture of smooth domains devoid of particles separated by very densely packed particulated regions.

The smooth areas consist of solely lipids containing two saturated fatty acid chains. Due to a greater lipid-lipid interaction and a decreased protein-lipid interaction in this region, the proteins are squeezed out and segregate from the saturated lipids into the unsaturated or partly unsaturated lipids. These lipids remain fluid and phase separate from the gelled saturated lipids. This is diagrammatically represented below.

**Key:**
- • Proteins
- + 2 unsaturated chains
- ▲ 1 saturated & 1 unsaturated chain
- ○ 2 saturated chains
in particular maintain fluidity in the wild type is not fully supported by other fatty acid analyses reported in the literature. Haest et al. (1974) reports 2.7% 14:1, 7.1% 16:1, 46.6% 18:1 and 12.7% 19:0 (cyclopropane fatty acid) in Staphylococcus faecalis membranes which undergo lateral phase separations. These authors state that E. coli K12 has a similar fatty acid composition and no record of 16:2 and 18:2 fatty acids is made. Yatvin himself, however, (1976) demonstrates that oleate grown K 1060 contains 5.6% 16:2 and 4.3% 18:2 fatty acids, and Shecter et al. (1974) quote 5% 18:2 in oleate grown K 1059. Overath et al. (1975) quote only 4.3% 16:1 in oleate grown K 1062 cells. Jackson and Sturtevant (1977) detected no sixteen carbon chain unsaturated fatty acids in either oleate grown or elaidate grown K 1060. Thus the presence of a minor component of fluid membrane appears to be the essential factor preventing radiation sensitivity changes below the phase transition temperature.

Although linolenate grown K 1059 have undergone complete phase transitions at 6°C (Shecter et al. 1974) Letellier et al. (1977) propose that the phospholipids containing two 18:3 fatty acid chains do not solidify at 0°C. Shecter et al. (1974) report that phase transitions have not been completed in linoleate grown K 1059 at 2°C.

Haest et al. (1974) obtained no lateral phase separations with the gram positive bacteria, Staphylococcus aureus, Bacillus subtilis, B megaterium and B. cercus, although breaks in the Arrhenius plot of NADH oxidase activity occurred between 4 and 40°C. The authors considered that the very high percentage of branched chain fatty acids present in these bacteria (up to 85%) prevented phase separation. No aggregation occurred even at -10°C. X-ray diffraction patterns suggested that branched chains were more loosely packed than straight chains in the gel phase. This difference in packing probably explains why membrane particles were not squeezed out of the lipids during phase separations.
Thus the presence of a small proportion of fluid lipid is sufficient to maintain normal sensitivity in bacteria.

Since pre-cooled bacteria irradiated at 37°C are not more sensitive, then one must conclude that the lateral phase separation results in higher levels of lethal lesions due to more interaction of damage to adjacent but different molecules or to a different type of lesion. Although the results of Chapter 3 do not indicate a change in the level of strand-breaks, the simultaneous DNA degradation prevents the witnessing of equivalent strand-break repair following 4°C and 37°C irradiations.

In Chapter 3 I chose to disregard the possible role of peroxidation in the difference in sensitivities of elaidate and oleate grown bacteria, stating that each fatty acid contained only one double bond. However, elaidate grown bacteria contain about 1.5 times more elaidic acid and hence 1.5 times more unsaturated bonds than oleate grown bacteria contain oleic acid and unsaturated bonds (Yatvin et al. 1979). Thus there is a potential for more peroxidation in elaidate cells. A simple explanation for the greater sensitivity of elaidate grown cells at any temperature below 35°C is that elaidate grown cells will be more below their phase transition temperature than oleate grown cells. Indeed Yatvin (unpublished data, and Yatvin et al. 1979) have demonstrated a continuing increase in radiosensitivity of elaidate K 1060 with decreasing temperatures between 35°C and 4°C.

I have reported in this thesis that E.coli K 1060 incubated in C/R buffer at 4°C does not release non-protein bound SH (GSH) into the buffer. I used this to support the statement that leakage was not responsible for the increased radiosensitivity below the membrane phase transition temperature. However, it could be that leakage of GSH only occurs following radiation and that this occurrence explains the loss
of viability of bacteria held in medium before plating. If Vitamin E, a potent membrane anti-oxidant, is present in K1060, and its function is impaired by lateral phase separation away from targets which can be oxidised, then this could result in the increase in radiosensitivity below the membrane phase transition temperature. Alternatively, if the sensitive target is DNA or protein connected to the membrane, then this type of molecule is likely to be situated in a more highly unsaturated environment following phase separations, since embedded particles will remain in the fluid phase. Law et al. (1963) state that bacteria do not contain lipid antioxidants. I have not found a more recent reference. Lateral diffusion, reduced by membrane gelling, would presumably be unimportant as wild type bacteria also undergo phase transitions. It might be rewarding to search for evidence of increased peroxidative damage in E.coli K1060 irradiated below its membrane transition temperature, by lysing cells post radiation and holding them for one hour at the temperature of irradiation, before analysis, to allow peroxidation to proceed. Mead (1976) suggests that lowering lipid fluidity and hence mobility should reduce radical chain propagation occurring in lipid peroxidation, however if the unsaturated fatty acids are closer together below the phase transition the situation could well increase radical chain propagation, and lipid peroxidation. Suzuki and Akamatsu (1980) have shown that the radiation sensitivity of K⁺ permeability in E.coli mutants depends on the fluidity change produced by the unsaturated fatty acid supplied. One effect of lipid peroxidation is the production of lyso products. These compounds are phospholipids with only one fatty acid side chain remaining, following oxidation and chain breakage of an unsaturated fatty acid in the other available position. These compounds stay in the membrane and act as a detergent, causing increases in permeability, whereas oxidised fatty acid chains
enter the aqueous environment (Dobretsov et al. 1977). It is interesting to note that Medvedev et al. (1977) discovered that lysodiphosphatidyl glycerol was one of the natural lipid components responsible for potassium transport in mitochondria. However, radiation of isolated mitochondria reduced $K^+$ transport and caused the loss of lysodiphosphatidyl glycerol and similar active lysoforms from the membrane (lysopolyglycerophospholipids).

These data appear to be contradictory. Jacobson and Yatvin (1976) reported a loss of 9% of the membrane lipid following $\gamma$-irradiation of *E. coli* B/r and Bs-1, which was recovered as a non-phospholipid fraction of unidentified composition. These could have been broken fatty acid chains from phospholipids. Konings (1981) has demonstrated an inverse dose rate effect from 0.3 to 3.0 Gy per minute, in $K^+$ ion leakage from bovine erythrocytes, presumably a consequence of lipid peroxidation.

Some comments can be made about the localization of any postulated membrane targets. Overath et al. (1975) demonstrated that although the transition temperatures were equivalent in the outer and cytoplasmic membranes of the *E. coli* auxotroph K 1062, 60-80% of the hydrocarbon chains took part in the phase transition of cytoplasmic membranes, whereas only 25% to 40% of the chains became ordered in the outer membrane. This makes the cytoplasmic membrane a qualitatively more likely target. However, 70% of the total phospholipid is in the outer membrane of the gram positive *Bacillus amyloliquefaciens* (Paton et al. 1978) including most of the phosphatidyl ethanolamine, although cardiolipin is in higher amount in the cytoplasmic membrane. Thus possibly an equivalent absolute amount of hydrocarbon chains take part in the phase transitions in the membranes of K 1062. Overath et al. (1975) also report that lipopolysaccharides contain mainly 12:0, 14:0, 16:0 and B-OH-14:0 fatty acids and Silbert et al. (1973) have observed
that the fatty acid composition of lipopolysaccharides remains constant in fatty acid auxotrophs. This suggests that lipopolysaccharides are unlikely targets in the radiation sensitivity changes discussed here.

Glycolipids have been shown to stimulate DNA polymerase activity in a DNA-membrane fraction extracted from *Pneumococci* (Zerial et al. 1978) and had the greatest effect on Pol III activity. This stimulation appeared to be partly in consequence of the creation of a favourable environment for the polymerase. Although only present in gram positive bacteria, an equivalent molecule in gram negative bacteria could be an important lipid target associated with DNA.

5.3.2. Mammalian cells

In contrast to the bacteria data, Goureau-Counis et al. (1974) demonstrated that the rate of DNA synthesis of liver nuclei in vitro was lower in the presence of phospholipids of higher unsaturation. However, results in this thesis have not produced radiation sensitivity changes following changes in the phospholipid fatty acid composition of LDV cells although under highly delipidated growth conditions, linoleic supplemented cells grew more slowly than oleic supplemented cells.

Peroxidation in mammalian cells is normally prevented by antioxidants such as vitamin E and glutathione (GSH). Vitamin E is hydrophobic and is contained in the cellular membranes whereas glutathione (a tripeptide) is mainly cytoplasmic. The distribution of vitamin E is not uniform; 60% being in the mitochondrial membranes, and 30% in the endoplasmic reticulum (Witting, 1980). This distribution reflects its biological function of preventing lipid damage caused by free radicals produced by biological enzyme systems such as oxidative phosphorylation in mitochondria. Lipid peroxidation has not been demonstrated in vivo, although phenomena associated with lipid damage, but not necessarily peroxidation, have. Konings (1981) reported that the release of haemo-
globin was greater at dose rates lower than 30 rads/minute in erythrocytes. However, there are many records of peroxidation in vitro, as reported in the introduction. Konings (loc. cit) observed that soluble SH (glutathione) disappeared rapidly during the post-radiation incubation of microsomes, however vitamin E (α-tocopherol) did not. Vitamin E proved to be much more efficient than GSH at protecting lipids from peroxidation, and this was only oxidised once the SH antioxidants had disappeared. The in vitro lag prior to peroxidation, reported by Wills and Wilkinson (1967) Watkins (1970) and others, proved to be coincident with the non-oxidation of vitamin E.

That sensitivity changes did not occur due to an increased potential for membrane lipid peroxidation damage in LDV cells, could be connected with the decline in the polyunsaturated fatty acid contribution in the phospholipids. Konings et al. (1979) showed that the polyunsaturated fatty acids are the most prone to peroxidation, at a dose rate of 80 rads/minute. However, very efficient protection of these polyunsaturated fatty acids (in liposome phospholipids) was obtained with low concentrations of vitamin E (10^{-5} M). It is therefore possible that vitamin E levels in the LDV cells can prevent any significant peroxidation following doses of irradiation used in experiments described in this thesis. In an attempt to accentuate lipid peroxidation, I intend to inhibit any role played by glutathione (GSH, antioxidant) by adding the highest non-toxic concentration of N-ethyl maleimide (N.E.M.) to the LDV cells. This binds to glutathione and prevents its action. Studies to date demonstrate that N-ethyl maleimide is equally toxic to control, oleic, and linoleic grown cells. I do not at present know the amounts of vitamin E present in LDV cells grown on foetal calf serum. No vitamin E is present in the RPMI medium used for growth. It may be possible to investigate the additional effects of reduced vitamin E, on radiation sensitivity.
Although experiments with the fluorescent probe diphenyl-hexatriene did demonstrate that membrane fluidity was increased in the order linoleic grown cells > oleic grown cells > control, it is possible that lipid and protein phase separations did not occur on cooling to 4°C. In fact the probe data indicated that cells containing greater amounts of linoleic acid and oleic acid would be more fluid at 4°C and therefore possibly more resistant than control cells, had sensitivity changes occurred. Berlin et al. (1980) showed that the relative fluidity of rabbit platelet membranes ascertained using DPH, was correlated with the polyunsaturated fatty acid contents of the membrane phospholipids. The higher polyunsaturates were 18:2, 20:4 and 24:1 (monounsaturated).

In contrast, Herring et al. (1980) could detect no change in the plasma membrane fluidity of Dictyostelium discoideum at 20°C following a substantial increase in polyunsaturated fatty acids. They reasoned that the high levels of linoleic acid (60%) in the membranes prior to alteration were already sufficient to produce a maximal fluidity. The combination of unsaturated fatty acids from 18:3 to 20:4 was zero in the unsupplemented cell membranes but rose to 34% in supplemented cell membranes.

Thus, according to the conclusions from the K 1060 section one would not expect any radiosensitivity changes due to the changes in fluidity in LDV cells reported here. Further experiments using saturated fatty acid supplementation would be more appropriate for studies on radiation sensitivity changes at 4°C dependent on membrane fluidity changes.

In considering particular lipids as potential targets, I note that cholesterol esters usually have low molecular weight fatty acids, and would not be expected to contain significant proportions of 20:4 and 22:6 unsaturated fatty acids. Schroeder (1980) has also demonstrated
that the outer monolayer of murine fibroblasts is more fluid than the inner monolayer, and this was due to the higher levels of the more fluid phosphatidylcholine. The less fluid phosphatidylethanolamine could therefore represent a site more vulnerable to fluidity changes by decreasing the temperature. The disproportionate level of phosphatidylcholine is not universal, however.

Dobretsov et al. (1977) used several membrane probes to monitor the increase in biological and artificial membrane viscosity, caused by lipid peroxidation. Despite the fact that the probes were distributed differently within the membrane bilayer, they indicated similar values for the increase in membrane viscosity. Thus fluidity changes caused by peroxidation do not appear to be spatially localized.

5.4. Sublethal damage and potentially lethal damage

Evidence supporting the importance of membrane fluidity is not entirely lacking in mammalian cell radiobiology. Nolan et al. (1981) demonstrated that the membrane lipid perturber, butylated hydroxytoluene (BHT) increased the level of sublethal damage repair in Chinese Hamster V79 cells held at 0°C to 25°C. Sublethal damage repair normally decreases as a function of temperature to about 20°C and then remains constant until declining again below 2.5°C. They concluded that the antioxidant activity of BHT was not responsible for the observed effects, and that sublethal damage repair probably involved a membrane bound enzyme. Potentially lethal damage, in contrast, was unaffected by BHT at temperatures of 5°C and 20°C. These holding temperatures normally reduced the repair of potentially lethal damage. The results also supported the hypothesis that PLD repair and SLD repair are independent.

It would be interesting to determine whether the membrane fatty acid changes obtained with LDV cells influenced the timing and extent of sublethal damage repair.
In bacteria, sublethal damage repair has not been reported, and attempts to measure it have proved negative in *E. coli* B/r (W.A. Cramp, personal communication). The short cell division times make such studies difficult. However, I can think of no other mechanism by which Yatvin's (1976) results show shallower and more curved survival curves at 1.8 Krad/minute, than described in this thesis at 20 Krad/minute; since the irradiation conditions were comparable; except for differences in potentially lethal damage. Potentially lethal damage is generally defined by changes in the slope of the survival curve.

Recent work by Leenhouts et al. (1981) supports the postulation that sublethal damage repair is dependent on single-strand break repair, whereas potentially lethal damage repair is due to an induced double-strand break repair system in *Saintpaulia ionantha* (plant). A similar induction of a potentially lethal damage repair system has been reported in *E. coli* (Koukalova and Reich, 1981), although it is speculative that this would be dose-rate dependent. If membrane fatty acid changes affect either sublethal damage repair or potentially lethal damage repair then one would expect a change in strand-break production and repair kinetics if the theories of Leenhouts et al. (1981) are correct.

5.5. Future Research

(1) Is the increase in radiosensitivity below the membrane transition temperature due to peroxidation? This could be investigated using N-ethyl maleimide to bind the glutathione SH groups. Alternatively a protective agent such as cysteine could be added. If peroxidation is relevant, a differential effect should be obtained above and below the membrane phase transition temperature.

(2) Does magnesium influence survival in K1060? Work by Sato and Takahashi (1969 III) suggests that DNA ligase activity could be
affected by cold shocking. I would use a test concentration of $5 \times 10^{-3}$ M magnesium acetate.

(3) If DNA damage is important in the K 1060 sensitivity change, then neutron irradiation may result in equivalent cell killing above and below the membrane phase transition temperature.

(4) DNA-membrane attachment. Worcel and Burgi (1972) only obtained DNA-membrane attachment at 4°C. Since this was prevented by amino acid starvation or chloramphenicol treatment, such treatments may negate the temperature effect if DNA membrane attachment is relevant. These authors and Pettijohn and Hecht (1973) also noted that after cold-lysis, more protein bound to DNA, and that this protein was derived mainly from the membrane fraction.

(5) Other mutations. Work on other membrane mutants of *E. coli* may be useful in investigations of the role of the membrane in cell killing, although auxotrophy for fatty acids or glycerol may be an extra requirement before such mutants could be used. Altenburg et al. (1970) report the production of extra cytoplasmic membrane formation in *E. coli O111a* above a 30°C growth temperature. Lusk and Kennedy (1972) pinpointed an altered phospholipid metabolism in a sodium-sensitive mutant of *E. coli*. More cardiolipin and less phosphatidylethanolamine were produced, which is the main post-irradiation effect reported by Jacobson and Yatvin (1976).

(6) The membrane phospholipid changes recorded in LDV cells in Chapter 4, proved insufficient to change radiation sensitivity with temperature. Supplying LDV cells with saturated fatty acids plus the minimum essential fatty acid requirement would be necessary to look for the type of phase transition effect recorded in *E. coli* K 1060. The lack of a radiosensitivity change following the incorporation of different unsaturated fatty acids may be due to several reasons -
a) Changes recorded in this thesis may be too small. b) The fatty acids used are not sufficiently sensitive to peroxidation. c) The low unsaturated fatty acid content of the nuclear membrane, the postulated radiosensitive site, is the reason for a lack of radiosensitivity change. If peroxidation is important, then either arachidonic acid (20:4) could be used as a supplement, or the inhibition of the antioxidant system could be attempted by using N-ethyl maleimide or a vitamin E deficient serum. I am aware that vitamin E is only present in low amounts in the nuclear membrane compared to the other membranes, possibly due to the lack of free radical enzyme systems in the nuclear membrane. The fact that the nuclear membrane is of low unsaturated fatty acid content may imply that this location is an undesirable site for potential lipid peroxidation, possibly due to the close juxtaposition of DNA.

Experiments using non-toxic levels of N-ethylmaleimide to inhibit the antioxidant activity of glutathione in LDV cells are already in progress. I expect the LDV control cells to be more sensitive and if lipid peroxidation is important then linoleic supplemented cells should be more sensitive still. The presence of a large triglyceride and fatty acid cytoplasmic pool in the supplemented cells will have to be taken into consideration if differential sensitivity changes are obtained.
5.6. Addendum

I have just completed a repeat experiment (Experiment 5), to determine the membrane fatty acid composition of total cell membrane phospholipid and nuclear membrane phospholipid, and the results are summarised in Table 5A. Cells were grown over 16-20 passages with oleic acid or 2-7 passages with linoleic acid at a constant \( \frac{1}{250} \) FCS. I expected the changes achieved to be at least equal to those obtained with cells grown in full RPMI plus fatty acid supplements, as shown in Table 4K. However, changes were less than obtained in Experiment 4 (Table 4K). Cells used for the air irradiations with X rays were grown under these conditions. In order to be sure of clean nuclei, extracted nuclei were washed two times with lysing solution minus the non-ident detergent, and microscopic studies were made at each stage. I am fully satisfied that the nuclei obtained were totally free of other membrane fractions. In addition, the following extra fatty acid methyl esters were used to assist with fatty acid identifications 17:0, 19:0, 20:2 w 6, 20:3 w 9, 21:0, 22:0, 22:1, and 24:0. Fatty acids between 20:0 and 22:2 were sometimes difficult to confirm with exact retention times, possibly due to their overlapping, and also to the possibility of isomers running at slightly different rates.

The major changes that occurred were an increase in oleic acid content in cell phospholipid from 20.9% to 29.6%, and in linoleic acid content in cell phospholipid from 1.7% to 11.5%. The polyunsaturates again declined in the fatty acid supplemented cells, and although a reason to explain this in Chapter 4 was the suggestion of homeostasis, it could be just the lesser amounts of these acids available in the medium and that they are not normally synthesised in significant amounts by LDV cells. No increase in 20:2 was obtained in linoleic supplemented cells but on this occasion 20:3 increased from 1.4 to 7.9%. This is
almost certainly the W6 fatty acid as the W9 standard used had a longer relative retention time indistinguishable from W6 arachidonic acid.

Nuclear membrane changes proved to be minimal. The nuclear membrane phospholipid from oleic supplemented cells appeared to be identical to that extracted from control cells, whereas the linoleic acid content of linoleic acid supplemented cells increased from 2.1% to 5% on average.

Three to five samples were used for each phospholipid class.

The unsaturation indices at the foot of Table 5 A again demonstrate that the nuclear membrane phospholipids are more saturated than total cell membrane phospholipids, and are also noticeably lacking in the polyunsaturated fatty acid components.

In conclusion, the fatty acid changes obtained in LDV cells are comparably less than obtained with K1060, and this fact coupled with the inflexibility of the nuclear membrane phospholipid composition, may well explain the lack of radiation sensitivity changes in this cell line, following supplementation with oleic and linoleic fatty acids.
<table>
<thead>
<tr>
<th>FATTY ACID</th>
<th>RRT</th>
<th>CELLS</th>
<th>NUCLEI</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>.287</td>
<td>4.1</td>
<td>11.3</td>
</tr>
<tr>
<td>14:1</td>
<td>.322</td>
<td>0.7</td>
<td>2.8</td>
</tr>
<tr>
<td>15:0</td>
<td>.361</td>
<td>3.6</td>
<td>5.4</td>
</tr>
<tr>
<td>15:1</td>
<td>.390</td>
<td>4.3</td>
<td>9.4</td>
</tr>
<tr>
<td>15:2</td>
<td>.444</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>16:0</td>
<td>.522</td>
<td>21.2</td>
<td>26.4</td>
</tr>
<tr>
<td>16:1</td>
<td>.594</td>
<td>4.2</td>
<td>3.1</td>
</tr>
<tr>
<td>16:2</td>
<td>.631</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>17:0</td>
<td>.722</td>
<td>1.0</td>
<td>1.3</td>
</tr>
<tr>
<td>17:1</td>
<td>.809</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>17:2</td>
<td>.920</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>18:0</td>
<td>1.0</td>
<td>14.5</td>
<td>15.6</td>
</tr>
<tr>
<td>18:1</td>
<td>1.126</td>
<td>20.9</td>
<td>14.9</td>
</tr>
<tr>
<td>18:2</td>
<td>1.408</td>
<td>1.7</td>
<td>2.1</td>
</tr>
<tr>
<td>20:0</td>
<td>1.970</td>
<td>0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>20:1</td>
<td>2.20</td>
<td>2.1</td>
<td>0.5</td>
</tr>
<tr>
<td>20:2</td>
<td>2.76</td>
<td>0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>21:0</td>
<td>2.817</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>20:3</td>
<td>3.15</td>
<td>1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>20:4</td>
<td>3.52</td>
<td>7.8</td>
<td>0.6</td>
</tr>
<tr>
<td>22:0</td>
<td>3.85</td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>22:1</td>
<td>4.17</td>
<td>0.5</td>
<td>1.8</td>
</tr>
<tr>
<td>20:5</td>
<td>4.78</td>
<td>1.1</td>
<td>0.3</td>
</tr>
<tr>
<td>22:2</td>
<td>4.90</td>
<td>0.2</td>
<td>1.75</td>
</tr>
<tr>
<td>22:4</td>
<td>6.77</td>
<td>2.3</td>
<td>0.15</td>
</tr>
<tr>
<td>22:5</td>
<td>9.30</td>
<td>6.6</td>
<td>0.3</td>
</tr>
<tr>
<td>22:6</td>
<td>10.40</td>
<td>4.7</td>
<td>0.3</td>
</tr>
</tbody>
</table>

No. of samples | 3 | 5 | 3 | 4 | 4 | 4

Saturated | 42.9 | 43.2 | 44.2 | 61.8 | 58.8 | 63.5
Unsaturated | 57.1 | 56.8 | 55.8 | 38.2 | 41.2 | 36.5
UI | 3.61 | 2.38 | 3.03 | 0.79 | 0.92 | 0.72
REFERENCES

ABE, M., BROWN, C., HENDRICKSON, W.G., BOYD, D.H., CLIFFORD, P.,
USA, 74, 2756-2760.

ACHEY, P.M., BILLEN, D., and BELTRANENA, H.P. (1971) Int. J.

Proc. of the Vth Symp. on Microdosimetry. Verbania-Pallanza,
(EUR, 5452 d-e-f).

12, 593-595.

26, 493-497.

Academic Press. 469-472.

289.

Biol. 34, 317-327.

ALEXANDER, P., DEAN, C.J., LEHMANN, A.K., ORMEROD, M.G., FELDSCHREIBER,
P., and SERIANNI, R.W. (1970) in Radiation Protection and
Sensitization, H.L. Moroson and M. Quintilliani, Eds., (Barnes


STI/PUB/286 Vienna 171-184.


J. Bacteriol. 98, 784-796.
553, 365-377.
HOLMAN, R.T. (1971) Prog. in the Chemistry of Fats and Other Lipids.
9, 275-348.
HOUVER, R.L., BHALLA, D.K., YANOVICh, S., INBAR, M., KARNOVSKY, J.
54, 360-366.
Acta. 555, 56-66.
Sci. USA. 72, 167-171.
58, 384-397.


LARK, K.G. (1972) J. Mol. Biol. 64, 47-60.


SHINITZKY, M., and HENKART, P. (1979) Int. Rev. of Cytology 60, 121-147.


