THE INFLUENCE OF VITAMIN E DEFICIENCY AND OF DIETARY POLYUNSATURATED FATS UPON THE STRUCTURE AND FUNCTION OF THE RAT PROXIMAL SMALL INTESTINE

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ABSTRACT

A vicious cycle of malabsorption and malnutrition has been implicated in the pathogenesis of protracted diarrhoeal disease (PDD) in infancy. Impaired antioxidant defences are common in malnourished children, and it is hypothesised that increased fluxes of free radicals and compromised antioxidant defences might have an influence in perpetuating PDD. Vitamin E (α tocopherol) is an important lipid soluble antioxidant, and polyunsaturated fatty acids (PUFA) a major substrate for oxidative attack in biological membranes. High PUFA diets are sometimes used in the nutritional rehabilitation of infants with PDD.

In order to study the effects of impaired antioxidant defences and of high PUFA diets a rat model was established. Four groups of rats were fed diets which were vitamin E sufficient or deficient and contained high or low PUFA, and jejunal structure and function studied after 24 weeks. Electrogenic secretion and absorption were measured in Ussing chambers and the maximal secretory responses (δIsc_{max}) and the concentration of secretogogue producing a half maximal response (EC_{50}) determined. Malondialdehyde (MDA) and thiobarbituric reactive substances (TBARS) were measured in mucosal scrapings as indices of lipid peroxidation, and the fatty composition of the apical membrane by gas chromatography.

A high PUFA diet resulted in a change in the fatty acid composition of the apical brush border membrane and was associated with a complex modulation of electrogenic secretion and absorption. Chronic vitamin E deficiency was associated with elevated indices of lipid peroxidation, net electroneutral jejunal secretion and a modified secretory response. The combination of vitamin E deficiency and a high PUFA diet resulted in an enteropathy, elevated indices of basal and secretogogue mediated secretion, and higher levels of lipid peroxidation.

The combination of vitamin E deficiency and high PUFA intake appears deleterious. This may have implications for the nutritional rehabilitation of malnourished infants with PDD.
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FOREWORD

A standardised format of data presentation has been adopted throughout the thesis in both text and figures. This is defined and explained in full in appendix 1.
CHAPTER 1

Protracted diarrhoea, malnutrition, antioxidants and free radicals. A unifying hypothesis?

1.1 Protracted diarrhoeal disease in infancy

It has been estimated that in 1980 close to 1 billion episodes of acute diarrhoea occurred worldwide in children under the age of 5 years and that in approximately 5 million the diarrhoea became protracted and resulted in death. Protracted diarrhoeal disease (PDD) of infancy is a syndrome rather than a specific disease and there are many recognised causes. It is likely, however, that prolonged injury to the small intestinal mucosa represents a final common pathway in the pathogenesis of PDD whatever the cause.

Acute diarrhoea is most commonly caused by specific enteric infections or enterotoxins. These illnesses are usually short lived with a median duration of less than one week. Prospective community based epidemiological studies in less developed countries have found that as few as 2% (figures range between 2-20%) of all diarrhoeal episodes will last for more than two weeks. Hence the persistence of diarrhoea for more than 2 weeks in the absence of continued enteric infection must be considered abnormal and indicative of a different pathology. Idiopathic protracted diarrhoea (IPD) of infancy and childhood is characterised by the passage of four or more loose or watery stools each day for more than 2 weeks during which time the child looses or fails to gain weight in the absence of continued gastrointestinal infection or a specific identifiable cause.

The duration of a diarrhoeal illness in malnourished children, including illness associated with specific pathogens, is significantly prolonged when compared with better nourished counterparts. This observation of a relationship between nutritional status and duration of diarrhoeal illness has been confirmed in animal studies. Whilst a link between malnutrition and protracted diarrhoea is widely accepted, there are many mechanisms whereby malnutrition may prolong the course of PDD. These may include reduced activities of intestinal mucosal enzymes, reduced pancreatic exocrine secretion, bacterial overgrowth within the small intestine, increased permeability to macromolecules and ensuing protein sensitivity, deconjugation of bile acids and loss of gut hormones. Delayed
mucosal healing in malnourished individuals is also likely to have an effect on mucosal morphology\textsuperscript{15} particularly following an acute diarrhoeal episode, and hence on the natural history of the diarrhoeal illness.

Nowhere has the link between malabsorption, malnutrition and infection been more elegantly illustrated than in the paper by Larcher et al of 1977\textsuperscript{16}. These authors reported their experience of 82 infants aged less than 1 year seen at the Hospital for Sick Children, Great Ormond Street (GOS) during the period 1970-1975. The authors were unable to arrive at a specific diagnosis in 28% of the infants in this series despite thorough investigation (figure 1,1). After excluding a (small) number of infants suffering from specific or inherited diarrhoeal disorders they reported their experience of the natural history and the nutritional rehabilitation of the remaining 17 infants (21%) with idiopathic protracted diarrhoea (IPD).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{Diagnosis of Larcher's infants with protracted diarrhoea (CMP = cows milk protein).}
\end{figure}

The authors were struck by a number of similarities between individuals in this group of infants with IPD. These included the extreme degree of malnutrition at presentation to GOS, the almost universal occurrence of an overt infective illness (usually, but not exclusively, within the GI tract) as a precipitant of the diarrhoeal illness, and the universal response of this group of infants to a period of nutritional rehabilitation with a comminuted chicken based feed (mean time 29 weeks in hospital) with an eventual return to normality. The authors suggested that a
"vicious cycle" of malabsorption and malnutrition was important in perpetuating the protracted diarrhoeal state [Figure 1,2].

The hypothesis of such a "vicious cycle" does little to advance our understanding of the precise pathophysiology of PDD. It does, however, make the point that an interruption of the cycle, for example by improving nutritional status, might perhaps lead to an improvement in the diarrhoea.

It has been realised for some time that deficiencies of a number of micronutrients are common in children with PDD although, with a few notable exceptions, the precise role of specific nutrient deficiencies in predisposing to or perpetuating the diarrhoeal illness is unknown. For example, increased rates of diarrhoea are recognised in vitamin A deficiency\(^\text{17}\) yet only recently has it been directly demonstrated that vitamin A deficiency per se may result in an increase in small and large intestinal secretion\(^\text{18}\). There are a small number of other reports in the literature which suggest that specific micronutrient deficiencies might perpetuate diarrhoea. These include deficiencies of folic acid\(^\text{19}\) and nicotinic acid\(^\text{20}\).

1.2 Free radicals in protracted diarrhoeal disease
A further perspective on the relationship between micronutrient deficiency and diarrhoeal disease arose from the observations of Golden et al\(^\text{21}\). Their studies of malnourished children with marasmus and kwashiorkor (in which protracted diarrhoea is ubiquitous) in the West Indies led to a hypothesis that increased fluxes of free radicals coupled with impaired antioxidant defences were of importance in the pathophysiology of kwashiorkor (Fig 1,3). Their work demonstrated that children with kwashiorkor had lower serum levels of vitamins E and A, zinc, copper, manganese and selenium than children with marasmus. These vitamins and
micronutrients all have important roles in an individual's defence against an oxidative stress (vide infra).

**Figure 1.3 Golden theory of kwashiorkor**

Key to figure 1.3; Increased fluxes of free radicals arising through a number of noxious stimuli coupled with impaired antioxidant defences following micronutrient depletion leads to lipid peroxidation, formation of cytotoxic aldehydes and induction of the hexose - monophosphate shunt. SOD - superoxide dismutase; ZnMTH - zinc metallothionen; GST - glutathione S transferase; GPX - glutathione peroxidase; GR - glutathione reductase; HMP - hexose monophosphate shunt; CAT - catalase.

Deficiencies of these micronutrients which are important in oxidatiive defence are also common in the severely malnourished infants with PDD seen at GOS (vide infra). Infection, a common precipitant of PDD, can give rise to a potent oxidising stimulus (for example the respiratory burst of activated phagocytes). The "vicious cycle" of Larcher et al might, therefore, be interpreted in the light of the observations of Golden as a vicious cycle of increased free radical fluxes in the face of impaired antioxidant defences which could expose the enterocyte to an increased oxidative stress which might exacerbate the malabsorption. Malabsorption will exacerbate the antioxidant deficiency and hence perpetuate the cycle. This parallel cycle is illustrated above in figure 1.4.
Figure 1.4 Increased fluxes of free radicals and depleted antioxidants may be implicated in a vicious cycle of malabsorption and malnutrition.

Whilst these arguments have been largely directed at children with idiopathic protracted diarrhoea, *malnourished* infants with *specific* causes of PDD (for example short gut syndrome) could, by analogy, also have the delicate balance between secretion and absorption perturbed in favour of net secretion if this hypothesis is corroborated.

1.3 **Free Radical Chemistry**

In the strictest sense a "free radical" is defined as "any chemical species capable of independent existence that contains one or more unpaired electrons". The unpaired electron may render the species highly reactive. This broad definition of a free radical includes the hydrogen atom, most transition metals and the oxygen molecule which has 2 unpaired electrons each located in a different $\pi^*$ antibonding orbital. Oxygen does not behave as a highly reactive free radical because its two unpaired electrons are located in differing $\pi^*2p$ orbitals and have parallel spins (i.e., they have the same spin quantum number).

Hence if oxygen attempts to oxidise another atom or molecule by accepting a pair of electrons from it, both of the new electrons must have parallel spins so as to fit into the vacant spaces in the $\pi^*$ orbitals. A pair of electrons in a molecular orbit will
normally have antiparallel spins. This imposes restrictions on the oxidations by molecular \( \text{O}_2 \) and tends to make it accept its electrons one at a time (Figure 1,5), so slowing its reaction rate with non radical species.

![Electron bonding configurations in the diatomic oxygen molecule](image)

**Figure 1,5** Electron bonding configurations in the diatomic oxygen molecule

A number of important molecular species may be generated during the reduction of molecular \( \text{O}_2 \) to water as illustrated in figure 1,6.

If a single electron is accepted by the ground state \( \text{O}_2 \) molecule, it must enter one of the \( \pi^* \) antibonding orbitals, and the product formed is the superoxide radical. Superoxide is formed in nearly all aerobic cells. One important source of superoxide is the respiratory burst of activated phagocytes (which include macrophages, neutrophils, eosinophils and monocytes). Addition of a second electron to the superoxide radical produces the peroxide ion which is not a free radical because it possesses no unpaired electrons. The pKa of the peroxide anion \( \text{O}_2^2^- \) favours its protonation at physiological pH to form hydrogen peroxide. The importance of \( \text{H}_2\text{O}_2 \) lies in its water solubility and ability to diffuse comparatively long distances in biological systems before decomposing (especially in the
Figure 1.6 Reduction of molecular oxygen to water by the sequential addition of 4 electrons

presence of transition metal ions - vide infra) to form the extremely reactive hydroxyl radical (•OH). The •OH radical reacts with extremely high rate constants with almost every type of molecule found in living cells including sugars, amino acids, phospholipids, DNA bases and organic acids.

Mitochondrial cytochrome c oxidase, which accounts for most of the oxygen consumed by respiring cells, reduces oxygen to water with the release of only small amounts of superoxide (•O₂⁻) or peroxide. This minimises the problem of •O₂⁻ toxicity by largely avoiding its production. There are several enzyme reactions which produce larger amounts of •O₂⁻. For example xanthine oxidase and aldehyde dehydrogenase produce substantial amounts of O₂⁻ as do enzymes within phagocytes during the killing of engulfed microorganisms. Whilst there is evidence to suggest that superoxide itself is able to damage membranes and kill cells, there is little doubt that the production of •OH from the superoxide radical and the peroxide ion, usually in the presence of transition metal ions is a far more potent oxidant.
The hydroxyl radical is amongst the most reactive species found in biological systems. Reactions of •OH may be divided into hydrogen abstraction, electron transfer (accepting or donating), and addition reactions. These reactions serve to illustrate an important point in radical chemistry - that reaction of a free radical with a non-radical species often results in the generation of a different free radical which may be more or less reactive. Given that •OH is highly reactive, then the radical formed by its reaction with a molecular species is likely to be less reactive.

Transition metals contain an unpaired electron in the 3d orbital and exhibit a variable valency which allows them to undergo changes in oxidation state involving one electron transfer with comparative ease (eg. iron II, iron III, and iron IV). Hence these metals are often found at the centre of oxidases and oxygenases in biological systems because of their ability to accept and donate electrons in an energetically favourable manner. Copper also shares this ability, although it is not a transition metal. In its unionised form its 3d orbitals are full and there is a single electron in a 4s orbital. However unionised copper it readily forms Cu^{2+} by the removal of two electrons (one from the 4s orbital and one from the 3d) and its valency is able to vary between Cu^{+} and Cu^{2+} by the filling and emptying of the remaining "space" in the 3d orbital (figure 1,7). This readiness to participate in redox reactions means that the transition metals may, under appropriate conditions, lead to the generation of highly reactive oxidising species following reactions with peroxides and superoxides.

The ability of a mixture of an iron (II) salt and hydrogen peroxide to form the •OH radical was noted as long ago as 1894 by Fenton and this reaction now bears his name. The "Fenton reaction" is shown below;

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot\text{OH} + \text{OH}^-
\]
Traces of Fe\(^{3+}\) can react further with \(H_2O_2\):

\[
Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^\cdot + H^+
\]

Copper (I) salts can react with hydrogen peroxide in much the same way with a higher rate constant:

\[
Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^- + OH^-
\]

In many systems which generate \(O_2^\cdot\), scavengers of the hydroxyl radical, \(OH\), have been shown to have a protective effect against any damage that might be caused. Fridovich\(^{35}\) suggested that this is because of a reaction of \(O_2^\cdot\) and \(H_2O_2\) to form the hydroxyl radical. This reaction is known as the Harber - Weiss reaction (first postulated by these workers in 1934\(^{36}\)).

\[
H_2O_2 + O_2^\cdot \rightarrow O_2 + OH^- + OH^-
\]

The rate constant for this reaction is, however, extremely small (near zero) in the absence of transition metal ions and this has led to the suggestion that these metal ions play an important part in the generation of the hydroxyl radical. The "iron catalysed Harber Weiss reaction" may be represented by the net result of the two

\[
Cu^+ + O_2 + H_2O_2 \rightarrow Cu^{2+} + O_2 + H^+
\]
The reactions shown below.

\[
\text{Fe}^{3+} - \text{complex} + \cdot \text{O}_2^\cdot \rightarrow \text{Fe}^{2+} - \text{complex} + \text{O}_2
\]

\[
\text{Fe}^{2+} - \text{complex} + \text{H}_2\text{O}_2 \rightarrow \cdot \text{OH} + \text{OH}^- + \text{Fe}^{3+} - \text{complex}
\]

The second of these reactions is, of course, a Fenton reaction. Similar reactions may be written for the reaction of copper (II) salts with the superoxide anion. The role of the superoxide ion in reducing iron (III) and copper (II) is not without contention and it has been argued that biological systems are full of reducing agents (e.g., ascorbate, GSH, NADH, NADPH, cysteine) which could equally well reduce Fe\(^{3+}\) to Fe\(^{2+}\) or Cu\(^{2+}\) to Cu\(^{+}\). It seems likely that in vivo the most important of these is ascorbate. The observation that \cdot OH generation can be inhibited by superoxide dismutase in many biological systems is evidence in favour of a role for Harber - Weiss type reactions in vivo.

This propensity of relatively unreactive peroxyde ions to form the highly reactive hydroxyl anion in the presence of transition metal ions may explain why in health these metals exist only in tightly protein bound forms in living organisms. Conditions in which the carrier proteins for these molecules are saturated with metal ions, as may be seen with iron in kwashiorkor, or disruption of tissues, as will accompany cell death, can lead to a decompartmentalisation of these metal ions and could lead to an increased flux of free radicals.

The hydroxyl radicals formed as a result of these reactions react with extremely high rate constants with almost every type of molecule found in living cells. It has been calculated, for example, that the \cdot OH radical will have a half life in biological systems of only a few microseconds\(^{37}\). In consequence the radius of diffusion of the radical from its site of production is estimated to be less than 100nm. Free radicals of lesser reactivity (for example the superoxide radical or organic products of hydrogen abstraction) and hydrogen peroxide will be able to diffuse further within the cell before reacting or undergoing the Harber Weiss reaction and hence an oxidising effect more distant from their initial site of production.

1.4 Lipid peroxidation

The major constituents of the cell membrane are lipids and protein. The lipid component of membranes frequently contains large amounts of polyunsaturated fatty acids. [Membrane structure is discussed more fully in chapter 8]. The
peroxidation of these polyunsaturated fatty acids, for example arachidonic (C
20:4\(\omega6\)) and docosahexaenoic (C 22:6\(\omega3\)) acids is a feature of many types of cell
injury in which free radicals are involved. In this process of lipid peroxidation a
primary reactive free radical (R\(\bullet\)) reacts with a polyunsaturated fatty acid to initiate
a complex series of reactions resulting in a variety of products (figure 1,8).

First chain initiation refers to the abstraction of a hydrogen atom from a
(polyunsaturated) fatty acid within a membrane or lipid system resulting in the
formation of a carbon centred lipid radical. The •OH radical and the protonated
form of the superoxide radical HO\(_2\bullet\) are strong enough oxidants to be able to do
this (although the latter is only able to do so with some fatty acids, for example
linoleic acid). Hydrogen abstraction is more likely to occur at the site of a double
bond in the fatty acyl chain. The carbon centred radical formed following abstraction tends to undergo a molecular rearrangement to yield a more stable molecule, a conjugated diene. The most likely fate of the conjugated diene under aerobic conditions is to combine with O₂ to form a lipid peroxyl radical, ROO•. Peroxyl radicals are again a highly reactive species which are able to abstract hydrogen from an adjacent lipid molecule and hence bring about a propagation of the lipid peroxidative process - hence the so called "chain reaction". The peroxyl radical combines with the hydrogen atom it abstracts to form a lipid hydroperoxide (L-OOH) some of which may go on to form cyclic endoperoxides.

Pure lipid peroxides are relatively stable at physiological temperatures. In the presence of transition metal complexes, however, the decomposition of these peroxides is greatly accelerated and this may result in a great diversity of products which include alkoxyl radicals (RO•), epoxides, carbonyls (including the aliphatic aldehydes malondialdehyde and 4 - hydroxy nonenal), and hydrocarbon gases. The role of transition metals in this series of reactions is similar to their role in the decomposition of hydrogen peroxide. For example:

\[
R-OOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^- + R-O\cdot
\]

and

\[
R-OOH + Fe^{3+}\text{-complex} \rightarrow Fe^{2+}\text{-complex} + ROO\cdot + H^+
\]

Thus an iron (III) complex can catalyse the generation of both peroxyl and alkoxyl radicals by being recycled between iron (III) and iron (II). Hydrocarbon gases may be produced (eg ethane, pentane) depending upon which carbon atom of the lipid hydroperoxide substrate (commonly linoleic acid or arachidonic acid) the peroxide radical is found on. This process is illustrated in figure 1,9.
The formation of aliphatic aldehydes from lipid hydroperoxides in the presence of metal ions is discussed in detail in chapter 3.

The importance of these end products of transition metal catalysed decomposition of lipid hydroperoxides lies in their reactivity with other components of the cell. The radicals, aldehydes and other products produced in this manner may damage the cell and its organelles. For example 4-hydroxy trans nonenal has been shown to crosslink membrane proteins, to inhibit protein synthesis and interfere with the growth of animal cells in culture, to exert chemotactic actions on neutrophils and also to possess mutagenic properties in bacterial test systems. Consequences of such damage upon the cell membrane may result in a decrease in membrane fluidity, and an increase in membrane "leakiness" (for example to calcium), and an alteration in the functioning of cell membrane associated proteins. The scenario of lipid peroxidation causing cell death is, however, probably an infrequent event, yet the scenario of lipid peroxidation accompanying cell death is undoubtedly more common.

Cholesterol, another important constituent of cell membranes, may also be attacked by free radicals to yield a variety of products including cholesterol-7-hydroperoxides, epoxides and cholestanediols.
There are, of course, other substances within the cell membrane which contain C=C double bonds which will be susceptible to oxidation. For example retinol (vitamin A) may be oxidised to retinal.

1.5 Generation of free radicals in biological systems
The generation of free radicals in health and disease may occur through a number of mechanisms. These will include:

- Normal oxidative metabolism
- Microsomal P₄₅₀ activity (inducible by a number of compounds)
- Defence against invading micro organisms and inflammatory diseases
- Ingestion of toxic foreign compounds
- Ischaemia reperfusion injury

Normal oxidative metabolism
It is generally accepted that the most important source of •O₂⁻ in vivo in most aerobic cells is the electron transport chain located on the inner mitochondrial membrane. The oxidation of NADH and FADH₂ produced by the Krebs (tricarboxylic acid) cycle and the β oxidation of fatty acids provides energy to synthesise ATP. The reduction of molecular O₂ to water is highly efficient and there is only a little leakage of electrons from the electron transport chain during this process. It has been shown that the principal site of this leakage is the NADH-coenzyme Q reductase complex. The small leakage of electrons will react with molecular oxygen to form the superoxide anion which is efficiently dismuted by the mitochondrial form of the enzyme superoxide dismutase which is found between the inner and outer mitochondrial membranes. The role of this small leakage of electrons in the pathophysiology of disease is unclear although "swollen" mitochondria are seen in a number of conditions in which antioxidant defences have been compromised.

Microsomal P-450 activity
The endoplasmic reticulum of the liver is particularly rich in a group of cytochromes known collectively as cytochrome P-450. Cytochrome P-450 is involved in the
oxidation of a wide variety of substrates using molecular oxygen. A number of substances are able to induce cytochrome P-450 activity including barbiturates and ethanol. The electrons required for the P-450 mediated oxidation in the liver are donated by NADPH via the activity of NADPH cytochrome P-450 reductase. This oxidising system also "leaks" a proportion of the electrons which go through the pathway and, by analogy to the mitochondrial electron transport chain, this may result in the generation of the superoxide anion. The amount of superoxide produced in vivo by this means is not easily measurable and it is likely to be small as the oxygen tension within the endoplasmic reticulum is low. In vitro, however, matters are likely to be different as the disruption of the microsomal fraction which occurs during fractionation will tend to allow electrons to escape from the system more easily, and the concentrations of oxygen in the fractionated microsomal preparation might also be higher than that found in the ER in vivo.

**Inflammatory diseases**

Cytotoxic oxygen species are used during phagocytosis to destroy invading microorganisms. When activated, polymorphonuclear leucocytes and macrophages immediately consume large quantities of oxygen to generate superoxide anion radicals through the action of the enzyme NADPH nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which is located on the exterior surface membrane of the cell. The superoxide radical is converted into hydrogen peroxide and the hydroxyl radical. H$_2$O$_2$ may be converted to hypochlorous acid, HClO, (an extremely powerful oxidant) by the enzyme myeloperoxidase which is released simultaneously by activated phagocytes. HClO is both bactericidal and cytotoxic. Reactive oxygen species produced by polymorphonuclear leucocytes are probably also important in the pathogenesis of non infectious diseases associated with inflammation such as autoimmune enteropathy. There is now good evidence that a number of inflammatory disorders of the intestine are associated with increased oxidative stress within the gastrointestinal mucosa.

Free radicals may also be generated from other sources, for example during prostaglandin metabolism by prostaglandin hydroperoxidase.

**Ingestion of foreign or toxic compounds**

Bacterial overgrowth is ubiquitous in kwashiorkor and is common in protracted
diarrhoea. It has been suggested that bacterial overgrowth within the small intestine results in an "uncontrolled biochemical powerhouse" capable of producing numerous products many of which may be toxic which could result in increased fluxes of free radicals within the gastrointestinal tract.

The ingestion of aflatoxins has been proposed as a means of producing kwashiorkor. These and other toxins might result in increased fluxes of free radicals (as for example one might find following ingestion of carbon tetrachloride, paraquat or bleomycin) although hard evidence for this seems to be lacking.

A high intake of polyunsaturated fatty acids may turn the normal antioxidant effect of primary bile acids (at physiological concentrations similar to those found within the intestinal lumen) into a prooxidant effect which could subject the intestinal mucosa to increased oxidative stress.

**Ischaemia / reperfusion injury**

It is well established that ischaemia - reperfusion injury is one mechanism of increasing free radical fluxes both within the gastrointestinal tract and within a number of other organs. There is increasing evidence that this type of injury may be important in the pathophysiology of necrotising enterocolitis. This issue is discussed further in chapter 10.

1.6 Protection against oxidants in biological systems - Antioxidants and other defences.

There are a number of stages at which the peroxidative process may be prevented or arrested. These include:

1. **Preventing first chain initiation** by scavenging initiating radicals such as •OH, or preventing the generation of •OH.
2. **Binding metal ions** in forms that will not generate these initiating species and / or will not decompose lipid peroxides to peroxyl or alkoxyl radicals.
3. **Decomposing peroxides** by converting them to non radical products such as alcohols.
4. **Chain breaking** i.e scavenging intermediate radicals such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction.

These defence mechanisms will be discussed in the following sections.
1.6.1 Catalase (EC 1.11.1.6) and Glutathione peroxidase (EC 1.11.1.9)

Hydrogen peroxide is damaging in biological systems because it can give rise to the •OH radical. It is important therefore for cells to control the amount of $H_2O_2$ which is allowed to accumulate. There are two mechanisms for this. Firstly catalases which convert hydrogen peroxide to water and $O_2$ and secondly peroxidases which oxidise $SH_2$ to $S$ and in the process reduce the peroxide to water.

Catalase is present in all the major organs and is particularly concentrated in liver and red cells. In the liver catalase activity is concentrated in the peroxisomes which generate $H_2O_2$ through enzymes such as the flavoprotein dehydrogenases involved in the β-oxidation of fatty acids. Mitochondria which perform most of the β oxidation of fats contain little or no catalase, and so any hydrogen peroxide generated by mitochondria in vivo cannot be disposed of in this manner.

Glutathione peroxidase is present in high concentrations within the liver and in smaller concentrations in erythrocytes, lung and kidney. The substrate for this enzyme is the thiol compound glutathione which is often found at millimolar concentrations in animal tissues. Most glutathione in vivo is present in its reduced form (GSH). Glutathione peroxidase catalyses the oxidation of GSH to its oxidised form (GSSG), and in so doing converts hydrogen peroxide to water (Fig 1,10). The enzyme is specific for GSH as a hydrogen donor but will accept other peroxides (for example lipid peroxides) as well as hydrogen peroxide. Selenium is present at the active site of this enzyme, probably as seleno-cysteine. Hence, a dietary deficiency of selenium results in a clinical deficiency of glutathione peroxidase.

The ratio of GSH / GSSG in cells is kept high by the action of glutathione reductase (EC 1.6.4.2) which utilises NADPH generated by the action of glucose 6 phosphate dehydrogenase (G6PD) in the hexose monophosphate shunt.
1.6.2 Superoxide dismutase

(i) Copper - zinc enzymes (EC 1.15.1.1)

The copper - zinc containing superoxide dismutases were described by McCord and Fridovich in the late 1960's. They are located within the cytosol of the cell and contain two protein subunits each of which contains an active site containing one zinc and one copper ion. Superoxide is dismuted by the enzyme to yield hydrogen peroxide and ground state oxygen.

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

The copper ions seem to function in the reaction by undergoing alternate oxidation and reduction (use being made of its properties as a transition metal ion). The zinc ion is thought to stabilise the enzyme and not to have any catalytic activity.

(ii) Manganese enzymes

Manganese superoxide dismutase (MnSOD) is found within the mitochondria, although in human liver a small amount may be found outside the mitochondrial matrix. Removal of manganese from the catalytic site causes loss of catalytic
activity.

1.6.3 Ascorbic acid (vitamin C)

Ascorbate is able to act as a reducing agent (an electron donor), and may be able to detoxify several organic radicals in vivo. Ascorbate is also able to function as a chain breaking antioxidant. Donation of one electron by ascorbate results in the formation of the semidehydroascorbate radical, which can further be oxidised to give dehydroascorbate. The semidehydroascorbate radical is not very reactive and may undergo a disproportionation reaction to yield ascorbate and dehydroascorbate or react enzymatically with dehydroascorbate reductase and GSH to yield GSSG and ascorbate. Ascorbate reacts rapidly with •O₂⁻ and HO₂•, and even more rapidly with •OH to yield semidehydroascorbate. It also scavenges singlet oxygen, reduces thyl radicals and combines quickly with hypochlorous acid. Hence, ascorbate is able to protect against oxygen derived species in aqueous phases in vivo.

Ascorbate may also, however, under the right conditions function as a promoter of free radical production in conjunction with transition metal ions. This is possible because ascorbate can react in a manner analogous to the superoxide radical in the Harber Weiss reaction (see page 40) to reduce iron (III) to iron (II) and hence allow the generation of hydroxyl radicals from hydrogen peroxide. In the presence of free transition metal ions, therefore, ascorbate may turn from a powerful scavenger of hydroxyl radicals into a catalyst for the production of this reactive species.

1.6.4 Glutathione

The role of GSH as a substrate for glutathione peroxidase (page 48) and dehydroascorbate reductase (page 50) has already been eluded to. Glutathione is the most important cellular thiol for the action of glutathione peroxidase.

Glutathione is also an important substrate for the action of glutathione S transferase (EC 2.5.1.18) in the detoxification of many toxins to form mercapturic acids in the liver which are then excreted in the bile (figure 1,11). These toxins include some of the products of lipid peroxidation. The formation of mercapturic acids by glutathione S transferase results in a net consumption of GSH (as opposed to a recycling of 2GSH = GSSG as is seen with glutathione peroxidase).
The resultant decrease in the ability of the liver to cope with hydrogen peroxide and other oxidising stimuli following a depletion of GSH may be of relevance in the pathophysiology of disease\(^2\).

\[
\text{SH} \\
RX + \text{glu-cys-gly (GSH)} \\
\downarrow \text{glutathione S transferase} \\
\text{SR} \\
\text{glu-cys-gly} \\
\downarrow \text{gammaglutamyltranspeptidase} \\
\text{R-S-cys-gly} \\
\downarrow \text{cysteinylglycinase} \\
\text{R-S-cys} \\
\downarrow \text{N-acetylase} \\
\text{R-S-cys-CH}_3 \\
\text{a mercapturic acid}
\]

Figure 1.11 Mercapturic acid formation by glutathione S transferase. RX represents a "foreign" compound.

1.6.5 Vitamin E

Vitamin E is a term for a lipid soluble group of molecules (the tocopherols) which have structural and antioxidant roles in biological membranes. \(\alpha\) tocopherol has been shown to be the most important lipid soluble antioxidant in biological membranes\(^6\), although the same may not, of course, be true for the aqueous phase of plasma. \(\alpha\) tocopherol (see chapter 2) is able to quench and react with singlet oxygen to remove this species, to quench superoxide, and to react with \(\cdot\text{OH}\) at a diffusion controlled rate. The major function of \(\alpha\) tocopherol in biological membranes is, however, as a chain breaking antioxidant which reacts with lipid peroxyl and alkoxy radicals, donating a hydrogen and in the process forming the
comparatively unreactive tocopheroxyl radical. The tocopheroxyl radical is insufficiently reactive to abstract H⁺ from adjacent membrane lipids (because the unpaired electron can be delocalised into the aromatic ring structure), and hence the chain reaction of lipid peroxidation is terminated. There is evidence, at least in vitro, that the tocopheroxyl radical can be recycled to tocopherol within the biological membrane following reduction by ascorbate (Fig 1,12). This recycling of the tocopheroxyl radical has recently been demonstrated to occur in vivo. Ascorbate may then be recycled as discussed previously (page 50). Hence α tocopherol is preserved within the membrane.

![Figure 1,12 Recycling of the tocopheroxyl radical to tocopherol by ascorbate](attachment:figure1.png)

The relative importance of α tocopherol in protecting membranes against oxidative stress depends upon the type of oxidative stress applied. For example the importance of α tocopherol may be diminished when peroxidation is initiated by complexes containing Fe²⁺ and enhanced when peroxidation involves peroxy radicals, especially those generated by propagation reactions.

### 1.6.6 Carotenoids

Lycopene and β - carotene are both able to quench singlet oxygen which may be generated enzymatically or during the process of lipid peroxidation. Carotenoids may also participate in free radical reactions - for example β - carotene can decrease the rate of formation of lipid hydroperoxides and may "quench" both carbon centred and peroxy radicals (figure 1,13). Carotenoids may therefore act as lipid soluble antioxidants. The available evidence suggests that the

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*Graham Burton, unpublished data*
The antioxidant effect of \( \beta \) carotene is especially good at low partial pressures of \( O_2 \) (less than 150 mmHg) similar to those which may be found within the tissues\(^{58}\). At higher \( O_2 \) concentrations \( \beta \) carotene loses its antioxidant effect and may even have a pro oxidant effect\(^{58}\).

Carotenoids may also function as efficient antioxidants within the aqueous phase of plasma\(^{56}\). For example, Packers group at UCLA\(^{59}\) have demonstrated in an aqueous system (based on the loss of fluorescence of phycoerythrin upon exposure to a diazo initiator of peroxyl radicals) that synthetic retinoids were able to trap peroxyl radicals with an efficiency which matched that of Trolox (a water soluble vitamin E analogue).

Despite the efficacy of this chain breaking antioxidant activity of vitamin A it seems likely that tocopherols are better placed within biological membranes to function as chain breaking antioxidants and, despite being present in lower concentrations in these membranes, they provide the most important antioxidant defence within this phase\(^{54}\). The observation that \( \alpha \) tocopherol is the only lipid soluble chain breaking antioxidant at high \( O_2 \) concentrations but that \( \beta \) carotene is effective at low \( O_2 \) concentrations has led to the hypothesis that the chain breaking antioxidant activity of these two compounds may be complementary\(^{58}\).

### 1.6.7 Compartmentalisation of metals

The important role of free metal ions in free radical reactions has already been discussed at some length. Transition metals within the body are present in a free form in only small amounts and for the most part are tightly bound to proteins both in the circulation and within the tissues. For example, an adult human contains about 4g of iron, about 65% of which is present in haemoglobin, 10% in myoglobin and a very small amount in various iron containing enzymes and in transferrin. The remainder is found in the intracellular storage proteins haemosiderin and ferritin. These are found mainly in the liver, spleen and bone marrow, although a small amount is found in other tissues and some ferritin is found in the blood. Transferrin is a glycoprotein which has two binding sites for iron (III) and transports iron from the gut to the tissues. Under normal conditions the transferrin in the blood is only about 30% loaded with iron and so the amount of free iron found in the blood plasma is virtually zero\(^{60}\). Any internal iron not required by the cells is stored in
ferritin. In theory the small amount of free iron moving between plasma transferrin and intracellular ferritin could be available for a Fenton type reaction although the evidence for this occurring in vivo in health seems to be lacking. In iron overloaded transferrin has been shown to catalyse •OH production\textsuperscript{61}, but the partially iron loaded protein does not\textsuperscript{62}.

Similarly copper, of which there is approximately 80mg in the adult, is absorbed in the upper intestine, complexed with amino acids or small peptides and on entering the blood stream most of the copper will be tightly bound to albumin. The liver takes up the copper bound to albumin and incorporates it into the glycoprotein caeruloplasmin which is then released into the circulation. About 95% of total plasma copper is found in this form. It seems, by analogy to the situation with iron, that in health there is no free copper available within the circulation to participate in a Fenton type reaction.

Caeruloplasmin is also able to oxidise Fe\textsuperscript{2+} to Fe\textsuperscript{3+} \textsuperscript{63} and hence function as an antioxidant by preventing the Fe\textsuperscript{2+} dependent production of •OH from H\textsubscript{2}O\textsubscript{2}.

These, and other mechanisms, of limiting the availability of free transition metals within the body to participate in free radical generating reactions constitute an
important defence against free radical attack and damage.

1.7 Measurement of antioxidant status

Stocks et al\(^6\) have studied the antioxidant capacity of plasma using a tissue preparation of ox brain in which the rate of malondialdehyde (a secondary product of lipid peroxidation - see chapter 3) production was used as an index of peroxidation. Caeruloplasmin and transferrin were reported in these studies as the major preventative antioxidants in plasma. Tocopherol had little effect as an antioxidant as measured by this test system. Both caeruloplasmin and transferrin are acute phase proteins which may be raised in malnourished populations\(^65,66\). These findings presumably reflect the capacity of caeruloplasmin and transferrin to bind metal ions. The production of malondialdehyde from the ox brain lipid in this test system will be dependent upon the presence of free metal ions and it is no surprise, therefore, that efficient metal chelators function as efficient "antioxidants" in this system.

Wayner et al\(^5\) have used a different approach. They measured the total radical trapping (TRAP) activity of plasma by studying its ability to prevent lipid peroxidation initiated by the water soluble azo compound 2,2'-azo-bis-(2-amidino propane hydrochloride) [ABAP]. Peroxidation was assessed by measuring oxygen uptake with a Clark type oxygen electrode. This reaction is NOT Fe dependent and hence is not inhibited by Fe chelating materials. The TRAP activity of plasma was mainly due to four components: \(\alpha\) tocopherol, ascorbate, urate, and the sulphhydryl content of proteins\(^66\). In addition, carotenes\(^69\) and bilirubin\(^70\) can also trap lipid free radicals. Using the TRAP technique, Burton and Ingold\(^54\) demonstrated that vitamin E is the only lipid soluble antioxidant in human blood plasma. In terms of concentration however, proteins are the largest single antioxidant component in blood plasma, but if trapping efficiency is taken into account, then urate has the largest capacity\(^71\). Ames' group in California have studied the temporal order of antioxidant consumption in human blood plasma exposed to a constant flux of aqueous peroxyl radicals\(^72\). The order of consumption of antioxidants was ascorbate = protein thiols > bilirubin > urate > \(\alpha\) - tocopherol, and lipid peroxidation (measured as the appearance of lipid hydroperoxides) did not start until after the ascorbate had been completely consumed. They were able to demonstrate that plasma which was depleted of ascorbate was extremely vulnerable to oxidative
stress.

1.7.1 Studies in malnourished children
Thurnham has reported the results of the TRAP assay in malnourished Nigerian children (with trivial infections) and Caucasian adults. The TRAP values were significantly lower in the malnourished children. Concentrations of urate and glutathione (GSH + GSSG) were similar in the two groups, whilst those of tocopherol and ascorbate were lower in the malnourished children.

Low levels of ascorbate are commonly found in malnourished populations, and the impact of this on the antioxidant capacity of plasma may previously have been underestimated. The recycling of ascorbate within the erythrocyte to maintain the antioxidant capacity of plasma has been discussed already. It is worth making the point here, however, that the recycling of ascorbate / dehydroascorbate across the erythrocyte membrane in vivo may constitute a pitfall of the TRAP assay - the system does not contain any erythrocytes.

Golden et al have measured CuSOD activity in erythrocytes from 93 severely malnourished children and found this to be significantly depressed in about 40% of individuals. There was no difference between the infants with marasmus and kwashiorkor, and the authors were unable to correlate this finding with any particular clinical feature. The concentration of glutathione peroxidase in erythrocytes is quantitatively related to the dietary content / absorption of selenium. Levels of this enzyme have been reported in malnourished children in the West Indies and were low in 45% of individuals studied, although there were no differences between individuals with kwashiorkor and marasmus. Manganese superoxide dismutase has not, to date, been measured in malnourished children, although low levels of hepatic manganese have been reported in South African children with kwashiorkor but not in non oedematous malnourished children.

Zinc, in the form of zinc metallothionein is a very effective free radical sink in vitro and probably in vivo, but levels have not been measured in malnutrition. Levels of zinc are however uniformly low in kwashiorkor.

Glutathione S transferase may be induced (almost two fold) in kwashiorkor. Detoxification of aliphatic aldehydes by this method results in the consumption of glutathione, and low levels of glutathione have also been reported in kwashiorkor. The ratio of oxidised glutathione / total glutathione [2GSSG / (GSH+2GSSG)] is
reported as being normal in malnourished children, although the ratio gives no information about the rate of glutathione recycling / turnover. The hexose monophosphate shunt (which reduces NADP to NADPH to provide reducing equivalents for the regeneration of GSH from GSSG [vide supra]) is induced in kwashiorkor. Golden has proposed that these findings represent a paradox in that although levels of GPX are low, the enzyme may be working at an increased capacity fuelled by the induced hexose monophosphate shunt. Confirmatory evidence for this is lacking.

There is very little information on levels of antioxidants in malnourished infants with PDD at presentation to hospital in the Western world. A prospective case controlled study is currently underway at the Hospital for Sick Children, Great Ormond Street, London. The preliminary data suggest that there are compromised antioxidant defences in over half of the children at presentation to this hospital.

1.8 A unifying hypothesis for the vicious cycle of malnutrition, malabsorption and infection

Three observations made in the above discussions may be emphasised;
1. Any "vicious cycle of malabsorption and malnutrition" is likely to involve a number of different mechanisms and bodily systems. This cycle is not necessarily confined to children with idiopathic protracted diarrhoea, and might also be of importance in children with symptomatic protracted diarrhoea of infancy (ie PDD of specific aetiology).
2. There is a considerable body of evidence that antioxidant defence mechanisms may become compromised in malnourished children and this might arise both through a decrease in absorption and an increase in consumption of these micronutrients.
3. Protracted diarrhoea commonly follows an acute infective episode (which might be considered an oxidising stress). The individual may therefore be subjected to increased fluxes of free radicals which, in the presence of compromised antioxidant defences, may have consequences on gastrointestinal function.

It is the purpose of this thesis to investigate the link between increased fluxes of
free radicals within the small intestine and gastrointestinal absorption and secretion. Vitamin E (α tocopherol) is the most important lipid soluble antioxidant in biological membranes and concentrations of this vitamin are commonly depressed in malnourished children, particularly those with diarrhoea. It is for these reasons that it was chosen to use a rat model of chronic vitamin E deficiency to study these relationships. The model is described fully in chapter 2.

A "free radical" hypothesis for protracted diarrhoea, however attractive it may seem, is unlikely to provide an all encompassing explanation for the many different observations linking malabsorption and malnutrition\(^\text{14}\). It may, therefore, be more appropriate to hypothesise that free radical derived stimuli might predispose to the \textit{perpetuation} of the diarrhoeal state.
CHAPTER 2

Vitamin E and vitamin E deficiency

2.1 Biochemistry and physiology of vitamin E

Vitamin E was discovered in 1922 when Evans and Bishop demonstrated the existence of a fat soluble factor which was necessary for normal reproduction in the rat\textsuperscript{81}. The name "vitamin E" is, therefore, derived from the Greek \textit{tokos} - childbirth, and \textit{phero} - to bear. By the late 1950's research into the role of vitamin E in human nutrition had been subject to numerous and exaggerated claims which led to the infamous description of vitamin E as "a shady lady to be approached gingerly by respectable or discreet investigators in human nutrition"\textsuperscript{82}. It is now clear, however, that vitamin E (\(\alpha\)-tocopherol) is the most important lipid soluble chain breaking antioxidant in biological membranes\textsuperscript{83} despite being present in these membranes in relatively small amounts when compared to the abundance of membrane lipid\textsuperscript{84}. Vitamin E has, therefore, a fundamental role in the preservation of cell membrane integrity and function and it should come as no surprise that claims about the medical benefits of vitamin E supplementation have abounded\textsuperscript{85,86}.

Vitamin E is a generic term for tocopherols and tocotrienols, a group of naturally occurring fat soluble substances of differing antioxidant activities which are present in high concentrations in plant oils\textsuperscript{87}. As a group the tocopherols consist of a chromanol ring (the antioxidant part of the molecule) and a phytol side chain which anchors the molecule in the membrane lipid bilayer (vide infra) providing an important structural element of the cell membrane(s)\textsuperscript{88}. The tocotrienols are similar in structure, with the side chain containing 3 double bonds. The general structure of tocopherols and tocotrienols is shown in figure 2.1. \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-tocopherols and tocotrienols differ in the number and position of methyl groups at positions 5, 7 and 8 on the chromanol ring. These differences confer differences in the antioxidant activity of these molecules. For example the antioxidant potency of \(\alpha\)-, \(\beta\)-, \(\gamma\)-, and \(\delta\)-tocopherols (compared with the potency of \(\alpha\)-tocopherol) are historically quoted as 1.0, 0.3, 0.15 and 0.03 respectively\textsuperscript{89}. 
and for α-, β-, γ-, δ- tocotrienols as 0.3, 0.05, 0.05 and 0.05. A more recent study by Diplock\textsuperscript{90} looking at the inhibition of thiobarbituric acid reactive substances [TBARS] (see chapter 3) in cultured fibroblasts and liver homogenates quotes the antioxidant potency of α-, β-, γ-, and δ- tocopherols and α- tocotrienol as 1.0, 0.75, 0.75, 0.55 and 0.50 respectively although the precise potency varied with both cell type and oxidising stress.

![Structure of tocopherols and tocotrienols](image.png)

**RRR - tocopherol**

![tocotrienol side chain](image.png)

**Figure 2.1** Structure of tocopherols and tocotrienols

**Table 2.1. Nomenclature and structural characteristics of tocopherols and tocotrienols**

<table>
<thead>
<tr>
<th>Tocopherol / tocotrienol</th>
<th>Methyl position on chromanol ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>5, 7, 8</td>
</tr>
<tr>
<td>β</td>
<td>5, 8</td>
</tr>
<tr>
<td>γ</td>
<td>7, 8</td>
</tr>
<tr>
<td>δ</td>
<td>8</td>
</tr>
</tbody>
</table>

The 3 dimensional orientation of the three methyl groups of the phytyl side chain of the tocopherols can vary (methyl group position at each of carbons 2 [on the
chromanol ring], 4', and 8' being designated R or S) giving rise to 8 possible orientations. The accepted term for the naturally occurring \(d\)-\(\alpha\)-tocopherol is RRR-\(\alpha\)-tocopherol\[^91\] (or more strictly 2R, 4'R, 8'R-\(\alpha\)-tocopherol). RRR-\(\alpha\)-tocopherol has a greater antioxidant capacity than its isomers\[^60\] and accounts for approximately 90% of the vitamin E found in mammalian tissues\[^92\]. Synthetic \textit{all rac} - \(\alpha\) - tocopherol is a mixture of approximately equal amounts of all the possible stereo isomers of \(\alpha\) - tocopherol.

Table 2. 2 Nomenclature for vitamin E\[^*\]

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Name</th>
<th>Designated name</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-tocopherol</td>
<td>5,7,8 trimethyl tocol</td>
<td></td>
</tr>
<tr>
<td>(d)-(\alpha)-tocopherol</td>
<td>2R, 4'R, 8'R - (\alpha)-tocopherol</td>
<td>RRR - (\alpha)-tocopherol</td>
</tr>
<tr>
<td>(l)-(\alpha)-tocopherol</td>
<td>2S, 4'R, 8'R - (\alpha)-tocopherol</td>
<td>2 - Epi - (\alpha)-tocopherol</td>
</tr>
<tr>
<td>(d,l)-(\alpha)-tocopherol (totally synthetic)</td>
<td>2DL, 4'DL, 8'DL - (\alpha)-tocopherol</td>
<td>All - rac - (\alpha)-tocopherol</td>
</tr>
</tbody>
</table>

\[^*\]International Union of Pure and Applied Chemistry and American Institute of Nutrition

In biological membranes, the chromanol head group of vitamin E is probably located close to the surface of the lipid bilayer\[^93\]. The flexible phytol chain is aligned (in a time averaged sense) with the acyl chains in the interior of the membrane lipid bilayer\[^84\] and is important in anchoring \(\alpha\)-tocopherol within the membrane\[^85\].

The positioning of the chromanol ring at or near the cell surface may at first sight appear idiosyncratic because the peroxidation of unsaturated lipids is likely to be occurring deeper within the hydrophobic interior of the cell membrane. It has been argued that abstraction of a hydrogen atom from an unsaturated fatty acyl side chain will result in the formation of a peroxyl group which will be hydrophillic. The peroxyl group will as a result be pushed from the hydrophobic interior of the membrane towards the hydrophillic surface, and hence towards the chromanol head group of \(\alpha\)-tocopherol. The distribution of \(\alpha\)-tocopherol within a biological membrane is probably not homogenous, and there is evidence to suggest that it may be concentrated in regions of the membrane in which higher amounts of polyunsaturated fatty acids are to be found\[^96\].

2.2 Absorption, transport, and delivery of vitamin E to the tissues

Vitamin E is lipid soluble and highly hydrophobic and is absorbed in the small intestine along with dietary fats. Its absorption is therefore dependent upon the
emulsification of dietary lipid and the formation of mixed micelles within the intestine (processes themselves dependent upon the production of pancreatic lipase and bile salts). The efficiency of absorption of tocopherol from the intestinal lumen may be influenced by the composition of dietary lipid. Medium chain triglycerides appear to enhance absorption, and long chain polyunsaturated fats to decrease absorption. The evidence for this is not unambiguous and these differences might also be explained by an increased rate of oxidation of vitamin E in vivo by virtue of a high dietary PUFA content. States of fat malabsorption and steatorrhoea (for example with exocrine pancreatic insufficiency or biliary tract pathology) will therefore be associated with a malabsorption of vitamin E. Absorption of tocopherol is passive occurring maximally between the proximal and middle thirds of the small intestine. Within the enterocyte tocopherol is incorporated into chylomicra and secreted into the lymphatics (probably without the need for a specific carrier). The (vitamin E containing) chylomicra are catabolised by the action of lipoprotein lipase which is located on the endothelial lining of capillary walls in a number of tissues. The resultant chylomicron remnants are taken up by the liver, which subsequently secretes α-tocopherol into the bloodstream in VLDL. Vitamin E partitions between different lipoproteins during the catabolism of VLDL and chylomicra. Vitamin E is, therefore, transported in the blood by plasma lipoproteins (mainly VLDL and HDL) and erythrocytes and not by a specific carrier protein. An important consequence of this will be that plasma vitamin E levels will depend not solely on dietary intake, but will vary with the concentrations of plasma lipoproteins, and hence correlate with plasma total lipids. Plasma levels of α-tocopherol are usually 5-10 fold higher in humans than γ-tocopherol despite the fact that most diets are considerably richer in γ-tocopherol. Early reports that γ-tocopherol was absorbed less efficiently than α-tocopherol have been disproven. This implies a postabsorptive discrimination between the two forms. Further discrimination between the isomers of α-tocopherol occurs within both plasma and tissues. For example, in the rat, RRR-, rather than SRR-, α-tocopherol accumulates in plasma, erythrocytes and brain. In humans it seems that the site of discrimination is during the secretion of VLDL from the liver and this specificity has been attributed to a (31 kD) hepatic α-tocopherol binding protein.

A variety of mechanisms have been identified for the movement of lipids (and
hence vitamin E) from lipoproteins to the tissues. These include the actions of lipoprotein lipase\(^{117}\), lipoprotein receptor mediated endocytosis\(^{118}\), receptor independent uptake, and spontaneous transfer and exchange. Evidence to date, suggests that regulation of these processes is dependent upon the energy and cholesterol requirements of the cells and that there are no regulated mechanisms specifically governing tissue uptake of vitamin E from the blood\(^{119}\).

2.3 Vitamin E deficiency

2.3.1 Observations in Humans

There are a number of recognised clinical situations in which a deficiency of vitamin E produces characteristic clinical features\(^{120}\).

2.3.1.1 Preterm infant.

This group of infants will be susceptible to a deficiency of vitamin E because of low body stores at birth, poor postnatal absorption, low concentrations of LDL, and exposure to oxidising stimuli (e.g., hyperoxia and multiple infections). There were a number of reports of a syndrome of haemolytic anaemia, thrombocytosis, and oedema in premature infants which resolved following the administration of vitamin E in the late 1960's\(^{121,122}\). Milk formulae for infants are now supplemented with vitamin E (>0.6 mg \(\alpha\)-tocopherol / g polyunsaturated fat) and vitamin E deficiency and the above syndrome are now rare. Controversy remains as to whether further vitamin E supplementation might prevent the anaemia of prematurity. A balanced review of the evidence available does not support a beneficial effect\(^{123}\).

Retinopathy of prematurity

A link between retinopathy of prematurity (ROP) and the use of oxygen in the treatment of sick preterm infants has been recognised for many years\(^{124,125}\), yet the place of vitamin E supplementation in the prevention of this disease is not without contention\(^{126,127}\). The American Academy of Paediatrics report "vitamin E and the prevention of retinopathy of prematurity" in the mid 1980's concluded that vitamin E supplementation was without significant benefit in preventing ROP\(^{128}\). This conclusion is at first sight confusing because the report predicted that for every 100 infants weighing < 1500g treated with prophylactic vitamin E one case of ROP
would be prevented. This apparent contradiction arose through concern about potential harmful effects of vitamin E administration in preterm newborns. A characteristic syndrome of unexplained thrombocytopenia, renal dysfunction, hepatomegaly, cholestasis, ascites, hypotension and metabolic acidosis\textsuperscript{129} had been documented in newborns following the widespread use of an IM preparation of vitamin E. It was subsequently established that this preparation of vitamin E contained high levels of polysorbate, and the syndrome has since been attributed to the polysorbate content of the preparation\textsuperscript{130}. This preparation of vitamin E is no longer available and hence the interesting association of the use of the polysorbate containing vitamin E preparation with a characteristic syndrome is not relevant in the present day calculation of the risk - benefit ratio of using vitamin E prophylaxis in the preterm infant. Further worries about the safety of vitamin E administration to the preterm infant arose following reports that its administration was associated with an increased incidence of necrotising enterocolitis\textsuperscript{131}.

A more contemporary set of conclusions support the prophylactic use of vitamin E in the LBW infant\textsuperscript{126}. The data forecasts that treating an average of 22,000 infants weighing < 1500g each year would result in 1029 fewer cases of ROP or intraventricular haemorrhage, but in 740 more cases of necrotising enterocolitis or severe sepsis. There are a number of other studies which have demonstrated that whilst the incidence of ROP was not reduced by vitamin E supplementation, the severity of the retinopathy was\textsuperscript{132}. The balance of evidence suggests therefore that vitamin E supplementation of preterm infants might be of benefit in the prevention of ROP, but that this might be at the expense of an increased morbidity from other complications of prematurity.

\textit{Bronchopulmonary dysplasia (BPD)}

The aetiology of this chronic lung disease seen in low birthweight infants is multifactorial and probably includes the effects of barotrauma and hyperoxia. Recent animal studies have shown that vitamin E supplementation is able to raise the levels of vitamin E in the lungs of preterm guinea pigs, but this does not prevent the \textit{acute} effects of hyperoxia\textsuperscript{133}. The overall consensus of opinion is that the efficacy of vitamin E in the prevention of BPD is not proven\textsuperscript{134}. 
Periventricular haemorrhage

Periventricular haemorrhage occurs frequently in the very low birthweight infant, and extension of the haemorrhage into brain parenchyma may be associated with a poor neurodevelopmental outcome. Chiswick\textsuperscript{135} made the observation that intramuscular vitamin E decreased the incidence of periventricular haemorrhage (PVH) at post mortem. A prospective randomised trial of vitamin E supplementation for 3 days (20 mg/kg IM) in preterm infants demonstrated an independent protective effect against PVH as demonstrated by ultrasound (9\% vs 34\%)\textsuperscript{138}.

2.3.1.2 Abetalipoproteinaemia

This inherited disorder usually manifests in early life with variable gastrointestinal symptoms (diarrhoea, failure to thrive, and eventually steatorrhoea) and later with a progressive and severe neurological syndrome characterised by progressive ataxia, areflexia, proprioceptive loss, generalised muscle weakness, ophthalmoplegia and pigmentary retinopathy\textsuperscript{137,138,139}. The neuropathology of this syndrome is characteristic with degeneration of the posterior columns, and a selective loss of large calibre myelinated sensory axons in spinal cord and peripheral nerves. A similar pathology can be produced by making rhesus monkeys chronically vitamin E deficient\textsuperscript{140}. Apolipoprotein B, the major apoprotein of LDL, is undetectable in abetalipoproteinaemia\textsuperscript{141}, resulting in a total absence of LDL from the plasma of affected individuals\textsuperscript{142}. Apolipoprotein B is also an essential component of VLDL and chylomicra, and these lipoprotein species are also absent from the plasma of individuals with abetalipoproteinaemia. Bearing in mind what we now know about the absorption of vitamin E and its distribution to the tissues (vide supra) then it is no surprise that vitamin E was undetectable in the plasma of these individuals\textsuperscript{143}. Muller et al\textsuperscript{144} reported the results of treating a group of children with abetalipoproteinaemia with large doses of vitamin E. Infants supplemented with vitamin E before the age of 16 months did not develop either the clinical or the electrophysiological findings characteristic of the neurological syndrome of abetalipoproteinaemia. In children treated at older ages the progress of the disease was halted by vitamin E administration. This experience has subsequently been shared by others\textsuperscript{145}.
2.3.1.3 Other chronic disorders of fat absorption.
Plasma concentrations of vitamin E may be subnormal in other groups of children with fat malabsorption (for example biliary atresia\textsuperscript{146}, cystic fibrosis\textsuperscript{147}, intestinal lymphangiectasia, coeliac disease, short bowel syndrome) although levels are usually low rather than \textit{absent}\textsuperscript{148}. There are a number of reports in the literature of neurological syndromes in association with fat malabsorption and vitamin E deficiency which improved following the administration of vitamin E\textsuperscript{149,150}.

2.3.1.4 Isolated defect in vitamin E absorption
There are in the literature a number of case reports of patients (\geq 9) with vitamin E deficiency and a neurological syndrome, but with no evidence of fat malabsorption\textsuperscript{151,152}. These patients differ from those with abetalipoproteinaemia by having high concentrations of serum cholesterol, triglyceride and LDL. They do, however, improve following supplementation with very high dose oral vitamin E. The absorption and transport of vitamin E has been studied in four of these patients using deuterated tocopherol\textsuperscript{153} and the aetiology seems likely to be a lack of or a defect in the hepatic tocopherol binding protein.

2.3.2 A rat animal model of chronic vitamin E deficiency

2.3.2.1 Basic diet and procedure
A well validated rat animal model of chronic vitamin E deficiency was used as previously described by workers from this laboratory\textsuperscript{154}.
Weanling (age 21-13 days) male Wistar rats were placed on pelleted semi synthetic diets supplied by Dyets Inc., Pennsylvania, USA. The composition of the basic vitamin E deficient diet is shown in table 2,3.
Table 2.3. Composition of lard based vitamin E deficient rat diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Grams / Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin free casein</td>
<td>200.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>653.2</td>
</tr>
<tr>
<td>Tocopherol stripped lard (stabilised with 0.02% BHT)</td>
<td>100.0</td>
</tr>
<tr>
<td>Choline bitartate</td>
<td>1.8</td>
</tr>
<tr>
<td>Vitamin mix 302362</td>
<td>5.0</td>
</tr>
<tr>
<td>Salt mix 200650</td>
<td>40.0</td>
</tr>
</tbody>
</table>

The vitamin E sufficient (control) diet was exactly the same but with the addition of \( \alpha \)-tocopheryl acetate (40mg / Kg feed).

Corn oil based diets were made with 100g / Kg of tocopherol stripped corn oil in place of the lard. The vitamin E supplemented diet contained \( \alpha \) tocopheryl acetate 40mg / Kg feed.

All diets were stored in the dark at 4°C until use.

The fatty acid composition of the corn oil and lard based diets determined by gas chromatography (as described in chapter 7) is shown in table 2.4.

Table 2.4. Fatty acid composition of diets (mol %) [mean of 3 estimations]

<table>
<thead>
<tr>
<th></th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>16:1</th>
<th>18:1</th>
<th>20:1</th>
<th>18:2</th>
<th>18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td>1.6</td>
<td>26.8</td>
<td>15.6</td>
<td>2.5</td>
<td>40.7</td>
<td>0.8</td>
<td>8.7</td>
<td>0.8</td>
</tr>
<tr>
<td>CORN OIL</td>
<td>0.3</td>
<td>8.5</td>
<td>1.7</td>
<td>0.4</td>
<td>29.8</td>
<td>0.6</td>
<td>59.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Animals were housed in grid bottomed cages to prevent coprophagia and allowed free access to food and water at all times. Uneaten diet was replaced every four days to reduce any effects due to auto oxidation of the fats in the feed.

2.3.2.2 Differences between lard and corn oil based diets

There are a number of issues which need to be raised about the differences between the lard and corn oil based diets.

Firstly the calorific values of the feeds were not identical. The lard source used had
a calorie density of 901 kcal / 100g of fat. The corn oil had a calorie density of 883 kcal / 100g of fat [data supplied by Dyets]. In terms of calories, 100g of lard would be equivalent to 102g of corn oil. The diets were not (quite) isocaloric, but the difference was sufficiently small that an increase in the fat content of the corn oil based diets to 102g / Kg was not warranted. Animals were also allowed to eat ad libitum (vide infra) rather than being pair fed and so adjusting the fat content of the corn oil based diet would not have guaranteed an isocaloric intake in the two groups.

Secondly, the lard based diets contained 0.1g cholesterol / 100g of fat, and the corn oil based diets no cholesterol. This is discussed more fully in chapter 13. Thirdly, the vitamin E / polyunsaturated fatty acid ratio of the vitamin E containing corn oil diet was considerably lower than that found in the lard based diet. (Lard diet 40mg α-tocopherol acetate / 10g polyunsaturated fatty acids; Corn diet 40mg α-tocopherol acetate / 56g polyunsaturated fatty acids. [Data supplied by Dyets Inc.]). It would have been interesting to have had a further group of corn oil based diets containing a normalised vitamin E / PUFA ratio. This issue is discussed further in chapter 13.

The characteristic neuropathology of the animals fed vitamin E deficient diets has been described in detail previously\(^\text{154}\), and has many similarities with that found in abetalipoproteinaemia. The poor coat condition, kyphoscoliosis, muscle wasting and ataxia seen in the vitamin E deficient animals is similar to that reported in a number of other rat models of vitamin E deficiency\(^\text{155,156}\). The animal model is, therefore, one of a severe, progressive and chronic vitamin E deficiency.

2.3.2.3 Validation of the rat model of vitamin E deficiency

The dynamics of tocopherol depletion were not studied in any of the cohorts of animals used in the present studies. Previous studies in this department with this same rat animal model (using deuterated tocopherols) have shown that this process is complex and involves the redistribution of tocopherol(s) between tissues (principally into neurological tissues) during the course of depletion\(^\text{157}\). These findings are in keeping with the observations of Goss Sampson et al\(^\text{154}\) that neurological tissues still contain about 10% of control levels of vitamin E after 12 months of vitamin E deficiency, whereas non neurological tissues (liver, adipose
tissue) contain no detectable vitamin E after this time period. In health the rat intestine contains amounts of vitamin E which compare with those found in other tissues\textsuperscript{158}. There are no published studies documenting the time course of the decrease in tissue levels of vitamin E within the intestine with vitamin E deficiency. If the intestine were to behave in an manner similar to adipose tissue, then one would expect that levels would have fallen below 10\% of control values within 6-8 weeks of vitamin E deficiency.
CHAPTER 3

Measurement of lipid peroxidation

3.1 Introduction
The peroxidation of lipids may be measured at different stages of the process, including (i) losses of unsaturated fatty acids, (ii) measurement of primary peroxidation products, (iii) measurement of secondary products including carbonyls and hydrocarbon gases. A fourth group of substances which might be measured are the carbon or oxygen centred radicals produced during the peroxidative process. These can be measured by electron spin resonance [ESR] techniques using a suitable spin trap. There are, therefore, a variety of methods from which to choose when measuring lipid peroxidation. The method chosen will be determined both by the sensitivity and specificity required and also by which stage of the peroxidative process is of greatest interest. The chemical composition of the end products of peroxidation varies according to the fatty composition of the lipid substrate and the quantities and species of metal ions present during the peroxidative process. It is in general, therefore, a good idea to use more than one method of measuring lipid peroxidation and, wherever possible, it is better to study the process at different stages.

3.2 Malondialdehyde as an Index of lipid peroxidation
Aldehydes are always produced when lipid hydroperoxides break down in biological systems. These provide a useful index of lipid peroxidation but are also of biological importance by virtue of their reactivity with amino acids and thiols, and their ability to cross link proteins and nucleic acids. Malondialdehyde (MDA) is in many instances the most abundant individual aldehyde resulting from lipid peroxidation. MDA is a volatile low molecular weight (formula C₃H₄O₂) secondary product of lipid peroxidation and is among the most widely quoted (and misquoted) indices of this process. It can be detected in small amounts in a variety of tissues although the amounts produced during peroxidation will vary considerably between different tissues and subcellular fractions.

The major source of MDA in biological systems is the oxidative decomposition of polyunsaturated fatty acids (PUFA) containing three or more double bonds. The
mechanism by which MDA is formed following the oxidation of polyunsaturated fatty acids is not clear. Pryor suggested that MDA is produced as a consequence of fragmentation of cyclic endoperoxides and peroxides produced during the peroxidation process (Figure 3.1). Fragmentation will require the presence of iron salts in the reagents used in the test or in the biological sample. Pryor's hypothesis has been supported by the observation that hydroperoxides with a double bond $\beta$-$\gamma$ to the peroxy-bearing carbon undergo cyclisation to form prostaglandin like endoperoxides following hydrogen abstraction.

![Diagram of MDA formation](image)

**Figure 3.1** Production of MDA from a lipid peroxyl radical via the fragmentation of cyclic endoperoxides (as suggested by Pryor)

When using MDA as an index of lipid peroxidation it is important to remember that it is a secondary product of lipid peroxidation which is only part of a complex mixture of products, that it is only formed from PUFA with three or more double bonds, and that only certain peroxidation products are capable of breaking down to yield MDA. Moreover, free MDA is rapidly metabolised in vivo.
3.3 Thiobarbituric Acid Reactive Substances (TBARS)

MDA exists in an uncharged enol form or as a conjugate base depending upon solvent and pH. In aqueous solution at pH >7.0 the conjugate base (enolate anion) will predominate because MDA is a weak acid with a pKₐ of 4.46. The uncharged enol form is much more reactive, particularly towards nucleophiles, than the conjugate base. This ready reactivity of MDA at low pH with nucleophiles forms the basis for the thiobarbituric acid (TBA) test of lipid peroxidation. The TBA test is one of the oldest and most frequently used methods of measuring lipid peroxidation, and was used in biology as long ago as 1944. The lipid material is heated with thiobarbituric acid (TBA) at low pH, with the formation of a pink chromogen which has been shown to be a (TBA)₂-MDA adduct (figure 3,2). In acid solution the (TBA)₂-MDA adduct absorbs light at 532nm and fluoresces at 553nm, and is readily extractable into butan-1-ol for measurement. Several other aldehydes formed in peroxidising systems yield different chromogens with TBA.

![Chemical structures](image)

**Figure 3.2** Formation of a fluorescent pink chromogen in the TBA test

A substantial proportion of the colour change generated in this test follows the decomposition of lipid hydroperoxides during the acid heating stage of the test. This process is dependent upon the presence of transition metal ions in the reaction mixture. Thus the addition of metal chelating agents, or of antioxidants will influence both peroxidation of lipids within the incubation medium and hydroperoxide decomposition during the heating process. Both the type and
concentration of acid added to reduce the pH in the TBA test will influence the rate of peroxide decomposition and hence the amount of TBA reactive material formed\textsuperscript{174,175}, as will the duration and the temperature of the heating stage\textsuperscript{176}. Other compounds, in particular other aldehydes, may interfere with the TBA test by producing yellow ($\varepsilon_{\text{max}} = 495\text{nm}$), orange ($\varepsilon_{\text{max}} = 552\text{nm}$) or other chromogens which have a significant absorption at $532\text{nm}$ after heating with TBA. Bile salts, for instance, will produce a different chromogen, but amino acids and carbohydrates will react with TBA to form an authentic (TBA)$_2$-MDA adduct. Authentic (TBA)$_2$-MDA adducts may be separated from non authentic TBA adducts by high performance liquid chromatography (HPLC)\textsuperscript{177}.

The advantages of the TBA test are that it is sensitive, cheap, and easy to perform. As a consequence it is probably the most widely used index of lipid peroxidation and many small modifications have been made to the test as originally described. The dependence of the composition of the products in the test on the conditions under which the test is performed, coupled with a widespread variability in experimental detail has meant that comparison of results from different laboratories may be unreliable.

It is worth emphasising that the TBA test does not measure MDA per se but rather total MDA plus MDA equivalents generated during the assay procedure. These are conveniently referred to as thiobarbituric acid reactive substances (TBARS).

I evaluated three methods of measuring TBARS using small intestinal mucosal homogenates. Two of these measured the pink chromogen by colorimetric methods\textsuperscript{178,179}. These were over 5 fold less sensitive than the fluorometric method described by Yagi\textsuperscript{180,181}. The latter method was therefore adopted routinely in these studies and is described in detail below.

3.3.1 Fluorometric determination of thiobarbituric acid reactive substances

Reagents used:

- 7% sodium dodecyl sulphate (SDS)
- 10% phosphotungstic acid
- 0.67% thiobarbituric acid
- 0.1M HCl
- 0.1M phosphate buffer pH 7.4
3.3.1.1 Preparation of MDA standard:
16.5μl of 1,1,3,3-tetramethoxypropane (TMP), which forms MDA on hydrolysis, was diluted to 1l with dH₂O. An aliquot was diluted 1/50 with phosphate buffer. 1ml of this solution was equivalent to 1.99 nmol of MDA. A standard curve for MDA was constructed by using appropriate dilutions of this standard (0, 0.5, 1.0, 1.5, 2.0 nmol / ml). A calibration curve was constructed each time a batch of samples was assayed.

3.3.1.2 Assay procedure
MDA standard, or 1ml of mucosal homogenate (10mg / ml) in phosphate buffer, was vortex mixed for 30 seconds with 0.2 ml SDS, 0.3 ml phosphotungstic acid and 1ml of TBA, and then incubated at 95°C in a water bath for 60 minutes. After cooling to room temperature, the pink colour was extracted into 5 ml of butan-1-ol by vortex mixing for 2 minutes, centrifuging at 3000 rpm for 10 minutes and then removing the upper (butanol) phase. The fluorescence (due to the (TBA)₂-MDA adduct) in this phase was measured in a Perkin Elmer LS-3 fluorescence spectrophotometer (excitation λ = 515nm, emission λ = 553nm).

![Graph](image.png)

Figure 3.3 TBA test standard curve. Fluorescence of (TBA)₂-MDA adduct extracted into butan-1-ol.
The assay was linear beyond 2 nmol/ml MDA (figure 3,3) although tissues were usually diluted sufficiently so that the homogenate contained 0.5-2.0 nmol MDA equivalents / ml.

The linearity of the assay with serial dilutions of mucosal homogenate is shown in figure 3,4.

![Figure 3,4 Fluorescence at 553nm of serial dilutions of mucosal homogenate in the TBA test](image)

**3.4 Measurement of free malondialdehyde by HPLC**

The problems with the TBA test, as described above, do not detract from its strengths as an easy, sensitive, and widely used index of lipid peroxidation. If MDA itself is to be used as an index of lipid peroxidation then a more specific method is required. HPLC technology has allowed the development of a number of different methods for measuring MDA. In the first instance I chose to measure MDA by the method of Esterbauer using a weak ion exchange aminophase column. Problems with this method when used with intestinal homogenates made it necessary to develop and validate a second method. The latter required derivatisation of MDA with dinitrophenylhydrazine (DNPH) before separation of the derivative by reverse phase chromatography. This method worked particularly well with intestinal tissues. Both methods are described below.
3.4.1 Non derivatised ion exchange chromatography with an aminophase column

This method was originally developed for the measurement of MDA in rat liver microsomes\(^{182}\) and makes use of the UV absorption of free MDA in its enolate anionic form which has an absorption maximum at 270nm\(^{184}\). The components of the HPLC system were as follows; column: Spherisorb S5NH\(_2\) aminophase column (25 cm x 4.6 mm, Hichrom, UK) with an aminophase guard column; mobile phase: 0.03M trizma, pH 7.4 / acetonitrile (15 / 85 \(\text{v/v}\)) at a flow rate of 1.5 mL / min (Perkin Elmer Binary LC Pump 250). 20\(\mu\)l aliquots of standard or sample were injected and uv absorption measured (\(\lambda=270\)nm, Spectromonitor 3100, LDC Analytical, UK) with the detector sensitivity set to 0.005 AUFS (arbitrary units full scale).

3.4.1.1 Preparation of MDA standards and procedure with tissues

MDA standards were prepared by the acid hydrolysis of 1,1,3,3-tetramethoxypropane (TMP). 200\(\mu\)l of TMP (equivalent to 1.206 mmol MDA) was diluted to 100ml with 1% (v/v) sulphuric acid and stood at room temperature for 2 hours. After this period an aliquot of the solution was further diluted 1/100 with 1% sulphuric acid to make a "stock MDA" solution. The MDA concentration of the stock solution was checked by measuring its UV absorbance in a 1cm cuvette at 245nm (molar extinction coefficient, \(\varepsilon = 13750\) L mol\(^{-1}\) cm\(^{-1}\)). This was stable for 1 week at 4\(^\circ\)C. Running standards of MDA were prepared freshly each day by diluting the stock MDA 1/100 with a mixture of 0.1M trizma pH 7.4 / acetonitrile (50/50 \(\text{v/v}\)). 20\(\mu\)l of this running standard contained 24.1 pmol
of MDA.

An example of the chromatography is shown in figure 3.5. A standard curve constructed using different concentrations of MDA was linear throughout the range of concentrations tested (5 - 100 pmol / 20μl injection). This is illustrated in figure 3.6.

![MDA standard curve. Aminophase ion exchange HPLC method](image)

Figure 3-6 MDA standard curve. Aminophase ion exchange HPLC method

Tissues were prepared by adding 50μl acetonitrile to 50μl of a 6% homogenate (w/v), mixing vigorously with a vortex mixer for 30 seconds, and centrifuging rapidly in an eppendorf minifuge for two minutes. This treatment deproteinised the sample, and is also reputed to arrest the peroxidative process. The supernatant was decanted and stored on ice until injection (as a 20μl aliquot) into the HPLC system for measurement.

MDA standards eluted from the column well after the solvent front with a retention time of 7.55 minutes (Fig 3,5). With intestinal homogenates, however, the MDA peak (which was not visible in unspiked tissues) would have eluted on the shoulder of the "solvent front" and would also have been obscured by a closely eluting broad peak at 8.05 minutes. This is more obvious when studying the chromatogram of homogenates spiked with MDA (figure 3.7). It proved difficult to separate the MDA peak (spiked homogenate) from the peaks eluting closely before
and after it.
Increasing the proportion of acetonitrile in the mobile phase delayed both peaks but did not aid their separation. Ascorbate, which has a retention time similar to MDA, exhibits strong absorption at 270nm, and is present in the cytoplasm of cells. To test whether the coeluting peak was ascorbate, hydrogen peroxide (final concentration 0.1 mM) was added to the mobile phase. This would convert ascorbate to dehydroascorbate which does not interfere with MDA detection by uv absorption\(^\text{182}\). This did not, however, improve the chromatography. These problems with intestinal tissues made the development and validation of an alternative method of measuring MDA necessary.

![Chromatogram of SI homogenate spiked with MDA. Aminophase HPLC method](image)
3.4.2 DNPH derivatisation and reverse phase HPLC methods

This method, which is an adaptation of methods formerly used to measure MDA in serum\textsuperscript{185} and urine\textsuperscript{186} makes use of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with α-keto acids to form a chromogen. The derivative formed with MDA in this reaction is probably 1-(2,4-dinitrophenyl)pyrazole formed by the cyclization and dehydration of the 2,4-dinitrophenylhydrazone product formed in the first instance\textsuperscript{183} (figure 3,8).

![Diagram of derivatisation of malondialdehyde with dinitrophenylhydrazine to yield 1-(2,4-dinitrophenyl) pyrazole](image)

**Figure 3,8** Derivatisation of malondialdehyde with dinitrophenylhydrazine to yield 1-(2,4-dinitrophenyl) pyrazole

The components of the system were as follows; column: Spherisorb ODS-5 (25 cm x 4.6 mm, Pharmacia, UK) reverse phase at room temperature fitted with a Spherisorb ODS guard column; mobile phase: acetonitrile / 0.1M HOI (43/57 v/v) at a flow rate of 1 ml/min (Perkin Elmer Binary LC Pump 250); detection: uv absorption at 310nm (Spectromonitor 3100, LDC Analytical, UK) with a detector sensitivity of .005 AUFS.

3.4.2.1 Preparation of MDA standards and procedure with tissues (free MDA).

MDA "stock" solution was prepared as described above. Working standards were prepared freshly each day by diluting the stock MDA 1/100 with 10mM phosphate buffered saline (PBS). Reagents were made as follows;

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPH</td>
<td>0.5 mg/ml in 1M HCl</td>
</tr>
<tr>
<td>Nitroresorcinol (internal standard)</td>
<td>0.0625μM stock</td>
</tr>
</tbody>
</table>
3.4.2.2 Final procedure

250μl MDA standard or 250μl homogenate in PBS (10% w/v)
250μl acetonitrile (ACN)
20μl DNPH
20μl nitroresorcinol
50μl 2M HCl

Mix for 30 seconds on a vortex mixer and then store on ice.

20μl aliquots were injected onto the column. Nitroresorcinol (IS) eluted at 4.6 minutes, DNPH at 5.3 minutes and the MDA-DNPH derivative at 9.1 minutes (figure 3,9). Calibration was linear in the range 5-100 pmol MDA / 20μl injection aliquot (figure 3,10).

Figure 3.9 Chromatogram of MDA standard. DNPH derivatisation and reverse phase HPLC
DNPH has a characteristic absorption spectrum with an absorption maximum at 355nm and significant, although considerably lower, absorption at 310nm. The MDA-DNPH derivative elutes from the column after DNPH and it was my early experience with this assay that the MDA-DNPH derivative eluted on the shoulder of the DNPH peak. At this stage of method development 200μl aliquots of DNPH were being added to the incubation mixture. The assay relies on there being an excess of DNPH to react with the MDA in the reaction mixture. The effects of reducing the amount of DNPH added from 200μl to 5μl in the presence of 600 pmol MDA (250μl of standard containing 48pmol / 20μl MDA) is shown in fig 3.11.

It was clear that reducing the amount of DNPH added lead to an increase in sensitivity which plateaued at about 20μl of DNPH. This resulted from an improvement in the separation of the MDA-DNPH peak from the shoulder of the preceding DNPH peak. Continued reduction in the amount of DNPH added should eventually lead to a reduction in the yield of MDA-DNPH. This was not demonstrated with volumes of DNPH as low as 5μl. As adequate separation of the peaks was obtained with the addition of 20μl of DNPH reagent, this volume was added in all the quantitative assays of tissue MDA. The assay remained linear in

Figure 3.10 MDA standard curve. DNPH derivitisation and reverse phase HPLC method
the range 5-100 pmol MDA / injection aliquot (figure 3,10).

Figure 3,11 Effect of reducing the volume of DNPH added to the incubation mixture on measured yield of MDA-DNPH

The MDA-DNPH adduct peak is surrounded by a number of other peaks which might potentially interfere with the quality of the chromatography. The composition of the mobile phase was adjusted to optimise the separation of MDA-DNPH from these adjacent peaks.

For this series of experiments, 125μl of MDA standard was added to 125μl of mucosal homogenate (in PBS) and then derivatised as outlined above. Reducing the proportion of HCl in the mobile phase shortened the retention time (figure 3,12). Optimum separation of the MDA - DNPH peak occurred with ACN / HCl mixed in proportions of 43 / 57 (v/v). This mobile phase was used thereafter.

3.4.2.3 Internal standard

There are two main drawbacks in using nitroresorcinol as an internal standard. Firstly, its UV absorbance does not rely on its derivatisation by DNPH, and secondly it was often partly or completely obscured by other peaks in chromatograms of mucosal homogenate (figs 3,13 & 3,14). It did however provide an element of quality control when comparing the derivatisation and
chromatography of MDA standards in different batches.

**Figure 3.12** Effect of altering the ratio of HCl / ACN in the mobile phase on the retention time of MDA-DNPH

**Figure 3.13** Tissue homogenate. MDA HPLC by DNPH derivatisation

**Figure 3.14** Tissue homogenate spiked with MDA. MDA HPLC after DNPH derivatisation
3.4.2.4 Coefficients of variation

"Intrabatch" coefficients of variation were determined by six consecutive measurements performed on the same MDA standard (24pmol / 20μl injection) during a four hour period (samples kept on ice). Whilst the samples contained nitroresorcinol, no corrections were made for this in calculating the coefficient of variation (cv) \[cv = \frac{sd}{mean}\]. The results are shown in table 3.1.

Table 3.1 Coefficient of variation of nitroresorcinol and MDA - DNPH measured by reverse phase HPLC.

<table>
<thead>
<tr>
<th>Nitroresorcinol (Pk area)</th>
<th>MDA-DNPH (Pk area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13976</td>
<td>23091</td>
</tr>
<tr>
<td>14280</td>
<td>22945</td>
</tr>
<tr>
<td>14068</td>
<td>23243</td>
</tr>
<tr>
<td>14227</td>
<td>22592</td>
</tr>
<tr>
<td>mean</td>
<td>14137</td>
</tr>
<tr>
<td>standard deviation</td>
<td>141</td>
</tr>
<tr>
<td>coefficient of variation</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The coefficient of variation and recoveries when running mucosal homogenates were studied by running MDA standards, mucosal homogenates and MDA spiked mucosal homogenates together. Samples were prepared as described above. The composition of each was as follows;

<table>
<thead>
<tr>
<th></th>
<th>Homogenate</th>
<th>Buffer (PBS)</th>
<th>MDA std</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA std</td>
<td>0</td>
<td>125μl</td>
<td>125μl</td>
</tr>
<tr>
<td>Homogenate</td>
<td>125μl</td>
<td>125μl</td>
<td>0</td>
</tr>
<tr>
<td>Homogenate + MDA</td>
<td>125μl</td>
<td>0</td>
<td>125μl</td>
</tr>
</tbody>
</table>

In this instance the peak areas for MDA-DNPH were normalised for the mean area of the nitroresorcinol peaks (13462) taken from the first two runs of MDA standard.
Table 3.2 Coefficients of variation of MDA standards and MDA in mucosal homogenates (spiked and unspiked) - raw data

<table>
<thead>
<tr>
<th></th>
<th>Nitroresorcinol measured</th>
<th>MDA-DNPH measured</th>
<th>Corrected measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA standards</td>
<td>13336</td>
<td>15380</td>
<td>15246</td>
</tr>
<tr>
<td></td>
<td>13587</td>
<td>15687</td>
<td>15543</td>
</tr>
<tr>
<td></td>
<td>14091</td>
<td>15993</td>
<td>15279</td>
</tr>
<tr>
<td>Mucosal homogenates</td>
<td>14468</td>
<td>21466</td>
<td>19973</td>
</tr>
<tr>
<td></td>
<td>15348</td>
<td>22999</td>
<td>20172</td>
</tr>
<tr>
<td></td>
<td>14594</td>
<td>21197</td>
<td>19552</td>
</tr>
<tr>
<td></td>
<td>15097</td>
<td>23682</td>
<td>21117</td>
</tr>
<tr>
<td>Homogenate + MDA spike</td>
<td>13330</td>
<td>33733</td>
<td>34067</td>
</tr>
<tr>
<td></td>
<td>14109</td>
<td>35378</td>
<td>33756</td>
</tr>
<tr>
<td></td>
<td>13581</td>
<td>33916</td>
<td>33618</td>
</tr>
<tr>
<td></td>
<td>14342</td>
<td>38594</td>
<td>36225</td>
</tr>
<tr>
<td></td>
<td>13366</td>
<td>37132</td>
<td>37398</td>
</tr>
</tbody>
</table>

The coefficients of variation for each of these groups were derived (table 3.3), and the recovery of MDA standard in the homogenates calculated as recovery = \( \frac{\text{MDA}_{\text{spiked homogenate}} - \text{MDA}_{\text{homogenate}}}{\text{MDA}_{\text{standard}}} \) using the means of each group.

Table 3.3 Coefficients of variation of MDA standards and MDA in mucosal homogenates (spiked and unspiked) - summary

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA standards</td>
<td>1.1%</td>
</tr>
<tr>
<td>Mucosal homogenates</td>
<td>3.3%</td>
</tr>
<tr>
<td>Homogenates with MDA spike</td>
<td>4.3%</td>
</tr>
</tbody>
</table>

The mean recovery of MDA standard from a mucosal homogenate during this series of experiments was 96.5% (n=5).
3.4.3 Measurement of total MDA (free and bound)

Any free malondialdehyde produced in vivo will be rapidly metabolised, for example to form malonic acid (by the actions of mitochondrial aldehyde dehydrogenase) which is then decarboxylated to CO₂ and acetate. It has been estimated that 60-70% of an oral dose of [¹⁴C] MDA will be excreted as ¹⁴CO₂. MDA is also found in hydrolysable bound forms in biological samples. These bound forms may include Schiff's base - type adducts to protein, nucleic acids, and other nucleophiles. Under acidic, basic or high temperature conditions these adducts may release MDA. Measurement of the bound MDA fraction may be of interest because the toxicity of MDA is thought to arise as a result of this reactivity with biological nucleophiles such as amino acids and thiols, and its ability to cross link proteins and nucleic acids. The TBA test includes a heating stage which will release bound MDA and hence this test will provide an index of free and bound MDA (although, of course, the test measures much more than just MDA as discussed in section 3.3, page 72).

Total MDA was measured in homogenates by subjecting them to alkaline hydrolysis in the presence of an antioxidant. An estimate of bound MDA can then be made by subtracting the value obtained for free MDA. The method requires the heating of the homogenate with a strong base. Both these conditions favour the peroxidation of PUFA within the sample. Promethazine was therefore used as an antioxidant to minimise this effect.

Reagents;
- saturated NaOH solution which is then filtered and diluted 1:4 with distilled water
- approximately 3M HCl
- promethazine 0.5mg / ml in 10mM PBS

3.4.3.1 Procedure

120μl of homogenate (10% w/v) was mixed with 20μl promethazine and 25μl NaOH. The mixture was incubated at 60°C for 60 minutes. Samples were allowed to cool and neutralised with 35μl of HCl. 200μl of acetonitrile was added and the homogenate mixed on a vortex mixer for 30 seconds. The samples were spun in an eppendorf minifuge and 125μl of the supernatant decanted. To this was added
12μl 2M HCl, 5μl DNPH, 5μl nitroresorcinol. This mixture was vortexed and then incubated at room temperature for 1 hour (in the dark, as promethazine is light sensitive). Thereafter samples were stored on ice in the dark until analysed. The chromatography was, in general, less satisfactory after this procedure because of the presence of interfering peaks. This was reflected in a higher intrabatch coefficient of variation (7.9% for whole homogenates, n=4 over 4 hours). Maximal yield of MDA was obtained within 50 minutes of starting the alkaline incubation with no further rises apparent after 120 minutes incubation (figure 3.15). An incubation time of 60 minutes was therefore adopted for the assay.

![Graph](image)

**Figure 3.15** Yield of MDA as a function of the duration of alkaline hydrolysis (mean of 4)

### 3.4.3.2 Promethazine as an antioxidant

The method described above includes an incubation at 60°C and there was the possibility (by analogy with the TBA test) that the MDA being measured was generated during this heating stage by the continuing oxidation of PUFA and the decomposition of preformed lipid peroxides. However, incubation of a sample with promethazine in the absence of NaOH (60°C for 60 minutes) did not result in an increase in MDA as compared with time zero, yet incubation of the sample in the absence of both promethazine and NaOH resulted in an approximately 3 fold
increase in MDA. This is suggestive that promethazine is functioning as an antioxidant and preventing peroxidation of the sample during the heating stage.

3.5 Measurement of lipid peroxidation by other methods.

In the introduction to this chapter it was stressed that, wherever possible, it is valuable to measure more than one index of lipid peroxidation. The quality of the data produced would be enhanced further if the measurements related to different stages in the peroxidative process. A number of alternative means of measuring lipid peroxidation were validated during the early stages of this work, although results were in general disappointing when used with intestinal tissues.

3.5.1 Oxygen uptake

The carbon centred radical formed following hydrogen abstraction from a polyunsaturated fatty acid undergoes a molecular rearrangement to form a conjugated diene which then takes up oxygen to generate a lipid peroxy radical (see figure 1,8). A number of the ensuing decomposition reactions also involve the uptake of oxygen. Thus oxygen uptake can provide an index of the progress of peroxidation and can be measured with a Clark type oxygen electrode\(^6^8\). In this study a thermolabile azo compound, ABAP, (2,2'-azobis-2-amidinopropane hydrochloride) which forms carbon centred radicals on decomposition was used to initiate the peroxidative process. I was able to demonstrate the peroxidative process using a solution of linoleic acid. 11mM ABAP was added to a 50mM solution of linoleic acid in 50mM phosphate buffer at 37°C, and after a short lag phase a period of linear O\(_2\) uptake followed. The lag phase could be lengthened or the rate of O\(_2\) uptake temporarily reduced by the addition of the water soluble vitamin E analogue TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), final concentration 7\(\mu\)M, to the system. This is shown in figure 3,16. A modification of this method has successfully been used to study the antioxidant capacity of serum\(^6^8\).

This method was evaluated with intestinal homogenates. Mucosal homogenate (approximately 120\(\mu\)g of protein) was added to 52mM linoleic acid and peroxidation initiated with 12mM ABAP. In both vitamin E deficient and sufficient tissues (12 month old animals) the lag phase was 20-30 seconds and was followed by a linear period of oxygen uptake which was comparable in both groups of mucosae
(control: mean 1.33µl min⁻¹, range 1.26-1.39, n=3; deficient: mean 1.33µl min⁻¹, range 1.30-1.37, n=3). The method was not, however, used to measure antioxidant capacity beyond this validation phase because of inherent difficulties with the use of whole homogenates.

Use of a total lipid extract from the tissues in lieu of the whole homogenates would have circumvented many of these technical problems but the results obtained under such circumstances might not have provided an accurate representation of the situation in whole tissues (in which there are proteins and in which the vitamin E has a defined orientation within the membrane). Such studies were not, therefore, performed.

3.5.2 Measurement of peroxides
Lipid peroxides are capable of oxidising iodide (I⁻) ions into iodine (I₂) which, in the presence of an excess of I⁻ will be converted to I₃. I₃ can be measured by
colorimetry using a suitable colour reagent. Use of a commercially available colour reagent for the enzymatic determination of cholesterol (CHOD-iodide, Merck, Darmstadt, FRG) enables the use of absorbance at $\lambda=365$nm against a reagent blank to be used as a measure of $I_3$ concentration\cite{note1}. No changes could be demonstrated with either control or vitamin E deficient small intestinal homogenates and this technique was not therefore adopted as a routine.

### 3.5.3 Measurement of conjugated dienes
The oxidation of unsaturated fatty acids is accompanied by the formation of conjugated dienes which absorb ultraviolet light in the wavelength range 230-235nm. Measurement of this uv absorbance when using anything other than pure lipid mixtures is, however, complicated by the fact that polyunsaturated fatty acids themselves absorb light at only a slightly lower $\lambda$. Haem proteins, purines and pyrimidines may also absorb light at these wavelengths and produce a high background absorbance. The use of second derivative spectroscopy has led to improved sensitivity\cite{note2} and the method has been used with some success in rat liver microsomes\cite{note3}. The "hump" which appears in the uv absorption spectrum as a result of peroxidation translates into a sharp minimum when one examines the second derivative spectrum. The size of this peak is an index of the conjugated dienes present. The technique may also allow discrimination between different conjugated diene structures which may be present.

I validated this technique using a solution of linoleic acid in phosphate buffered saline (100mg linoleate in 10ml of PBS) with / without peroxidation induced by 50mM ABAP. Lipids were extracted into hexane, dried down and then taken up into cyclohexane for spectroscopy. UV absorbance was measured with a Perkin Elmer computerised spectrophotometer against a cyclohexane blank using the following settings to obtain the second derivative ($d^2$) spectra;

- slit 2nm
- scan 300 $\to$ 215 nm
- scan speed 60 nm/min
- response time 10 secs
- $\delta \lambda = 1$ nm
- scale = -0.65 - +0.65 A units
It has been demonstrated\textsuperscript{191} that the second derivative minima at 233 and 242 nm correspond to mixtures of \textit{cis}, \textit{trans} and \textit{trans, trans} diene hydroperoxides. UV absorption and second derivative spectra for linoleic acid are shown in figures 3.17 and 3.18.

Whilst these minima were clearly visible in experiments using linoleic acid (figure 3.18), they could not be visualised with any certainty in total lipid extracts (100\(\mu\)g lipid ml\textsuperscript{-1} in cyclohexane) from mucosal scrapings (figure 3.19). The clarity of these recordings might have improved if a purified membrane fraction had been used. Difficulties were envisaged in obtaining sufficient lipid from such fractions with the small amounts of tissue available, and on confirming the identities of the absorption minima. No further studies of this nature were performed.
3.5.4 Measurement of free radical formation in a lipid phase by the hydrolysis of dichlorofluorescin diacetate.

2′7′-dichlorofluorescein diacetate (DCFDA) can be oxidised to dichlorofluorescein (DCF) by free radicals\textsuperscript{192} and other oxidising stimuli. DCFHDA is not fluorescent, yet DCF has a characteristic fluorescence spectrum. This property of DCFHDA has been used to measure the generation of free radicals in subcellular fractions of the brain\textsuperscript{193}. The method was used in a number of preliminary studies on the ability of oxidative stress to initiate lipid peroxidation in intestinal mucosal homogenates.
A calibration curve of fluorescein in methanol (0.1 - 1.0 μM L⁻¹) was constructed using fluorescein standard (figure 3.20). Fluorescence was measured in a Perkin Elmer LS3 fluorimeter in a 1ml cuvette with a light path of 1cm. Excitation was at 525nm (bandwidth 5nm) and detection at 525nm (bandwidth 20nm). The curve was linear in the range 100 - 600 pmol ml⁻¹.

1 ml of a homogenate of mucosal scrapings (100μg protein / ml) in 10mM phosphate buffered saline was mixed in a vortex mixer with 200μl of 0.30μM DCFHDA in methanol and incubated at 37°C for 15 minutes to load the membranes. At the end of this period the homogenate was pelleted by spinning in an eppendorf minifuge at 4°C for 5 minutes, washed by resuspension in 1 ml of PBS and a further period of centrifugation and finally resuspended in 1 ml of PBS.

Fluorescence was measured at 37°C with regular mixing before and after the application of an oxidative stimulus. Autofluorescence of the homogenate (with no DCFDA loading) was subtracted from the reading obtained. The initial rate of increase in fluorescence of 5μM DCFDA incubated at 37°C for 60 minutes (reflecting spontaneous hydrolysis of DCFHDA) was negligible, although fluorescence increased 10 fold over the subsequent 120 minutes.
The oxidising stimuli used were either 0.6mM ABAP (final concentration) or a final concentration of 20μM CuSO₄ / 500μM H₂O₂. Parallel experiments were performed with ABAP or CuSO₄ / H₂O₂ and PBS (no tissue), and the fluorescence measured in these blanks was subtracted from the readings obtained with the homogenates. The results are shown graphically in figure 3.21.

A "lag phase" was not visible with ABAP in either vitamin E deficient or control homogenates. There was no accelerated phase of peroxidation with CuSO₄ / H₂O₂. Experiments were not continued beyond 15 minutes because of rapidly rising background counts in the reagent blanks in the presence of an oxidising stimulus. Because of these high background counts, and difficulties in ensuring consistent loading of the tissues and reproducibility of the assay the method was not pursued further.

The lack of a lag phase in the ABAP treated homogenates could be explained by the peroxidative stimulus being too powerful. Experiments were conducted with concentrations of ABAP as low as 30μM but a lag phase could not be

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b A "lag phase" refers to the period of time between oxidative challenge and the onset of rapid lipid peroxidation in the so called propagation phase of peroxidation. The length of the lag phase is dependent upon the type and the concentrations of antioxidants present in the biological test system and the nature of the samples being peroxidised
demonstrated. Lower concentrations were not tested. It should be noted that with 12μM ABAP a clear lag phase was demonstrable during the peroxidation of linoleate in the presence of TROLOX during the validation of the oxygen electrode methodology. The lack of an accelerated phase of peroxidation in the CuSO₄ / H₂O₂ experiments may have related to the ability of the free fatty acids in rat intestinal membranes to act as a "metal ion sink"³²³,³²⁴. Higher concentrations of copper were not used in an attempt to overcome the metal ion binding capacity of the tissues. These issues were not addressed further because the method was unsatisfactory for comparative work.
CHAPTER 4

Measurement of vitamin E

4.1 Introduction

α-tocopherol is the most important\textsuperscript{195,196}, though probably not the only\textsuperscript{197,198} lipid soluble chain breaking antioxidant in biological membranes and is present in high concentrations within the small intestine\textsuperscript{158}. Vitamin E is a first line defence against lipid peroxidation through its free radical quenching and chain breaking antioxidant activities. Vitamin E is also able to quench singlet oxygen and has an important structural role within the cell membrane\textsuperscript{199}.

There are other potent inhibitors of in vitro lipid peroxidation, specifically metal ion catalysed peroxidation, present within the small intestine\textsuperscript{322}. These include a group of nonesterified long chain mono-unsaturated fatty acids\textsuperscript{323,324} which act by sequestering free transition metal ions and hence negating their catalytic effect on free radical generation and propagation.

4.2 Measurement of α-tocopherol by HPLC

α-tocopherol may be measured in a number of different ways. These include colorimetry, fluorimetry, paper, column and thin layer chromatography, and gas chromatography\textsuperscript{200}. In this study α-tocopherol was measured by high performance liquid chromatography (HPLC) with fluorometric detection using a modification\textsuperscript{201} of the method of Buttriss and Diplock\textsuperscript{202} which was established and validated in this department.

200 μl of a 6% (w/v) homogenate of mucosal scrapings in 0.1M phosphate buffer was mixed vigorously for 1 minute on a vortex mixer with 1ml of 75% ethanol in water to precipitate the protein. The lipid phase was then extracted by mixing vigorously for 1 minute on a vortex mixer with 1.2 ml of HPLC grade hexane and centrifuging the mixture at 3000rpm for 10 minutes. 100μl of the upper hexane phase was injected onto a 25cm x 4.9mm id 5mm direct phase silica HPLC column (Jones Chromatography) using an injector port fitted with a 100μl loop.
The mobile phase was 1% methanol in hexane, dried with 0.4nm molecular sieves, and the flow rate 2 ml min⁻¹. Fluorescence detection of α-tocopherol in the eluent was performed with a Perkin Elmer LS1 fluorimeter using an excitation wavelength of 280nm and an emission wavelength of 310nm. Quantitative measurement was achieved by comparison of the peak area of the α-tocopherol peak with that of a previously run external standard (50 pmol / 100μl). The standard used was 5μM α-tocopherol (Sigma, Poole, UK). 5mM stock was made by dissolving 0.0205g of α-tocopherol in 10mls of hexane and working standard made by a further 1/1000 dilution. This was stored at 4°C.

α-tocopherol eluted from the column with a retention time of about 3.5 minutes, and the peak was free of interfering peaks (figure 4.1). Peak areas in the range 5 - 50 pmol per 100μl injection were linear (figure 4.2). The vitamin E content of tissues was expressed in terms of the total tissue lipid. This assay is described in the following section.
4.3 Total lipid extraction.

Total lipids were extracted from the intestinal homogenates by the method of Folch[203]. The remainder of the above sample (ie both aqueous and hexane phases after removal of the 100µl aliquot for measurement of vitamin E) was blown down under nitrogen and taken up into 3 mls of 2:1 chloroform: methanol (v/v). This was vortex mixed for 2 minutes and then washed with 0.75 mls of 0.1% NaCl (w/v) by vortex mixing for a further 1 minute. After standing for 10 minutes, the upper aqueous phase was aspirated and discarded and the remaining (lower) phase blown down under nitrogen. This was taken up in 1 ml of hexane for the hydroxamic assay of lipid esters[204].
4.4 Hydroxamic acid assay of lipid esters

4.4.1 Reagents
Reagents used in the assay were made as follows;

Stock ferric perchlorate
Dissolve 5g ferric perchlorate in 10 mls 70% perchloric acid and 10 mls dH₂O. Dilute to 100ml with cold absolute alcohol and store at 4°C.

Ferric perchlorate reagent
Add 3 ml of 70% perchloric acid to 4 ml of stock ferric perchlorate and dilute to 100ml with cold absolute alcohol.

Alkaline hydroxylamine
Solution A. 2.0g of hydroxylamine hydrochloride dissolved in 2.5ml of dH₂O and diluted to 50 ml with absolute alcohol.
Solution B. 4g sodium hydroxide dissolved in 2.5 ml of dH₂O and diluted to 50 ml with absolute alcohol.
Mix equal volumes of A and B, centrifuge at 2000 rpm for 5 minutes, and use the supernatant as the alkaline hydroxylamine reagent.

Both the ferric perchlorate reagent and the alkaline hydroxylamine were made fresh each time the assay was performed.

4.4.2 Procedure
The lipid extract (in hexane) was blown down under nitrogen. This was taken up again in approximately 0.5ml of acetone, and blown down to dryness (to ensure complete removal of the chloroform). This was taken up again in 1ml of hexane, and a 250µl aliquot removed which was dried down for the assay. 1 ml of alkaline hydroxylamine was added to each of the dried lipid samples and the tubes placed in a water bath at 65°C for 2 minutes (being gently agitated all the time). Samples were cooled to room temperature in cold water and mixed with 0.75ml of ferric perchlorate reagent. The samples were stood at room temperature for 30 minutes (colour development taking between 15 - 20 minutes) and the purple colour
measured as the absorbance at 530nm in a 1cm light path cuvette against a reagent blank.

A standard curve was constructed on each occasion the assay was performed using 2mM glycercyl trioleate [10 - 100 μl = 1.82 - 18.2 μg of lipid].

The assay was linear in this range (figure 4,3) and the absorbances of the lipid extracts from tissues generally fell within the range 0.15 - 0.3.

![Graph](image_url)

**Figure 4,3** Standard curve for glycercyl trioleate in the hydroxamic acid assay of lipid esters

### 4.5 Intrabatch coefficient of variation of vitamin E / lipid ratio

Intestinal mucosal scrapings prepared from the whole small intestine of 3 vitamin E sufficient rats were pooled and a 6% homogenate in phosphate buffer prepared. 200μl aliquots (X5) of the homogenate were assayed for α tocopherol and total lipid esters as described in the foregoing sections. The results are shown in table 4.1 overleaf.
Table 4.1 Coefficient of variation for α tocopherol, total lipids, and α tocopherol / lipid ratio.

<table>
<thead>
<tr>
<th>lipid / µg</th>
<th>α tocopherol / µmol</th>
<th>α toco / lipid ratio nmol / mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.3</td>
<td>.83</td>
<td>38.2</td>
</tr>
<tr>
<td>52.5</td>
<td>.74</td>
<td>41.0</td>
</tr>
<tr>
<td>53.9</td>
<td>.87</td>
<td>46.9</td>
</tr>
<tr>
<td>55.4</td>
<td>.86</td>
<td>45.5</td>
</tr>
<tr>
<td>59.5</td>
<td>.80</td>
<td>39.4</td>
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<tr>
<td>mean</td>
<td>54.1</td>
<td>42.2</td>
</tr>
<tr>
<td>sd</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>cv</td>
<td>6.9</td>
<td>9.1</td>
</tr>
</tbody>
</table>
CHAPTER 5

Measurement of apical brush border membrane hydrolase activities

5.1 Assay of disaccharidases
This assay is based on the method of Dahlqvist\textsuperscript{206} as described by Phillips et al\textsuperscript{206}. There are two parts to the assay. Firstly an incubation in which the mucosal homogenate is incubated with an appropriate disaccharide substrate for the particular disaccharidase being measured. This reaction is stopped after a period of time and the glucose liberated by hydrolysis of each disaccharide measured by a colour reaction involving glucose oxidase\textsuperscript{207}. The Tris in the glucose oxidase reagent inhibits both the disaccharidases in the homogenate and those present as contaminants in the glucose oxidase preparation.

5.1.1 Reagents

(i) Buffer.
0.1M phosphate buffer pH 6 at 37°C.

(ii) Substrate.
0.056M disaccharide in 0.1M phosphate buffer (lactose or sucrose)

(iii) 0.1% phenol in water (stored in dark)

(iv) Tris / glucose oxidase reagent
0.5M Trizma base adjusted to pH 7.0 with HCl is used to make the following reagents up to a total volume of 100ml.
- 100mg sodium azide
- 30mg 4- amino antipyrene
- 3mg horseradish peroxidase (4500 units)
- 1500 units glucose oxidase (Sigma type V)

"colour reagent" comprises a 75:25 (v/v) mixture of Tris / glucose oxidase reagent with 0.1% phenol.
Glucose standards were made by dissolving 0.045g of anhydrous glucose in 100ml of saturated benzoic acid. 2.5, 5, 7.5, and 10 ml aliquots were made up to a total volume of 10 ml with saturated benzoic acid. These standards contained 2.5, 5.0, 7.5, and 10 nmol of glucose per 40μl.

5.1.2 Procedure

5.1.2.1 Terminology (see table 5,1):
(a) Tests
For each homogenate a 20μl of each of the disaccharide solutions was pipetted into a separate tube.

(b) Blanks
Contained 20μl of buffer in place of the disaccharidase substrate

(c) Controls
Contained 20μl of buffer in place of homogenate

(d) Standards
Contained 40μl of the glucose standard solutions but no homogenate or disaccharidase substrate.

5.1.2.2 Assay
Test, blank and control eppendorf tubes were warmed to 37°C in a water bath for 2-3 minutes, and then either "homogenate" (2% homogenate in 0.1M phosphate buffer pH 7.4 or a bushborder membrane preparation) or buffer was added to "start" the incubation as shown in table 5,1 with thorough mixing on a vortex mixer. After exactly 40 minutes, 600μl of colour reagent was added to each tube and the incubation continued for a further 40 minutes. After this period the tubes were centrifuged in an eppendorf minifuge for 10 minutes. At the end of this time the colour change produced by the liberated glucose was measured @ 515nm in a 1cm light path microcuvette. A blank was not required in the spectrophotometer (vide infra).
Table 5.1. Summary of disaccharidase assay.

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>Control</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homogenate</td>
<td>20</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
</tbody>
</table>

Incubate at 37°C for 40 minutes

| Colour reagent | 600 | 600 | 600 | 600 |

Incubate at 37°C for 40 minutes

Centrifuge 10 minutes

Aliquots in **bold italic** are added to start the incubation

### 5.1.2.3 Calculations

A standard curve was constructed (as in Figure 5.1) and the regression line extrapolated to the y axis to obtain a zero reading (0). The absorbance of the substrate control (C) was added to that for the homogenate blank (B) for each substrate and homogenate and the standard zero subtracted to obtain a value \( \alpha \) [ie \( C + B - 0 = \alpha \)]

Hence there was one \( \alpha \) for each substrate for each homogenate.

The appropriate \( \alpha \) value was then subtracted from each "test" absorbance (T-\( \alpha \)).

The amount of glucose liberated in the assay (nmol) is obtained by reading (T-\( \alpha \)) off the standard curve or by using the equation for the regression line \( x = (y - \alpha) / b \).

2% homogenate usually generated an absorbance between 0.75 and 1.15 which was within the linear part of the calibration curve.

The amount of glucose liberated per minute was equivalent to the amount of sucrose or lactose hydrolysed (each of these disaccharides contains one glucose molecule).
Figure 5.1 Standard curve for colour reaction in disaccharidase assay.

5.2 Determination of Alkaline Phosphatase (EC3.1.3.1.)

This was measured by a colorimetric assay as described by Wilkinson et al.\textsuperscript{208}. The method relies on the liberation of p-nitrophenol from p-nitrophenylphosphate through the action of alkaline phosphatase\textsuperscript{209}. The accumulation of p-nitrophenol phosphate was measured as the increase in absorbance at 405nm. Reagents for the assay were bought as a commercially available kit (Sigma Kit No. DG 1245-K).

5.2.1 Reagents

Reagent A: diethanolamin buffer pH 9.8
magnesium chloride

Reagent B: p-nitrophenylphosphate

Start reagent: 2 tablets of reagent B in 5 mls of water made up to 30 mls with reagent A.
5.2.2 Procedure
The spectrophotometer cuvette holder was warmed to 37°C by a water circulation system connected to a water bath, and the "start reagent" was warmed to 37°C in the water bath.

3 ml of the start reagent was placed in a prewarmed cuvette (light path 1.0 cm) and the reaction started by the addition of 20μl of a 2% homogenate of mucosa (in 0.1M phosphate buffer pH 7.4) and thorough mixing.

The change in absorption at 405nm was measured against a water blank. Absorbances were measured at one minute intervals for 3 minutes in a recording spectrophotometer and an average reading taken.

5.2.3 Calculations

\[
\text{ALP activity (U)} = \frac{\delta A \times TV}{18.45 \times SV}
\]

where;

\(\delta A = \text{change in absorbance @ 405nm min}^{-1}\)

\(TV = \text{total volume / ml}\)

\(SV = \text{sample volume / ml}\)

18.45 = millimolar absorptivity of p - nitrophenol @ 405nm cm\(^{-1}\)

One unit (U) of alkaline phosphatase activity is defined as the amount of enzyme which will catalyse the formation of 1μmol of p - nitrophenol min \(^{-1}\) under the conditions of the assay.

A 2% homogenate usually produced a value for \(\delta A\) of less than 0.25 min\(^{-1}\). If for any reason this was exceeded then the sample was diluted 1:5 and the assay repeated. The increase in absorption at 405nm due to liberation of p - nitrophenol was linear beyond three minutes with a 2% homogenate (Figure 5,2), but only the first three minutes of the reaction were routinely measured to determine alkaline phosphatase activity.

The linearity of the assay was assessed by making serial dilutions of a 3% homogenate and repeating the measurements (on each occasion a 20μl aliquot of homogenate was added to 3 mls of start reagent). It can be seen (Figure 5,3) that the decrease in alkaline phosphatase activity with the serial dilution was linear over
Figure 5.2 Increase in absorption at 405nm with time in the ALP assay

the range $\delta$ absorbance min$^{-1}$ of 0.05 - 0.25. Samples with $\delta A$ min$^{-1}$ greater than 0.25 were therefore diluted (see above) so that $\delta A$ fell in this range.

Figure 5.3 Effect of diluting homogenate on measured ALP activity
5.3 Protein assay

Protein was measured by a colorimetric method using a commercially available kit (Pierce BCA protein assay kit No. 23225) using bicinchoninic acid. 2% homogenates were diluted 1/20 with phosphate buffer (0.1M, pH 6.0). 100μl aliquots of homogenate were then thoroughly mixed with 2ml of freshly made up protein assay reagent (a 50 + 1 mixture of reagents A and B as per manufacturers instructions). Samples were then incubated at 37°C for 30 minutes and the colour change measured as the absorbance at 562nm in a 1cm light path against a reagent blank.

Bovine serum albumin standards (in phosphate buffer) were made (containing 10 - 50 μg / 100μl) and a standard curve constructed each time the assay was performed. The assay was linear over this range. Homogenates were diluted so that the protein content lay within this range.

Figure 5.4 Protein assay standard curve
CHAPTER 6

Isolation and purification of small intestinal brush border membranes

6.1 Introduction

The methods most frequently used to isolate brush border membrane vesicles are modifications of the divalent cation precipitation methods introduced by Schmitz et al\textsuperscript{210}. Vesicles produced by these methods, in particular if magnesium precipitation is used, are, however, not homogenous because of contamination from the basolateral membrane and by membranes from incompletely differentiated enterocytes\textsuperscript{211}. This contamination of an apical membrane fraction with components from the basolateral membrane has in the past led to confusion when interpreting transport data\textsuperscript{212} from vesicle studies. Basolateral membranes differ in composition from the apical membrane as a result of cell polarisation. It is important, therefore, that fractions should be as pure as possible. Yakymyshyn et al have described a modification of the method of Schmitz which utilises a Percoll\textsuperscript{TM} density gradient to separate the apical brush border membranes and which results in no discernable Na\textsuperscript{+}K\textsuperscript{+}ATPase activity (a basolateral membrane marker) in the final apical membrane preparation\textsuperscript{213}. This method was evaluated and subsequently utilised to prepare brush border membranes from the proximal small intestine of the rats studied.

6.2 Procedure

Mucosal scrapings were prepared from the proximal small intestine of a rat by flushing the small intestine with ice cold normal saline, opening the tissue along its antimesenteric border, blotting the mucosa dry with lint-free tissue and then scraping lightly with a microscope slide. The scrapings were placed in ice cold Tris-mannitol-EGTA buffer (2mM TRIS·HCl, 50mM mannitol, 0.8mM EGTA, pH 7.1) to make a 4% (w/v) homogenate and the mixture kept on ice. This was homogenised in a "Polytron" blender for 15 seconds at a setting of "8" and the mixture filtered through buffer soaked gauze and then a nylon sieve (40μm mesh) to remove cell debris and mucus. Homogenates were then diluted 1:2 with Tris mannitol EGTA buffer to make a 2% homogenate. 1ml aliquots were removed at this stage for measurement of alkaline phosphatase and disaccharidase activities.
in the non enriched homogenates, and each of these samples was then divided into smaller aliquots and frozen at -70°C until use. Pre-weighed solid magnesium chloride was added to the remainder of each of the homogenates to give a final concentration of 10mM. On addition of MgCl₂ homogenates were mixed in the Polytron mixer for 10 seconds at a setting of "6" to aggregate the endoplasmic reticulum, basolateral membrane and mitochondria. Samples were then centrifuged at 11700g for 5 minutes at 4°C in a Beckman ultracentrifuge [12600 rpm with a 70Ti rotor]. The supernatant (S₁) was decanted and spun at 41000g for 20 minutes at 4°C [23600 rpm with a 70Ti rotor]. The pellet (P₂) was resuspended in 0.5 ml of Tris-mannitol-EGTA buffer and sonicated at 4°C for 5 seconds (5 bursts of 1 second each) to form apical brush border vesicles. The P₂ fraction was layered onto 15mls of 40% Percoll™ (in 0.12M sodium chloride) and centrifuged at 27000g for 30 minutes at 4°C [19300 rpm with a 90Ti rotor]. The brush border membrane (BBM) fraction formed a well defined band approximately 1cm from the meniscus between densities of 1.015 and 1.055 g/ml (vide infra) which was aspirated with a syringe and needle in a volume of 2mls of Percoll™. The Percoll™ was removed by further centrifuging this sample at 100,000g for 30 minutes. This precipitated the Percoll™ whilst leaving the brush borders in suspension. The brush border suspension was then mixed well and divided into aliquots for freezing at -70°C until use.

The Percoll™ gradient centrifugation step has been shown to largely eliminate contamination by basolateral membranes (as measured by Na⁺K⁺ATPase activity) and nuclear DNA²¹³.
MUCOSAL SCRAPINGS
Homogenise - Polytron (15 sec / 8)
Filter
Solid MgCl₂ + Polytron mix
Centrifuge 11700g 5 mins
SUPERNATANT S₁
PELLET P₁
Centrifuge 41000g 20 mins
PELLET P₂
SUPERNATANT S₂
Resuspend
Layer on Percoll™
Centrifuge 27000g 30 mins
remove BBM FRACTION
Centrifuge 100000g 30 mins

Figure 6.1 Outline of fractionation procedure for apical brush border membranes
Percoll™ forms a gradient during centrifugation. This was checked each time the gradient was used by running in tandem with the samples a tube in which 0.5 mls of Tris mannitol EGTA buffer containing an assortment of density marker beads had been layered onto 15mls of 40% Percoll in 0.12M NaCl.

![Graph](image)

**Figure 6.2** Percoll™ density gradient illustrated with density marker beads

The gradient formed is illustrated in figure 6.2.
6.3 Enrichment of apical membrane markers

The enrichments of alkaline phosphatase and of the disaccharidases sucrase and lactase were determined as described previously (chapter 5). These are shown in table 6.1 below.

Table 6.1. Enrichment of apical membrane markers in the brush border membrane enriched preparation.

<table>
<thead>
<tr>
<th></th>
<th>Homogenate (n=12) mean (± sem)</th>
<th>BBM enriched fraction (n=12)</th>
<th>enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>396 (± 76 )</td>
<td>2416 (± 93 )</td>
<td>6.1</td>
</tr>
<tr>
<td>(U g⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrase (μmol glucose</td>
<td>55.2 (± 7.9 )</td>
<td>397 (± 15.7 )</td>
<td>7.2</td>
</tr>
<tr>
<td>released min⁻¹ g⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactase (μmol glucose</td>
<td>25.0 (± 4.2)</td>
<td>152 (± 6.2)</td>
<td>6.1</td>
</tr>
<tr>
<td>released min⁻¹ g⁻¹ protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.4 Discussion

The enrichments in apical membrane markers are low when compared with those reported using other methods. For example, Semenza's group report a 22X enrichment of sucrase and a 16X enrichment in alkaline phosphatase using calcium precipitation with rabbit ileum, and others have documented a 20 fold enrichment in alkaline phosphatase in rat ileum using sucrose gradient centrifugation. Magnesium precipitation is, however, less efficient than calcium precipitation and enrichments in the literature are quoted as 8 fold for alkaline phosphatase and 9 fold for maltase. In their description of the Percoll method of purifying rabbit small intestinal brush border vesicles, Thompson's group report a 7 fold enrichment of alkaline phosphatase and an 8 fold enrichment in sucrase. They argue that Tris buffer has an inhibitory effect on sucrase activity and that this might account for the lower enrichments than those quoted in the literature. It is not evident whether Percoll has any residual effect on these hydrolase activities but it is clear that the lower enrichments are due to either nuclear DNA or basolateral membrane contamination of the brush border membrane fraction.

There are objections to the use of both calcium and magnesium based methods of microsomal aggregation. A recent balanced review has recommended...
the use of Mg$^{2+}$ precipitation methods (rather than Ca$^{2+}$) for the purification of BBM for biochemical studies$^{219}$ and a Mg$^{2+}$ based method was therefore adopted in these studies.
CHAPTER 7

Measurement of fatty acid composition of the apical brush border membrane by gas chromatography.

7.1 Introduction

There are numerous methods available for determining the fatty composition of biological samples including thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and gas chromatography (GC). Gas chromatographic methods for the measurement of fatty acids are well established and this method was chosen because of the availability of both a gas chromatogram and a mass spectrometer in our laboratory. The "classical" method of measuring fatty acids by gas chromatography involves the preparation of fatty acid methyl esters from the native fats, separation on an appropriate column, and detection by flame ionisation. Methylation of the fatty acids renders them more volatile and improves the quality and hence the separation of the peaks on the chromatogram. In recent years, however, columns have become available which allow the chromatography of free fatty acids directly without derivatisation. These specialist columns are expensive and were not easily available in our laboratory. Fatty acids were therefore analysed as methyl esters. The procedure adopted for methylation and the technical aspects of sample introduction and separation on the column will be discussed.

There are a number of methods of detecting derivatised fatty acids after separation by the gas chromatogram. The "classical" tried and tested method has been by flame ionisation detection. This method relies on the burning of organic compounds in a hydrogen flame as they elute off the column and measurement of the resultant ions produced during the combustion by collecting them on a set of polarised electrodes. This is probably the most widely used method of detection in use with GC and combines good linearity \((10^{-9} - 10^{-2} \text{g})\) with high sensitivity. The identity of eluting peaks is ascertained by comparing the retention times with those of known standards.

The gas chromatograph in our laboratory was equipped with a mass spectrometer. Detection was by electron impact ionisation (EI). A clear advantage of having a mass spectrometer attached is the ability to identify eluting substances both by
retention time and by their characteristic mass spectrum. Ionisation of volatised sample molecules is induced in the ion source of an EI spectrometer by collision with a beam of energetic electrons (approximately 20-70 eV) produced from a heated rhenium filament. Removal of an electron from the sample molecule after the collision produces a molecular ion. The energy of the ionizing electrons and the stability of the chemical bonds in the molecule determine whether the molecular ions subsequently undergo decomposition by internal bond cleavage and atomic rearrangements to yield a plasma of ions, radicals and neutral species. Both positive and negative ions are produced, but the former are usually many orders of magnitude more numerous. The anions produced are generally of low mass and of little value to the interpretation of structure. The decomposition of the molecular ion by fragmentation is dependent upon its structure. This fragmentation pattern, the mass spectrum of the molecule, is unique to each compound. Ion source conditions, such as electron energy and temperature influence the relative abundance of individual ions.

Electron impact ionisation is, perhaps, not the best method for the measurement of highly unsaturated fatty acids. This is because of the reduced thermal stability of these compounds during volatisation in the ion source and also because of the low stability of the molecular ion of these compounds (vide infra). As the molecular ion is potentially the most informative ion in a spectrum, its absence reduces the utility of the spectrum. In this respect, chemical ionisation (CI) would be a better detection system for suitably derivatised polyunsaturated fatty acids. CI was developed to ionise molecules with a much reduced energy transfer and produce a more stable molecular species ion.

7.2 Outline of analytical procedure

7.2.1 Extraction of lipids and transmethylation

120 µl of a suspension of brush border membranes (approximately 150-200µg protein) or 120µl of a calibration mixture of saturated and unsaturated fatty acids (vide infra) was spiked with 30µl of nonadecanoic acid (internal standard) in methanol (5mg / 10 ml). Lipid was extracted from all samples by a Folch total lipid extraction as follows. 1ml of 75% (v/v) ethanol in water was added and samples vortex mixed for 2 minutes. Samples were then spun at 3000 rpm for 15 minutes
and blown down under N$_2$. When completely dried down 3mls of chloroform / methanol (2:1 v/v) was added and samples vortex mixed for 2 minutes. 0.75 ml of 0.1% NaCl was added and samples vortexed for 2 minutes and then left to stand at room temperature for 5 minutes. The upper (aqueous) layer was aspirated (and discarded) and the lower layer dried down thoroughly under N$_2$. When completely dry the lipid extract was taken up into 1 ml of hexane and an aliquot (approximately 0.75ml) removed and dried down under N$_2$ ready for transmethylation. Samples were often stored overnight at -20°C at this stage. In this case samples were again thoroughly dried down under N$_2$ the following morning before commencing the transmethylation process.

Methylation and transesterification was achieved by incubating the total lipid extracts with dry methanolic HCl as described by Balasubramanian$^{323}$. Completely dry samples were taken up into 1 ml of 3M methanolic HCl and incubated in a water bath at 65°C for 4 hours$^{222}$. The incubation was carried out in bottles with teflon seals on the tops so as to avoid contamination of the samples with plasticiser which interfered with the chromatography. After methylation, samples were extracted into 1ml of hexane, dried down completely under N$_2$ and then taken up into 100µl of hexane for analysis by gas chromatography.

7.2.2 Chromatography

Chromatography was performed on a Hewlett Packard 5890 gas chromatograph coupled to a Hewlett Packard 5970 mass selective detector. The column was a 15m long 0.248 mm internal diameter capillary column with a 0.25µM DB-1 bonded liquid phase (J & W Scientific, Folsom, CA, USA). 1µl samples were injected using a HP 7673 automatic liquid sampler. Carrier gas was helium, flow rate 1ml min$^{-1}$.

Before each run the glass liner of the injection chamber was replaced with a clean liner (cleaned with "glass treat", rinsed with methanol and dried at 100°C for at least 1 hour). With each change of liner, fresh glass wool was inserted into the liner. This ensured more efficient heat transfer within the injector and significantly improved the chromatography (vide infra).

Samples, in hexane, were injected into the injector with the injector port in a "splitless" configuration. The purge valve was off for the first two minutes post injection, and so throughout this period the sample was being deposited on the first
parts of the column. Concentration of the sample on the column into a narrow band was achieved by having a low initial oven temperature (50°C) approximately 10-20°C below the boiling point of the solvent (hexane). After 2 minutes the purge valve was opened so expelling the remaining sample within the injector and thereby preventing a broadening of the chromatogram peaks by continued deposition of sample on the column.

The temperature zones used for the chromatography are detailed in table 7.1.

**Table 7.1 Temperature zones for gas chromatography of fatty acid methyl esters (FAME).**

<table>
<thead>
<tr>
<th>Initial temperature</th>
<th>Initial time</th>
<th>Rate of rise / °C min⁻¹</th>
<th>Final temperature</th>
<th>Final time</th>
<th>Total time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2.00</td>
<td>50</td>
<td>190</td>
<td>0</td>
<td>4.8</td>
</tr>
<tr>
<td>2.5</td>
<td>213</td>
<td>0</td>
<td></td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>260</td>
<td>0</td>
<td></td>
<td>23.4</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>300</td>
<td>10</td>
<td></td>
<td>34.07</td>
<td></td>
</tr>
</tbody>
</table>

The last 10 minutes of the run was a "burn off" period in which less volatile compounds which were not of interest were allowed to come off the column.

Scan acquisition parameters were as follows;

**Table 7.2 Scan acquisition parameters for FAME**

<table>
<thead>
<tr>
<th>Start time / mins</th>
<th>Low mass</th>
<th>High mass</th>
<th>Threshold</th>
<th>Scans sec⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>50</td>
<td>360</td>
<td>1000</td>
<td>1.38</td>
</tr>
<tr>
<td>23.40</td>
<td>548</td>
<td>550</td>
<td>1000</td>
<td>209</td>
</tr>
</tbody>
</table>

A solvent delay of 7 minutes was allowed before mass spectrometer turn on to allow the solvent to elute from the column.

The injector temperature was maintained at 250°C and the detector temperature set at 300°C.

These settings produced acceptable chromatography with good separation of the peaks of interest and little tailing of peak height / areas for compounds eluting later off the column (figure 7,1).

Quantitative measurements were made by peak area integration of the total ion chromatogram. Peak areas were adjusted after reference to the internal standard
(nonadecanoic acid - C19:0) peak and then compared with a calibration mixture containing known quantities of fatty acids. Peaks were identified by their retention time and the identity of each peak checked by referring to its mass spectrum. A library of mass spectra of saturated and unsaturated fatty acids (C14 - C24) was made by preparing solutions of commercially available standards (approximately 0.5 μg / ml in methanol) [Sigma, Poole, Dorset], running these individually after derivatisation, and saving the ion abundance profile (mass spectrum) for the fatty acid methyl ester (FAME) to file in an appropriate "library".

Table 7.3. Composition of calibration mixture

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Molecular weight</th>
<th>μg / 5 mls methanol</th>
<th>nmol / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>228</td>
<td>2.35</td>
<td>2.06</td>
</tr>
<tr>
<td>16:1</td>
<td>254</td>
<td>2.35</td>
<td>1.85</td>
</tr>
<tr>
<td>16:0</td>
<td>256</td>
<td>2.75</td>
<td>2.15</td>
</tr>
<tr>
<td>18:2</td>
<td>280</td>
<td>2.35</td>
<td>1.68</td>
</tr>
<tr>
<td>18:1</td>
<td>282</td>
<td>2.90</td>
<td>2.06</td>
</tr>
<tr>
<td>18:0</td>
<td>284</td>
<td>2.45</td>
<td>1.60</td>
</tr>
<tr>
<td>20:4</td>
<td>304</td>
<td>2.65</td>
<td>1.74</td>
</tr>
<tr>
<td>20:1</td>
<td>310</td>
<td>2.70</td>
<td>1.74</td>
</tr>
<tr>
<td>20:0</td>
<td>312</td>
<td>2.40</td>
<td>1.54</td>
</tr>
<tr>
<td>22:6</td>
<td>328</td>
<td>2.45</td>
<td>1.49</td>
</tr>
<tr>
<td>22:4</td>
<td>332</td>
<td>2.40</td>
<td>1.45</td>
</tr>
<tr>
<td>22:0</td>
<td>340</td>
<td>2.45</td>
<td>1.44</td>
</tr>
</tbody>
</table>

As is evident in the total ion chromatogram shown in figure 7.1, approximately equimolar amounts of unsaturated fatty acid produced smaller peaks than the corresponding saturated fatty acid of the same chain length. This is a characteristic of the chromatography when measuring total ion EI chromatograms.
Figure 7.1 Total ion chromatogram of a 2µl injection of a calibration mixture of fatty acids - see table 7.3

Concentration - response curves for individual fatty acids are shown in figures 7.4 - 7.14, and these are discussed in a later section.
7.2.3 Choice of internal standard

7.2.3.1 General considerations
Nonadecanoic acid (C19) was chosen as an internal standard (IS) because it is not naturally found in the total lipid extract from the small intestinal mucosa. There is, however, no reason to expect that C19 will be a good IS for unsaturated fatty acids of longer chain length (eg docosahexaenoic acid, 22:6). Polyunsaturated fatty acids with an odd number of carbons (eg 21:4 or 23:6) are not commercially available for use as an IS. An alternative approach would be to use a stable isotope of a naturally occurring polyunsaturated fatty acid (deuterated for example). The isotopes can (in theory) be differentiated from the natural fatty acid by a higher molecular ion. These are commercially available, although they are extremely expensive. The abundances of the molecular ion of the polyunsaturated fatty acids (when measured by EI) are, however, comparatively small when compared with a saturated fatty acid because of fragmentation. Hence the option of using selective ion monitoring and using a stable isotope as an internal standard for the polyunsaturated fatty acids is not practical. Other methods of detection, for example Cl, might have provided a better opportunity to use this type of IS. This approach has been used with saturated fatty acids with EI detection. For the needs of the present studies it seemed acceptable to use an odd numbered carbon saturated fatty acid as an internal standard. Ideally a number of IS should have been used varying in both chain length, saturation and in amount (there is up to a 30 fold difference between the quantities of fatty acids occurring in the mucosal lipid extract - vide infra).

7.2.3.2 Nonadecanoic acid versus triheptadecanoin
The liberation of free fatty acids from the corresponding glycerol or choline lipid ester occurs during the process of methylation with methanolic HCl. Choice of an internal standard which requires transmethylation in this manner might, therefore, have been more appropriate. In the course of developing the method the triglyceride triheptadecanoin was used in addition to nonadecanoic acid as an internal standard. The ratio of the C17 / C19 peak areas achieved its maximum within the first 3 hours of the incubation in methanolic HCl demonstrating that hydrolysis of the fatty acid esters in triheptadecanoin was complete within this
period.

![Graph showing time of methylation vs. C17/C19 peak area ratio.](image)

**Figure 7.2** Hydrolysis and methylation of triheptadecanoin as compared to methylation of nonadecanoic acid (each point = mean of 3).

This is illustrated in figure 7.2. All data is normalised to the area of the C19 peak at 4 hours, and does not, therefore, address the issue of incomplete methylation of C19 (for which vide infra). On the basis of this study, nonadecanoic acid was adopted as IS, principally because its solubility in methanol at room temperature was better than that of triheptadecanoin. Nonadecanoic acid was therefore both easier to use and provided more reproducible chromatography.

### 7.2.4 Efficiency of transesterification and methylation

There are a number of methods which may be used to saponify and methylate lipids. These employ differing reagents which include diazomethane, sodium methoxide, methanolic H$_2$SO$_4$, and methanolic HCl. The diazomethane methodology is laborious because it requires the hydrolysis of lipids by phospholipase before methylation with diazomethane, which has to be generated freshly on each occasion. It has the advantage of avoiding the exposure of polyunsaturated fatty acids to high temperatures which might result in their degradation. Methods using methanolic H$_2$SO$_4$ are known to result in losses of
polyunsaturated fatty acids. This may result from the oxidising effect of 20% sulphuric acid. The methanolic HCl and sodium methoxide methods were validated during the course of the present studies. The rationale for the comparison was to see if losses of polyunsaturated fatty acids were occurring during the heating stage of the methanolic HCl method.

The optimal duration of incubation at 65°C with methanolic HCl was assessed by incubating aliquots of the calibration mixture. The internal standard used in this instance was a commercially available nonadecanoic acid methyl ester. The peak areas obtained after differing incubation periods for a saturated fatty acid (stearic acid C18:0) and a polyunsaturated fatty acid (arachidonic acid C20:4) are illustrated in figure 7,3. In this graph the peak areas have been normalised for the C19:0 peak and each data point represents the mean of three samples. This demonstrates that methylation of C18:0 is complete within 120 minutes, but that the polyunsaturated fatty acid C20:4 requires up to three hours incubation to achieve maximal peak area. The longer incubation time of 16 hours, as has been used by others, is also shown. Surprisingly, losses of 20:4 are small.

![Figure 7,3](image)

**Figure 7,3** Effect of duration of methylation at 65°C on peak areas of stearic and arachidonic acids

To evaluate losses of polyunsaturated fats during the "hot" incubation, a comparison was made between calibration mixture methylated with methanolic HCl
for 4 hours at 65°C and methylation with sodium methoxide (which requires a 30 minute incubation at 30°C under nitrogen). The protocol for the sodium methoxide was as follows. 120μl of calibration mixture spiked with 30μl of nonadecanoic acid methyl ester IS (approximately 5 mg / 10 ml methanol) was vortexed with 1 ml of chloroform:methanol 2:1 for 1 minute and then dried down under nitrogen at 30°C. The residue was taken up in 50μl dry methanol. 50μl of sodium methoxide was added, the mixture vortexed thoroughly for 1 minute, and then incubated at 30°C in a sealed tube under nitrogen for 30 minutes. At the end of this period, 50μl of acetyl chloride was added, the mixture again vortexed for 1 minute, and allowed to stand at room temperature for 5 minutes. 250μl of hexane was added, the mixture vortexed for a further minute and then spun in an eppendorf minifuge for 4 minutes. Approximately 200μl of the hexane phase was carefully removed, dried down thoroughly under nitrogen and then taken up into 100μl of hexane. 1μl aliquots were injected into the GC. The peak areas obtained with the two methods of methylation were compared after normalising for the size of the nonadecanoic acid methyl ester peak. The data is shown in table 7.4.

Table 7.4. Comparison of sodium methoxide and methanolic HCl methods of transmethylation of fatty acids (mean of three samples for each data parameter)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Methanolic HCl (MeHCl)</th>
<th>Sodium methoxide (NaOMe)</th>
<th>Ratio (MeHCl / NaOMe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>267428746</td>
<td>256385598</td>
<td>1.04</td>
</tr>
<tr>
<td>16:1</td>
<td>357603129</td>
<td>334367988</td>
<td>1.07</td>
</tr>
<tr>
<td>16:0</td>
<td>417919180</td>
<td>379971523</td>
<td>1.10</td>
</tr>
<tr>
<td>18:2</td>
<td>321831161</td>
<td>324848726</td>
<td>0.99</td>
</tr>
<tr>
<td>18:1</td>
<td>477658656</td>
<td>476646486</td>
<td>0.99</td>
</tr>
<tr>
<td>18:0</td>
<td>474187376</td>
<td>442690556</td>
<td>1.07</td>
</tr>
<tr>
<td>19:0</td>
<td>306847178</td>
<td>306847178</td>
<td>1.00 *IS</td>
</tr>
<tr>
<td>20:4</td>
<td>318888539</td>
<td>342381329</td>
<td>0.93</td>
</tr>
<tr>
<td>20:1</td>
<td>472426326</td>
<td>456830054</td>
<td>1.03</td>
</tr>
<tr>
<td>20:0</td>
<td>459008405</td>
<td>444770913</td>
<td>1.03</td>
</tr>
<tr>
<td>22:6</td>
<td>238307758</td>
<td>283170916</td>
<td>0.85</td>
</tr>
<tr>
<td>22:4</td>
<td>252691234</td>
<td>247173053</td>
<td>1.02</td>
</tr>
<tr>
<td>22:0</td>
<td>489959197</td>
<td>483390817</td>
<td>1.01</td>
</tr>
</tbody>
</table>
This data demonstrates that the two methods of methylation gave very similar results, although there was a tendency for lower yields of the methyl esters of the polyunsaturated fatty acids arachidonic (20:4) and docosahexaenoic (22:6) acid with the methanolic HCl method. The significance of this difference is unclear, particularly as the coefficients of variation for the measurement of these more unsaturated fatty acids are greater (vide infra). The methanolic HCl methodology produced consistently "cleaner" chromatography and this method was therefore adopted routinely in preference to sodium methoxide.

7.2.5 Linearity of the method
This was assessed by methylating different amounts of the calibration mixture (10 - 300 µl aliquots) each sample containing 30 µl of C19:0 internal standard. Peak areas were plotted against nmol of fatty acid after standardising for the internal standard peak area. All fatty acids in the calibration mixture exhibited a linear calibration curve in the range 0.8 - 12.5 ng of fatty acid in the initial sample. The calibration curves are illustrated in figures 7,4 - 7,14.

7.2.6. Coefficients of variation
These were estimated by methylating five 120µl aliquots of calibration mixture and measuring the fatty acid composition of these consecutively. Results were adjusted to standardise for the peak area of the nonadecanoic acid internal standard. The coefficient of variation for each of the fatty acids was determined by dividing the standard deviation of the peak area by the mean peak area. The data is shown in table 7.5.

It is evident from the standard curves and from the coefficients of variation shown in table 7.5 that quantitative analysis of the polyunsaturated fatty acids is, in general, more variable than that of the saturated fatty acids. The reasons for this include the observations that a saturated fatty acid is not necessarily the best internal standard for the measurement of unsaturated fatty acids, that EI detection of methyl esters is not the method of detection of choice for polyunsaturated fatty acids, and that the fatty acids elute from the column over a 15 minute period (8-23 minutes) whilst the internal standard elutes at 17 minutes. These matters have been discussed previously and will not be expanded on further here.
Table 7.5. Peak area and coefficient of variation for 5 sequential measurements performed on the calibration mixture.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mean peak area $[\times 10^9]$ (n=5)</th>
<th>Standard deviation</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.89</td>
<td>0.08</td>
<td>4.4%</td>
</tr>
<tr>
<td>16:1</td>
<td>2.56</td>
<td>0.04</td>
<td>1.6%</td>
</tr>
<tr>
<td>16:0</td>
<td>2.96</td>
<td>0.10</td>
<td>3.4%</td>
</tr>
<tr>
<td>18:2</td>
<td>2.27</td>
<td>0.11</td>
<td>4.9%</td>
</tr>
<tr>
<td>18:1</td>
<td>3.43</td>
<td>0.05</td>
<td>1.5%</td>
</tr>
<tr>
<td>18:0</td>
<td>3.42</td>
<td>0.04</td>
<td>1.2%</td>
</tr>
<tr>
<td>20:4</td>
<td>2.23</td>
<td>0.18</td>
<td>8.2%</td>
</tr>
<tr>
<td>20:0</td>
<td>3.37</td>
<td>0.07</td>
<td>2.1%</td>
</tr>
<tr>
<td>22:6</td>
<td>1.76</td>
<td>0.16</td>
<td>9.1%</td>
</tr>
<tr>
<td>22:4</td>
<td>1.64</td>
<td>0.21</td>
<td>12.8%</td>
</tr>
<tr>
<td>22:0</td>
<td>3.51</td>
<td>0.07</td>
<td>2.0%</td>
</tr>
</tbody>
</table>
7.2.5.1 Calibration curves for individual fatty acids

Figure 7.4 C14:0 Myristic acid

Figure 7.5 C16:1 Palmitoleic acid

Figure 7.6 C16:0 Palmitic acid

Figure 7.7 C18:2 Linoleic acid

Figure 7.8 C18:1 Oleic acid

Figure 7.9 C18:0 Stearic acid
Figure 7.10 20:4 Arachidonic acid

Figure 7.11 C20:0 Behenic acid

Figure 7.12 C22:6 Docosahexaenoic acid

Figure 7.13 C22:4 Docosatetraenoic acid

Figure 7.14 C22:0 Behenic acid
7.2.7 Choice of temperature ramp for optimal chromatography

The very long chain polyunsaturated fatty acids, which were of particular interest, elute off the column late and are found in the latter half of the chromatogram. It was striking in the early stages of method development that these peaks were smaller and broader than the peaks of the shorter chain fatty acids eluting at an earlier stage (although all were present in approximately similar amounts in the injectate). There were two reasons for this. Firstly the temperature ramp was not steep enough and secondly the longer chain fatty acids were not getting onto the column as efficiently as the shorter fatty acids. These effects are illustrated in the chromatogram in figure 7.15 in which a mixture of equal quantities (0.5µg / ml) of saturated and unsaturated fatty acids (C16 - C22) were derivatised and injected in splitless mode onto the GC.

![Figure 7.15 Tailing of peaks of longer chain fatty acids due to inefficient deposition on the column](image)

The shortening and widening of peaks which eluted off the column later was demonstrated by injecting an isomolar mixture of C19 - C26 alkanes (which do not need derivatisation) under isothermic conditions. [Injector temperature 250°C, oven temperatures; 50°C for 2 minutes, ramp of 50°C min to 200°C, hold at 200°C until end of run; purge valve open at 2 mins.] The peaks became smaller and wider with
time as is illustrated by the integration data for the peaks shown in table 7.6.

Table 7.6. Fall off in peak area with longer chain alkanes injected in splitless mode under isothermic conditions

<table>
<thead>
<tr>
<th>Identity</th>
<th>Retention time</th>
<th>Peak width</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td>C19</td>
<td>10.35</td>
<td>0.094</td>
<td>6.48 x 10^6</td>
</tr>
<tr>
<td>C20</td>
<td>12.25</td>
<td>0.087</td>
<td>5.93 x 10^6</td>
</tr>
<tr>
<td>C21</td>
<td>14.91</td>
<td>0.108</td>
<td>4.75 x 10^6</td>
</tr>
<tr>
<td>C22</td>
<td>18.65</td>
<td>0.126</td>
<td>3.76 x 10^6</td>
</tr>
<tr>
<td>C23</td>
<td>23.94</td>
<td>0.176</td>
<td>3.29 x 10^6</td>
</tr>
</tbody>
</table>

The introduction of a temperature ramp during the run improved the chromatography significantly (in that the later eluting peaks were no longer wider) but did not normalise the areas of these later eluting peaks. This suggested that there may have been a problem with getting the longer chain alkanes onto the column (ie losses in the injector). To investigate this matter further I chose, in the first instance, to work up the FAME chromatography methodology using the technique of "on-column" injection. Injecting 1μl of the alkane mixture directly on column under isothermic conditions [initial temp 40°C, ramp at 2 minutes to 200°C at 50°C min⁻¹ and hold at 200°C] again produced widening and broadening of later eluting peaks. This was abolished by incorporating a continuing ramp throughout the run time [initial temp 40°C, ramp at 50°C min⁻¹ to 175°C, then 2.5°C to 250°C]. The peak heights were now comparable for all the alkanes (C19 - C26) in the mixture. A methylated fatty acid calibration mixture was run under the same conditions and demonstrated a preservation of peak height and area in later eluting fatty acid methyl esters indicating that good chromatography was possible if the FAME were deposited on the column. "On column" chromatograms of the calibration mixture and a total lipid extract of the apical brush border membrane are shown in figures 7.16 and 7.17.
Figure 7.16 "On column" chromatogram of a mixture of saturated and unsaturated FAME

Figure 7.17 "On column" chromatogram of a total lipid extract of a brush border membrane fraction
Having demonstrated that adequate preservation of sensitivity with the longer chain polyunsaturated FAME was possible with a direct on column injection, attention was turned to improving the efficiency of getting the longer chain FAME from the injector port onto the column in splitless mode. This was achieved with the use of glass wool within the liner which serves to distribute the temperature more evenly within the injection chamber allowing the longer chain FAME to get onto the column before precipitating. Judicious modification of the temperature ramp was also undertaken and the final ramp is as described in section 7.2.2. A representative total ion chromatogram of a total lipid extract from a brush border membrane preparation is shown below in figure 7,18.

![Figure 7,18 Total ion chromatogram of a total lipid extract from a brush border membrane preparation injected in splitless mode.](image)

A chromatogram for a mixture of FAME standards run under similar conditions has already been shown in figure 7,1.
7.3 Conclusions
This chapter has outlined the development and validation of a simple method for the measurement of the fatty acid composition of biological samples by gas chromatography - mass spectrometry and has eluded to a number of problems with this methodology which are particularly evident with the long chain polyunsaturated fatty acids. In many respects these shortcomings are in the areas of most interest, as the depletion of PUFA in a biological sample when exposed to an oxidising stress is a recognised method of monitoring lipid peroxidation\(^24\). This information could be obtained with other methods of derivatisation and detection (see section 7.1).

Determining the total fatty acid composition of a subcellular membrane by the methods described above only provides a limited amount of information about the structure of that membrane. For example, the major structural backbone of the cell membrane is provided by phospholipids and this methodology does not provide any information about either the amounts of each phospholipid class present or about the fatty acid composition of each of these classes.
CHAPTER 8

Steady state fluorescence polography as a tool to measure membrane fluidity.

8.1 The structure of biological membranes
The mammalian plasma membrane is composed of a lipid bilayer approximately 5 nm thick comprising phospholipids, glycolipids and sterols with protein molecules either partially embedded in or completely traversing the lipid matrix (Singer and Nicholson, 1972)\(^{27}\). All three of the major classes of membrane lipid are amphipathic, with a hydrophillic polar end and a hydrophobic nonpolar end.

8.1.1 Phospholipids
Phospholipids are the major class of lipid in mammalian plasma membranes and form the backbone of the membrane comprising a central hydrophobic core of fatty acyl chains surrounded on each surface by hydrated polar head groups abutting onto the aqueous environments of the cell interior and exterior. For example, with phosphatidylcholine the choline, phosphate and glycerol moieties will form the polar head group and the two fatty acyl chains the non-polar tail (Figure 8.1).

Figure 8.1 Amphipathic structure of phosphatidylcholine
Biological membranes are dynamic structures$^{228}$ and the variety and proportions of saturated and unsaturated lipids and sterols within the membrane will have an influence on the physical state or "fluidity" (vide infra) of the membrane$^{229}$. Biological membranes are therefore far more complex structures than the simple scenario of an artificial bilayer of a single phospholipid type (e.g., dipalmitoyl phosphatidylcholine - DPPC) suspended in an aqueous phase where the acyl chains may pack tightly together to form a "rigid" crystal-like structure (vide infra). Phospholipids commonly possess one unsaturated and one saturated fatty acyl chain. The presence of cis-double bonds in an unsaturated fatty acid will produce a kink in this part of the lipid tail$^{230}$ which will alter the ability of the lipids to pack together in the bilayer and consequently modulate the physical characteristics of the lipid bilayer.

The physical state of phospholipids in a synthetic bilayer is also dependent upon temperature. On warming phospholipids reach an endothermic transition temperature at which a change of state occurs from "crystalline" (or gel) state to the "liquid crystalline" state, which is associated with increased conformational freedom of the fatty acid chains. The transition temperature, $T_t$, increases with increasing length of the fatty acyl chains and decreases with increasing unsaturation of the acyl chains. $T_t$ also depends upon the chemical nature of the polar lipid head group$^{231}$. Hence, mixtures of phospholipids in an artificial bilayer with varying degrees of saturation (and therefore different phase transition temperatures) exhibit phase separations where phospholipids in different phases tend to aggregate together. This is clearly an oversimplification of the situation in biological membranes both because of the bonding of saturated and unsaturated fatty acids to the same phospholipid molecule and because of the presence of sterols and proteins within the membrane which will also influence fluidity. Nevertheless distinct "domains" which are more or less "fluid" do exist in biological membranes$^{232}$.

### 8.1.2 Asymmetry of lipid composition between membrane leaflets

Mammalian plasma membranes contain four major phospholipids - phosphatidylcholine, sphingomyelin, phosphatidylserine, and phosphatidylethanolamine - which commonly account for approximately 85% of membrane phospholipid. The distribution of these phospholipid classes is asymmetric between the two halves
of the bilayer in contrast to cholesterol which is usually equally distributed between the two. The outer leaflet contains most of the phosphatidylcholine and sphingomyelin and is enriched in saturated fatty acids when compared with the inner leaflet in which most of the phosphatidylethanolamine and phosphatidylserine are found. This asymmetry gives rise to a difference in fluidity between the two halves of the lipid bilayer. This asymmetry in structure is generated by phospholipid translocators in the endoplasmic reticulum when synthesising the plasma membrane.

Phosphatidylserine (PS) carries a net negative charge on its polar head group whereas the other principal membrane phospholipids have an equal number of negative and positive charges. It is interesting therefore that PS is concentrated in the inner half of the membrane imparting a significant difference in charge between the two halves of the bilayer. This is of functional importance, as may be illustrated by the activation of protein kinase C which is dependent upon the presence of negatively charged phosphatidylserine in the cytoplasmic face of the plasma membrane to increase the enzymes affinity for Ca$^{2+}$ and hence facilitate its action. Phosphatidyl inositol is also predominantly localised in the inner leaflet of the cell membrane where it has an important function in intracellular signalling. Glycolipids contribute further to the asymmetry and are found exclusively in the outer leaflet with their sugar groups exposed at the cell surface. These molecules have two fatty acyl chains linked to a serine molecule (as opposed to a glycerol molecule typical of the major phospholipids) to which one or more carbohydrate residues are linked so completing the polar head group.

8.1.3 Movements of hydrocarbon chains within a membrane

The bulk of the hydrocarbon chains of the membrane lipids are in a fluid state under physiological conditions in which there is considerable flexion and rotation of the C-C bonds and lateral diffusion within the bilayer (Figure 8.2). For example in erythrocytes the lateral diffusion coefficient of a phospholipid is such that it might move as far as 2μM in one second. Flip-flop movements of lipids between the two halves of the bilayer are, however, infrequent as is testified by the asymmetric distribution of lipids across the cell membrane. The fluidity of the membrane is essential for normal functions of the membrane to occur - for example biogenesis, trafficking, exocytosis and endocytosis. This will be discussed later.
8.1.4 Sterols

Cholesterol (which is mostly nonesterified in biological membranes) is a major determinant of membrane fluidity. The -OH group on carbon 3 forms the polar head group and the hydrocarbon chain arising from carbon 17 (carbons 20-27) forms the hydrophobic tail (Figure 8.3).
The steroid rings act as a rigid planar structure between the hydrophilic hydroxyl head group and the hydrophobic hydrocarbon tail. The rigid steroid rings may therefore immobilise those regions of the fatty acyl chains closest to the polar head group resulting in a stiffening of this region of the membrane. Whilst this would tend to make the membrane less fluid, the high concentrations of cholesterol found in mammalian cell membranes will tend to keep adjacent acyl side chains apart and hence stop them "crystallising". Hence cholesterol has a considerable influence on membrane fluidity.

8.1.5 Membrane proteins

The proteins of biological membranes vary considerably in abundance in different membranes (for example protein: lipid ratios of ~ 3.6 are found in the protein rich inner mitochondrial membrane but of only ~ 0.25 in the myelin membrane of peripheral nerve). In addition to their roles as specific functional molecules, their presence has an influence on the physical state of the membrane.

Figure 8.4 Classes of membrane proteins

Five categories of membrane proteins exist when classified by their method of anchoring in attachment to the cell membrane and by the number of transmembrane domains (Figure 8.4). Class I proteins are peripheral or extrinsic
proteins which are bound mainly by ionic forces to the polar head groups of phospholipids or to other proteins. Class II proteins are anchored into a part of the lipid bilayer by a hydrophobic peptide, for example subunits of the nucleotide binding G-protein family. Class III and IV proteins are integral (transmembrane) proteins in which one (class III) or more (class IV) hydrophobic peptide domains span the lipid bilayer. Some class IV proteins function as transmembrane channels and ion pumps, for example the sodium coupled glucose and amino acid transporters (SGLT1 and SLAAT1) and the cystic fibrosis transmembrane regulated (CFTR) chloride channel. Transmembrane proteins have a defined orientation in the membrane and may as a consequence display functional asymmetry. For example the K⁺ and ouabain-binding sites of Na⁺-K⁺-ATPase are located on the extracellular surface while the Na⁺ and ATP hydrolysing sites are on the cytoplasmic surface. Class V proteins are peripheral membrane proteins which are attached to the lipid bilayer by a covalently attached glycolipid (for example alkaline phosphatase, 5'-nucleotidase, and many aminopeptidases) or by a thioester bond between the protein and membrane lipid (for example palmitic acid is attached to a cysteine residue of the transferrin receptor).

Proteins are, in general, able to diffuse laterally in the plane of the membrane, with rates 100 - 100,000 times slower than that of lipids. These lateral movements are influenced by the cell cytoskeleton, the cell surface glycocalyx and, in polarised epithelia such as the intestine, limited by the presence of tight junctions between cells which separate the plasma membrane into distinct apical and basolateral domains. The complex "rigid" structure of tight junctions limit the movements of proteins between these domains but may permit the lateral movement of phospholipid molecules in the cytoplasmic leaflet of the lipid bilayer. The apical and basolateral membrane domains have, as a consequence, distinct differences in membrane structure and physical characteristics.

8.2 Measurement of membrane fluidity
There are a variety of methods available to study the biophysical characteristics of biological membranes. The theory and practice of fluorescence polarisation techniques will be discussed in detail in this chapter. Other methods which may be used to study membrane "fluidity" include electron spin resonance, nuclear magnetic resonance, differential scanning calorimetry, freeze fracture electron
microscopy\textsuperscript{240}, infrared and Raman spectroscopy\textsuperscript{241} and X ray diffraction\textsuperscript{242}. Electron spin resonance (ESR) spectroscopy has utilised spin label analogues of phospholipids, cholesterol and other membrane constituents (coupled to a nitroxide radical\textsuperscript{243} or containing specifically synthesised paramagnetic groups\textsuperscript{244}) to study the local viscosity of membranes. This technique relies upon the tendency of a magnetic dipole arising from the spin of an unpaired electron to align either parallel or antiparallel (a high energy state) to an applied magnetic field. Transitions between these two spin states are induced by applying a second oscillating magnetic field at a frequency which induces a state of spin resonance. The population in the more stable parallel orientation will exceed those in the less stable high energy antiparallel orientation and hence there will be a net absorption of energy from the oscillating field. This absorption is the source of the electron spin resonance spectrum\textsuperscript{245}. Nuclear magnetic resonance (NMR) techniques of studying the motions of molecules within the membrane have the advantage that they do not require the introduction of highly modified molecules into the membrane which might themselves perturb or distort the normal structure of the membrane. These techniques have been used with some success in the study of phospholipid and membrane structure\textsuperscript{246}. By analogy with ESR techniques the method relies on the application of two oscillating magnetic fields to study energy shifts in nuclei with non zero magnetic moments. The disadvantage of NMR spectroscopy is the need for "NMR probes" which are enriched in $^{13}$C or $^2$H, and also that the interpretation of $^{13}$C or $^2$H relaxation times in terms of molecular dynamics is complex and sometimes controversial.

8.2.1 What is membrane fluidity

The notion that the cell membrane is a dynamic "fluid" structure in which lipids and proteins move about, albeit in a constrained manner, is neither novel or contentious. Membrane "fluidity" is however an imprecise term which may have different meanings in different contexts. In the following discussion fluidity will be considered to relate to the molecular behaviour of lipid molecules within the membrane. If one envisages the motions of a molecule which is representative of a lipid within that membrane then its motions are a result of its interaction with the other molecules of the membrane.
Two sets of parameters are normally considered to describe these motions; (i) structural, thermodynamic parameters which define the average orientation of the molecule relative to the local membrane surface. These are commonly referred to as "order" parameters. (ii) dynamic parameters defining the rates of reorientational motions of the molecule.

Before these arguments can be developed it should be emphasised that; (a) lipids do not intermix well to form homogenous layers and hence membranes are composed of numerous coexistent microenvironments or "domains", and (b) dynamic (reorientational) processes within a membrane can only be studied accurately with techniques whose intrinsic timescale is comparable to the timescale of the process itself\(^{247}\). In membrane systems the rotational correlation time (\(\tau_c\) - vide infra) is likely to be of the order of \(10^{-8}\) seconds. Analytical methods which operate within these timescales include \(^2\)H - NMR and ESR spectroscopy, and time resolved fluorescence anisotropy. These will not be discussed further here as the techniques were not used in the current studies.

### 8.3 Theoretical aspects of fluorescence polarisation experiments

Excitation of a fluophore in solution with polarised light will result in the emission of light which is also polarised. This polarisation results from photoselection of fluophores according to their orientation relative to the direction of the polarised excitation. Since the emission of a photon by the excited fluophore requires a much longer time than does absorption, the fluophore can often reorientate before emission occurs. If such a situation occurs (i.e. the rotational correlation time [vide infra] of the excited molecule is less than or of the order of the excited state lifetime), the emitted photon will no longer be polarised parallel to the exciting photon (assuming that the molecular excitation and emission dipoles of the fluophore are parallel). Rotation of the fluophore during the lifetime of the excited state will therefore result in a depolarisation of the emitted light. Hence polarisation (or anisotropy) experiments can provide a measure of the average angular displacement of a fluophore which occurs between the absorption and subsequent emission of a photon.

The measurement of fluorescence polarisation is illustrated in figure 8.5. The
\[ I_F = I_V + 2I_H \]

*Figure 8.5* Fluorescence of a cylindrically symmetric fluorophore whose excitation and emission dipoles are parallel to the symmetry axis.

Fluorophore (incorporated into the lipid phase of the membrane) is excited with polarised light (\( I_E \) - incident along the Y axis and polarised parallel to the Z axis). The intensity of emission is measured at right angles to the incident light (along the X axis) through a polariser. When the observing polariser is orientated parallel to the Z axis (the plane of the incident exciting light) the observed emission intensity is termed \( I_V \), and when the polariser is perpendicular to the excitation the emission intensity is termed \( I_H \).

Consider a rod shaped fluorophore rigidly orientated parallel to the Z axis (position (a)). If one assumes that the excitation and emission dipoles of the fluorophore are parallel to the longitudinal symmetry axis of the fluorophore, \( I_H \) will equal zero and the total intensity of emitted light will be \( I_V \). If, however, the fluorophore is symmetrically orientated about the Z axis (as in the so called "wobbling cone" model) then the emitted light will also be symmetrically orientated about the Z axis. In this instance the total intensity of emitted light, \( I_F \), will equal \( (I_V + 2I_H)^{248} \).
8.3.1 Anisotropy

By definition an anisotropic substance does NOT have the same physical properties in all directions. If one therefore defines anisotropy as used in the fluorescence polarisation studies as the ratio of the polarised component of the emitted light to the total intensity of emitted light, then:

\[
\text{Steady state fluorescence anisotropy } (r) = \frac{I_{V} - I_{H}}{I_{V} + 2I_{H}} \quad \ldots \ldots (1)
\]

It can be demonstrated that, for a fluophore with parallel excitation and emission dipoles that when the fluophore is orientated with an angle \( \beta \) to the Z axis (see figure 8.5) then:

\[
I_{V} = \cos^{2} \beta \quad \ldots \ldots (2)
\]

and

\[
I_{H} = \frac{1}{2} \sin^{2} \beta \quad \ldots \ldots (3)
\]

If one now assumes that a collection of fluophores are being excited which are randomly distributed relative to the Z axis with a probability \( f(\beta) \), then:

\[
I_{V} = \int_{0}^{\pi/2} f(\beta) \cos^{2} \beta \, d\beta = \frac{\cos^{2} \beta}{\cos^{2} \beta} \quad \ldots \ldots (4)
\]

and

\[
I_{H} = \int_{0}^{\pi/2} f(\beta) \sin^{2} \beta \, d\beta = \frac{1}{2} \sin^{2} \beta \quad \ldots \ldots (5)
\]

where \( f(\beta) \, d\beta \) is the probability that a fluophore is orientated between \( \beta \) and \( (\beta + d\beta) \).

If equations (4) and (5) are substituted into equation (1) then;

\[
r = \frac{3}{2} \cos^{2} \beta - 1 \quad \ldots \ldots (6)
\]

From this argument, anisotropy is a function of the average angle \( \langle \beta \rangle \) of the emission dipole relative to the Z axis, and higher values of \( r \) are associated with smaller values of \( \beta \).
This consideration of anisotropy is oversimplistic for three reasons:

(1) Excitation and emission dipoles are seldom co-linear, though this is nearly the case with diphenylhexatriene (vide infra)

(2) The argument takes no account of photoselection of fluophores (although in the equation (not shown) taking this into account \( r \) remains a function of \( \beta \) as in equation (6) above).

(3) The orientational function \( f(\beta) \) is better described as an infinite series of goniometric functions of the angle \( \beta \) rather than a normal distribution. The coefficients of the expansion are known as "order parameters" and are denoted \( \langle P_L \rangle \) \(^{248} \) where \( L \) is an integer.

For example:

\[
\langle P_2 \rangle = \frac{(3 \cos^2 \beta) - 1}{2} \\
\text{and} \\
\langle P_4 \rangle = \frac{(35 \cos^4 \beta - 30 \cos^2 \beta) + 3}{8}
\]

It is worth noting from equation (7) that the order parameter \( \langle P_2 \rangle \) is identical with the derived expression of steady state fluorescence anisotropy in equation (6), and indeed this is the only parameter which can be measured using steady state fluorescence techniques. This order parameter is sometimes referred to as the order parameter \( S \) or else \( r_0 \). The latter term \( r_0 \) will be used hereafter.

### 8.3.2 Maximal limiting anisotropy \( (r_0) \)

The excitation and emission dipoles of a fluophore, as eluded to above, are seldom parallel. Maximal limiting anisotropy, or \( r_0 \), is a term used to refer to the anisotropy observed in the absence of other depolarising influences, such as rotational diffusion or energy transfer. In vitrified solutions, such as propylene glycol at -70°C, fluophores remain immobile during the lifetime of the excited state. Under these conditions the measured anisotropy values \( (r_0) \) provide a measure of the angle between the absorption and emission dipoles. The orientation of the absorption dipole will differ for each absorption band and so the angle between absorption and emission dipoles \( (\alpha) \) will vary with wavelength and hence \( r_0 \) will vary with excitation wavelength. The maximum theoretical value for \( r_0 \) is 0.4\(^{248} \) which
occurs when $\alpha=0$. With diphenylhexatriene [DPH] (vide infra) measured $r_0$ has been reported to be as high as 0.39, implying a value for $\alpha$ of 7.4° although lower values of $r_o$ are reported\textsuperscript{260}.

8.3.3 Limiting hindered anisotropy ($r_\infty$)

In time resolved experiments, the fluorescence anisotropy ($r_\ell$) of the fluorescent probe DPH in a lipid bilayer does not decay to zero after a flash excitation but reaches a constant non-zero value ($r_\infty$ - limiting hindered anisotropy), indicating that the rotational diffusion of the probe is hindered and that the equilibrium orientational distribution function is anisotropic. The decay of $r_\ell$ from its initial value, $r_0$, to its final value, $r_\infty$, could be adequately described by a single exponential decay characterised by a rotational correlation time $\tau_c$\textsuperscript{251}.

Hence;

$$I_\ell(t) = r_\infty + (r_0 - r_\infty) e^{-\frac{t}{\tau_c}} \quad \ldots \ldots (9)$$

Where $\tau_c$ is defined as $\tau_c = (6R)^{-1}$, and $R$ is the rotational rate in radians sec$^{-1}$.

The decay of the vertical and horizontal components of fluorescence intensity may be described by the double exponential functions.

$$I_{V(t)} = \frac{1}{3} e^{-\frac{t}{\tau_F}} + \frac{2}{3} r_0 e^{-\frac{1}{\tau_F} + 6R \frac{t}{\tau_F}} \quad \ldots \ldots (10)$$

$$I_{H(t)} = \frac{1}{3} e^{-\frac{t}{\tau_F}} - \frac{1}{3} r_0 e^{-\frac{1}{\tau_F} + 6R \frac{t}{\tau_F}} \quad \ldots \ldots (11)$$

where;

$r_0 = \text{anisotropy in absence of any rotation (maximal limiting anisotropy)}$

$\tau_F = \text{fluorescence lifetime of the probe}$

Steady state anisotropy measurements are the integration of the time response functions over time from $t_0 \rightarrow t_\infty$.

Therefore in steady state:

By integrating equations (10) and (11) as in (12) and (13) and by defining $r_s$ as in (1)
\[ I_V = \left( \frac{1}{\tau_F} \right) \int_0^\infty I_V(t) \, dt \quad \ldots (12) \]

\[ I_H = \left( \frac{1}{\tau_F} \right) \int_0^\infty I_H(t) \, dt \quad \ldots (13) \]

\[ \tau_* = r_s - r_0 \frac{r_s - r_0}{6R\tau_F} \quad \ldots (14) \]

by defining rotational correlation time, \( \tau_c = \frac{1}{6R} \)

then

\[ r_s = r_0 - r_* \left[ \frac{\tau_c}{\tau_c + \tau_F} \right] + r_* \quad \ldots (15) \]

This is a modified form of the Perrin equation\textsuperscript{252}.

8.3.4 Relationship between steady state fluorescence anisotropy, membrane order and rotational correlation time

On further consideration of equation (15) it will be obvious that when \( \tau_c \ll \tau_F \), then \( r_s \) will approximate to \( r_* \). However, when \( \tau_F \ll \tau_c \), then \( r_s \) will approximate to \( r_0 \) because probe motion is effectively frozen on the scale of fluorescence lifetime.

Use has been made of the different properties of different fluophore probe molecules to extrapolate the information obtained from the relatively simple measurement of steady state fluorescence anisotropy by applying the above equation.

For instance, diphenylhexatriene (DPH) in natural membranes exhibits high values for \( r_* \), which largely determine \( r_s \).\textsuperscript{253} In contrast, 12, anthroyloxy stearic acid (12-AS) and other anthroyloxy probes exhibit low values of \( r_* \) in bilayer membranes,\textsuperscript{254} and in this instance \( r_s \) is largely determined by \( \tau_c \). By these means inferences can be made about both "static" [i.e. acyl chain order] \( r_* \) and "dynamic" \( \tau_c \) components of membrane "fluidity". Precise measurement of these parameters does, however, require the use of time resolved fluorescence\textsuperscript{255,256} or other methods.
Early studies of membrane fluidity\textsuperscript{257,258} using steady state fluorescence methods made use of the notion that in theory, (with a spherical probe), the rotational correlation time could be related to viscosity by the equation;

\[ \tau_c = \frac{\eta V}{RT} \quad \ldots \quad (16) \]

where;
\( \eta = \text{sample viscosity} \)
\( V = \text{effective rotational molar volume} \)
\( R = \text{gas constant} \)
\( T = \text{absolute temperature} \)

There are a number of shortcomings with this methodology\textsuperscript{259}, not least that most probes are not spherical and that membranes are not homogenous.

### 8.4 Characteristics of specific fluorescent probes

#### 8.4.1 Diphenylhexatriene\textsuperscript{260}

Diphenylhexatriene (DPH) is a linear lipophilic fluophore (Fig 8.6). In common with other hydrophobic probes DPH is insoluble in water and so partitions into the hydrophobic interior of biological membranes and lipid bilayers. Most of the DPH molecules will orientate themselves parallel to the phospholipid acyl chains, but a minority will be found orientated approximately parallel to the membrane surface\textsuperscript{260}. Its depth within the bilayer is, however, not as well defined as with some other probes (for example TMA-DPH has a hydrophillic head group and is therefore likely to be found at the hydrophobic - hydrophillic interface of the lipid bilayer). The fluorescence characteristics of DPH will depend upon which solvent and lipid system they are measured in. In hexane the excitation maximum is 355nm and emission maximum 425nm\textsuperscript{261}. Quoted fluorescent lifetimes in biological membranes are of the order of 8 nsec\textsuperscript{262}. Quoted maximal limiting anisotropy (\( r_0 \)) values range from 0.365\textsuperscript{250} to 0.39\textsuperscript{263}. The latter implies that the

\[ \text{Figure 8.6} \]

Structure of diphenylhexatriene
absorption and emission dipoles for this molecule are roughly colinear (see above).
The introduction of DPH into a membrane suspension via an organic solvent is quick, and is associated with a rapid increase in fluorescence as the quantum yield of these probes in water is negligible\textsuperscript{264}. DPH partitions equally into all lateral domains of the membrane\textsuperscript{265}, a comparatively unique property amongst membrane probes (TMA-DPH for example exhibits a preference for fluid phase domains).

### 8.4.2 Anthroyloxy fatty acids

These probes contain an anthroyloxy group attached to a saturated fatty acid chain, and are named according to the number of carbon atoms separating the carboxyl group from the anthroyloxy-substituted carbon\textsuperscript{266}. The emission spectrum of this group of probes is dependent upon the viscosity of the non-polar hydrocarbon solvent in which the probe is dissolved. This behaviour has been suggested to be a means of estimating the fluidity gradient through a lipid bilayer using probes with the anthroyloxy group located at different positions along the fatty acid chain\textsuperscript{267}.

\[
\begin{align*}
H_3C(CH_2)_m & \quad \text{CH} \quad (CH_2)_nCOOH \\
& \quad \text{O} \\
& \quad \text{C}=\text{O}
\end{align*}
\]

**Figure 8.7 General structure of anthroyloxy fatty acid based fluorescent probe**

\(r_0\), the maximal limiting anisotropy, has been noted to vary from 0.087 with excitation at 316nm to 0.325 with excitation at 381 nm, implying at least two excitation bands with different orientations of their transition dipoles relative to the emission dipole. This clearly complicates the interpretation of anisotropy measurements.

These probes are not taken up into membranes as readily as DPH\textsuperscript{268}. The
incorporation of the anthroyloxy fatty acid group of probes into a lipid bilayer may also result in a substantial perturbation of bilayer structure\textsuperscript{269} which makes them unsuitable for a number of purposes including the measurement of phase transitions.

The mathematical model to describe the isotropic rotation of this group of probes will be more complex than the simple cylinder approach derived above which is applicable to DPH. The minimum appropriate description of isotopic rotation will involve two rotational diffusion coefficients (in plane and out of plane) and therefore at least three exponentials to describe the decay of fluorescence anisotropy\textsuperscript{270,271}. Vincent et al\textsuperscript{271} found that the out of plane rotation of the anthracene group was essentially unhindered ($r_o \approx 0$). Thus, out of plane rotation should provide a measure of fluidity gradients throughout a bilayer.

8.5 Protocols adopted and experimental details

The studies were performed using apical membrane brush border fractions (BBM) which were isolated on a Percoll\textsuperscript{TM} gradient as described in chapter 6.

8.5.1 Diphenylhexatriene

A 2mM stock solution of DPH was made in tetrahydrofuran [THF] (46.5 mg / 100ml). This was stored in a -20°C freezer in the dark. A 5µM working solution was made up fresh for each assay by diluting the stock DPH solution 1:400 with phosphate buffered saline. This was kept in the dark at 4°C and was stirred for at least 2 hours before use to ensure adequate mixing of the THF.

A 200µl aliquot of brush border membranes (equivalent to approximately 150µg protein) was incubated in 2 ml of the 5µM DPH solution in the dark at 37°C for 1 hour. Control samples of DPH with no BBM and of BBM with no DPH were treated in an identical manner.

Fluorescence was measured in a 1cm light path cuvette preheated to 37°C in a Perkin Elmer LS3 fluorimeter. Excitation wavelength was 360nm (bandwidth 10nm) and emission wavelength 430nm (bandwidth 10nm). One polarising filter (Perkin Elmer) was placed between the exciting light source and the sample and a similar polarising filter between the sample and the detector. Four measurements were made with each sample (ie vertically [0°] and horizontally [90°] polarised excitation with either horizontal [0°] or vertical [90°] polarisation of the emission filter).
The endogenous fluorescence produced by a suspension of BBM in phosphate buffered saline (no DPH) was subtracted from the readings obtained for each of the four possible orientations of the filters. The fluorescence produced by DPH without BBM was negligible.

Steady state anisotropy was determined using the equation:

\[ I_s = \frac{\begin{pmatrix} 0 & 0 \\ 0 & 90 \end{pmatrix} \cdot \begin{pmatrix} 90 \\ 0 \end{pmatrix} - \begin{pmatrix} 90 & 0 \\ 0 & 90 \end{pmatrix}}{\begin{pmatrix} 0 & 0 \\ 0 & 90 \end{pmatrix} + 2 \begin{pmatrix} 90 & 0 \\ 0 & 90 \end{pmatrix}} \] ........ (17)

This has the same general form as equation (1). [The general syntax used in equation (17) is (excitation / emission)].

8.5.2 12 anthroyloxy stearic acid

2mM stock solution in THF was made and stored as with DPH. Membranes were loaded in a similar manner, but the incubation was continued for 2 hours as the probe is considerably less lipophilic than DPH. Care was taken throughout to minimise exposure to light as the probe is particularly susceptible to photobleaching. Excitation and emission wavelengths were 360nm and 471nm respectively.

8.5.3 Fluophore content of membranes

This was not routinely measured. Cogan and Schachter have described a method to estimate the fluophore content of membranes. This involves clarification of the sample in 1% SDS at 100°C for three minutes, and comparison of the fluorescence intensity with a set of standards. The interpretation, however, requires assumptions about the lipid content of the membrane fraction being studied. The approach used in these studies has been to standardise the protein concentration of the BBM suspension being used, and to use a comparatively long incubation time to load the membranes. This approach has been used by Schachter et al when working with BBM.

\[ \begin{pmatrix} 0 & 0 \\ 0 & 90 \end{pmatrix} \cdot \begin{pmatrix} 90 \\ 0 \end{pmatrix} - \begin{pmatrix} 90 & 0 \\ 0 & 90 \end{pmatrix} \]
8.6 General comments

This largely theoretical introduction to steady state fluorescence has aimed to provide some insight into how these measurements may be used to make inferences about membrane biophysical characteristics. It has been emphasised that the measurements do not provide precise information about molecular motions and that the interpretation of the data is dependent upon the fluorescence characteristics of the probes used and how these are altered by perturbations to the membrane. For example, lipid peroxidation may alter the fluorescence lifetime of DPH which may influence the results of these measurements (see chapter 11). Furthermore the measurements in my studies are averages which take no account of membrane microdomains. Lastly, perturbations in membrane structure may have opposing effects upon fluorescence anisotropy. For example vitamin E deficiency per se and lipid peroxidation have opposing effects upon fluorescence anisotropy measured with DPH (see chapter 11).

Hence, despite the rather precise mathematical derivations made in the chapter, caution is needed in interpreting fluorescence anisotropy data and overinterpretation should be avoided.
CHAPTER 9

Theory and validation of Ussing chamber techniques for the study of intestinal transport in vitro

9.1 Introduction

Intestinal absorption and secretion may be studied by a number of techniques which include \textit{in vivo} perfusion studies of isolated intestinal segments, and \textit{in vitro} studies of isolated intestinal sheets, isolated enterocytes and apical and basolateral enterocyte membrane vesicles. All have advantages and disadvantages and provide complimentary information. At an early stage in the present studies it was decided to use an in vitro method to study intestinal secretion and absorption in sheets of jejunum with or without prior stripping of the external (serosal) muscle layers. This technique has been used to study electrically overt movements of ions across actively transporting epithelia for over 50 years\textsuperscript{273}. It has also been used to measure fluxes of sodium and chloride across epithelia following the classic papers of Hans Ussing in the 1940's and 1950's\textsuperscript{274}. The pioneering studies of small intestinal secretion and absorption by Schultz\textsuperscript{275} and Field in the 1960's\textsuperscript{276} have given this technique a place in history as an extremely powerful tool to study the transport of solutes across the intestinal epithelium.

9.2 Design of the modified Ussing chambers

The Ussing chambers used in these studies consisted of two milled perspex half chambers between which the intestinal tissues could be mounted. These were built to my own specification after examining the chambers used in the Department of Biomedical Science at the University of Sheffield. The aperture across which the intestine was mounted was rectangular with rounded ends. The width of the aperture was 0.7cm and length 3cm, providing a cross sectional surface area of 2cm\textsuperscript{2}. There are sound theoretical reasons\textsuperscript{274} why this aperture should be as small as possible so as to minimise any potential difference between the centre of the tissue and the edges which might be produced by the passage of an electrical current across the tissue. In the early validation experiments smaller chambers were used\textsuperscript{277} with a circular cross section providing an exposed area of 0.64cm\textsuperscript{2}. In practice, however, results were more reproducible and tissue viability better
preserved with the larger aperture of the rectangular chambers. This may have been because the rectangular chambers did not require the tissues to be stretched on mounting. The rectangular chambers were used for all the experimental work described hereafter.

Each half chamber contained two small side ports through which salt agar bridges were passed to function as current passing and pd sensing electrodes. The pd sensing electrode tip was positioned 2 mm from the plane of the mounted tissue and the current passing electrode tip 5 cm away from the plane of the tissue. Two further bevelled side ports allowed the connection of each half chamber to an independent buffer circulating system. A schematic representation of the chambers is shown in figure 9,1.

![Schematic diagram of a cross section (coronal view) of an Ussing chamber](image)

**Figure 9,1** Schematic representation of a cross section (coronal view) of an Ussing chamber

Bathing solutions were circulated independently through each half chamber (vide infra) and transepithelial potential sensed and short circuiting current passed via salt agar bridges (vide infra).

When the perspex chambers were opposed the tips of the potential sensing agar bridges were approximately 3-4 mm apart to minimise the contribution of fluid
resistance to the *measured* transepithelial potential resistance when passing a current between the current passing agar bridge tips at the opposite ends of the chambers. In practice the contribution of fluid resistance was always taken into account (vide infra). This was particularly important with muscle stripped jejunal preparations because of the low tissue resistance of these preparations.

### 9.2.1 Composition of the bathing solutions

Krebs Ringer bicarbonate solution was made freshly each morning. The following constituents were made up to 998 mls with deionised water.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>7.8g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.37g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.1g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.3g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.17g</td>
</tr>
</tbody>
</table>

This solution was gassed vigorously with O₂ / CO₂ (95:5 v/v) for 1 hour and then 2mls of 1M CaCl₂ solution (BDH 19046) was added and mixed thoroughly. The pH of this gassed solution was 7.4. In the Ussing chambers the solution was maintained at 37°C by heated water jackets and oxygenated and circulated by an O₂ / CO₂ (95:5 v/v) gas lift system. When tissues were mounted glucose (10 mmol l⁻¹) was present in the serosal bathing solution and mannitol (10 mmol l⁻¹) in the mucosal bathing solution. The mucosal and serosal solutions were applied simultaneously (15 mls on each side) to minimise mechanical distortion of the tissue.

### 9.2.2 Manufacture of agar bridges

The transepithelial potential difference (VT) sensing and current passing agar-salt bridges comprised 3% agar made with 3 M KCl. The solution was heated near to boiling point until the agar became clear. A 5 metre length of prewarmed Portex tubing (Baird and Tatlock, catalogue no 275/1256/25) was filled with this solution using a 20ml syringe, and when cool the tubing was cut into 30cm lengths. These bridges were soaked in Ringer solution at 4°C for at least 1 week before use to allow "leachable" KCl to diffuse out of the bridges. Bridges were carefully checked for the presence of air bubbles or cracks in the agar before use and were changed regularly.
9.2.3 Manufacture of Ag / AgCl electrodes

Current passing electrodes were of the silver / silver chloride (Ag / AgCl) type. These are both reliable and simple to manufacture. 99.9% silver wire was cut to length and bent to a corkscrew shape around a pencil so as to fit neatly inside a universal container. This was cleaned with 2M HCl to remove any iron contamination which might have arisen during the cutting. Copper wire leads were soldered to the silver wire and araldite placed around the junction to avoid the copper lead coming into contact with electrolyte solution. The silver wire was cleaned with acetone to degrease the surface and so ensure even etching of the final chloride layer. The electrodes (several grouped together) were etched by placing them in 2 M HCl (used collectively as an anode), with a piece of platinum wire as a cathode. A current (density 20 mA cm\(^{-2}\)) was passed for 15 minutes with sonication every 3 minutes (to facilitate the etching process). After etching the electrodes adopted a dull grey appearance. Electrodes were then washed in dH\(_2\)O and chlorided by repeating the procedure in 0.1M HCl with a current density of 0.5 mA cm\(^{-2}\) again subjected to intermittent sonication as above. Electrodes were washed in dH\(_2\)O and kept in 3M KCl until use.

9.3 Electrical measurements

Spontaneous transepithelial electrical potential difference (Vt) was measured through the salt-agar bridges positioned close to the mucosal and serosal surfaces of the tissue via matched calomel electrodes (asymmetric potential ≤0.2 mV) immersed in 3 M KCl. Combined electrode / bridge junction potentials were always ≤0.3mV. Bridges were replaced if this tolerance was exceeded. Asymmetric potential (Vass) and fluid resistance (Rf) were measured in all experiments before mounting a tissue.

9.3.1 Manual methods

In the early experiments [the studies using unstripped jejunum in 12 month old animals - chapters 10 & 11], Vt was measured using a high impedance digital voltmeter (Analogic AN 2570). These tissues were not routinely short circuited as flux studies were not performed on this group of tissues. Intestinal short circuit current was calculated by estimating tissue resistance (Rt) [by passing a current pulse of 100μA, measuring the change in Vt and then applying Ohm's law] and
then calculating intestinal short circuit current \((I_{sc})\) by dividing open circuit \(V_t\) by \(R_t\) after taking into account the asymmetric potential and fluid resistance.

\[
\frac{V_{tc}}{R_t} = \frac{V_{ass}}{R_f}
\]

**Figure 9.2** Theory behind the open circuit method of estimating intestinal short circuit current

An understanding of this approach will be obtained by the following reasoning in conjunction with figure 9.2. Assume that on setting up the chambers in the absence of tissue the combined asymmetric potential of the calomel electrodes and any junction potentials at the tips of the agar bridges is measured as \(V_{ass}\), and that passing a 100\(\mu\)A pulse of current between the current passing electrodes increases this potential to \(V_{fc}\). On mounting a tissue the measured potential across the tissues is \(V_t\) which rises to \(V_{tc}\) on passing a current of 100\(\mu\)A across the tissue. By plotting the changes in measured potential in the manner shown in figure 9.2, one can obtain two lines the slopes of which (by Ohm's law) are a measure of fluid resistance \((R_f)\) and tissue resistance \((R_t)\) respectively. One may then use simple mathematics to predict intestinal short circuit current as follows;
Equation for a straight line is \( y = a + bx \)

Therefore

\[ y = \text{Vass} + (R_f \times \text{Isc}) \quad \text{and} \quad y = V_t + (R_t \times \text{Isc}) \]

Substituting for \( y \)

\[ V_t + (R_t \times \text{Isc}) = \text{Vass} + (R_f \times \text{Isc}) \]

Rearranging these

\[ \text{Isc} = \frac{V_t - \text{Vass}}{R_f - R_t} \]

Where

\[ R_f = \frac{V_{fc} - \text{Vass}}{0.1} \quad \text{and} \quad R_t = \frac{V_{tc} - V_t}{0.1} \]

In practice, the values Vass, Vfc, Vt and Vtc were noted at 30 second intervals throughout the experiment and subsequently entered into a spreadsheet (Smartware v1.02) which automatically calculated values for Isc and Rt.

Isc estimated in this manner was compared with that obtained by manually short circuiting the tissues and found to be similar.

Manual short circuiting\(^{279}\) was achieved by constructing a plot of current vs pd (I \( \times \) Rf) before mounting a tissue. Once the tissue was mounted, Isc was determined by changing the current passed until the pd measured was the same with the tissue in place as with the tissue absent. This value of I with the tissue in place (Isc) gave a value for pd equal to (-I \( \times \) Rf). This method of measuring Isc was both laborious and slow and was not well suited for measuring the rapid changes in Isc following the application of a secretogogue. The open circuit method of estimating Isc was therefore adopted.

9.3.2 Automatic voltage clamping

In later experiments using stripped tissues transmucosal pd was measured under open circuit conditions with a DVC 1000 automatic voltage clamp (World Precision Instruments Inc, New Hampshire, USA). The combined electrode / bridge junction potentials were manually offset with a rheostat and voltage source within the DVC 1000 circuitry, and corrections were automatically made for the resistance of the
fluid gap between the tips of the pd sensing electrodes and the tissue surfaces. This was achieved by passing 100µA pulses in the absence of tissue and adjusting a rheostat within the clamp until there was no resultant change in pd. The electrical resistance of the tissue was again determined under open circuit conditions by passing a current pulse of 100 µA, correcting the subsequent change in pd for fluid resistance and then applying Ohm's law.

9.4 Radioisotope fluxes

9.4.1 Outline of method
Transmucosal fluxes of Na and Cl were measured on paired tissues taken from adjacent segments of jejunum. Tissues were only paired if their electrical resistance differed by less than 25%. 15 minutes after mounting, 2.2 µCi of ^{22}Na and 2.2 µCi of ^{36}Cl (Amersham International Ltd) were added to the mucosal surface of one tissue and to the serosal surface of the paired tissue under short circuit conditions. After a further 15 minute equilibration period a 2 ml sample was removed from each unlabelled bathing solution and a 100 µl sample from each labelled solution. Samples removed from the unlabelled solutions were replaced with an equal volume of fresh unlabelled Ringer bicarbonate. Duplicate samples were taken 20 minutes later in order to determine baseline flux rates. Half of each sample was counted for ^{22}Na in an automatic well type gamma counter (LKB Wallace). 10ml of scintillant (Ria Luma) was added to the other half of each sample for determination of the combined β emissions of ^{22}Na and ^{36}Cl in a liquid scintillation counter (LKB Wallace). The activity of ^{22}Na assayed in the gamma counter multiplied by a factor determined by the relative efficiency of the two counters for ^{22}Na, was subtracted from the total beta counts to give the ^{36}Cl counts. Corrections were made for the dilution of the "unlabelled" solution after the serial removal of aliquots for counting and the replacement of these aliquots with fresh Ringer - bicarbonate.

9.4.2 Calculations
Unidirectional fluxes of Na and Cl were determined by dividing the steady state rates of radioisotope transfer by the specific radioactivities of the labelled solutions and by the surface area of the exposed tissue (2 cm²) as described by Schultz and
The formula used was as follows:

\[ J = \frac{v (p_2 - p_1)}{A \times t \times c} \]

where:
- \( J \) = unidirectional flux
- \( v \) = volume of bathing solution
- \( p_1 \) & \( p_2 \) = counts min\(^{-1}\) cm\(^2\) of isotope in "cold" reservoir at time t\(_2\) and t\(_1\) (after dilution factor taken into account).
- \( t \) = time between samples (ie t\(_2\) - t\(_1\)) in hours
- \( A \) = area of tissue exposed in chamber (2 cm\(^2\) in all cases)
- \( c \) = counts \( \mu \)mol\(^{-1}\) of Na or Cl in "hot" reservoir

"hot" denotes the reservoir into which the isotope was originally placed

The net fluxes of Na and Cl (\( J_{\text{net}} \)) were calculated as the difference between the flux from mucosa to serosa (\( J_{\text{ms}} \)) and from serosa to mucosa (\( J_{\text{sm}} \)). The mean Isc value of the tissues in each pair were summed and averaged to give a single value for the pair. This value was converted from \( \mu \)A to \( \mu \)mol hr\(^{-1}\) cm\(^{-2}\) by multiplying by a factor of 0.01865 (3.6 \times 10\(^{3}\)/A\( F \), where A is the surface area of exposed tissue and F the Faraday constant\(^6\)). Residual ion flux (\( J_{\text{R net}} \)) was calculated as \[ \text{Isc} - (J_{\text{Na net}} - J_{\text{Cl net}}) \].

9.4.3 Validation of steady state flux methodology and practical details of protocol

9.4.3.1 Dilution of radioisotopes;
3.5 mls of \(^{22}\)NaCl (activity 18 \( \mu \)Ci ml\(^{-1}\)) and 800\( \mu \)l of Na\(^{36}\)Cl (activity 78\( \mu \)Ci ml\(^{-1}\)) were added to 2.85 ml of fresh Ringer bicarbonate. 250\( \mu \)l of this solution contained 2.2\( \mu \)Ci of \(^{22}\)Na and 2.2\( \mu \)Ci of \(^{36}\)Cl, and this volume was added to the mucosal surface of one tissue and the serosal surface of the paired tissue.

\(^6\) 1 amp = 1 coulomb second

by using Faradays constant;
1 amp = 1.036 \times 10\(^{-5}\) moles / second of a univalent ion
1 amp = 0.0373 moles / hour
if surface area exposed is 2 cm\(^2\), then
1 amp = 0.0186 moles cm\(^{-2}\) hr\(^{-1}\)
9.4.3.2 Time to achieving steady state;
To determine the time to achieve steady state with regard to fluxes of $^{22}\text{Na}$ and $^{36}\text{Cl}$, 2.2µCi of each isotope was added to the mucosal side of a preparation and 2 ml aliquots of serosal solution removed at regular intervals (and replaced with an equal volume of non labelled Ringer) for counting of $^{22}\text{Na}$ and $^{36}\text{Cl}$. The volume of the mucosal and serosal solutions were 15 mls. Results are shown below for each isotope after correction for dilution.

![Graph showing time to steady state after addition of $^{22}\text{Na}$ to the mucosal reservoir (stripped jejunum).](image)

The linear portion of the graph was reached within 15 minutes of adding the isotope to the mucosal reservoir with both isotopes. The slope of each graph reflects the steady state mucosal to serosal flux of each isotope when account is taken of the volume of bathing solution, the cross sectional surface area of exposed tissue and the activity of Na and Cl isotopes expressed in terms of counts / µmol of Na or Cl present.

The Na and Cl counts in figures 9.3 and 9.4 have been corrected for the dilutional effects of adding unlabelled ringer solution to replace the aliquots removed for counting. A 15 minute equilibration period after the addition of radioisotopes to the
"hot" reservoir was therefore chosen and this period was adhered to in all experiments.

9.4.4 Radioisotope counting

9.4.4.1 Scintillation counting of β emissions
Scintillation counting of β emissions requires the addition of a liquid scintillant (containing a solvent and a scintillant or fluor). Energy from an emitted β particle is absorbed by the solvent molecules, causing them to become excited. The excited solvent transfers this energy to the fluor. When the excited fluor molecules return to the ground state they will release this energy as photons and heat. The photons can be detected by a phototube - photomultiplier system which converts the photons into amplified numbers of electrons which can be measured as a voltage pulse. β particles with different energies produce voltage pulses of different sizes. These are analysed with a pulse height analyser which can be adjusted to accept electrical pulses within a selected range of pulse heights by the use of upper and lower discriminators. Different β emitting isotopes have different energy spectra. The discriminator
settings used for β counting in the flux experiments were optimal for counting $^{36}\text{Cl}$. The energy spectrum for $^{22}\text{Na}$ β emissions also falls within this window. [Maximum energy $^{22}\text{Na}$ β$^-$ emission = 0.55 MeV; maximum energy of $^{36}\text{Cl}$ β$^-$ emission = 0.71 MeV]

### 9.4.4.2 Quenching of Isotopes on scintillation counting

Quenching is any process which causes a reduction in the amount of fluorescence produced by a β emission in a liquid scintillation cocktail or a reduction in the amount of light reaching the photomultiplier tube. Quenching will lead to a decrease in counting efficiency. It is usually caused by the presence of materials which interfere with the transfer of energy from solvent to fluor molecules so decreasing the amount of fluorescence, or else by the presence of coloured compounds in the sample which absorb the emitted photons. Quenching will therefore account for much of the difference between the number of disintegrations per minute (dpm) and the measured counts per minute (cpm). In the flux experiments the relationship between dpm and cpm is less important because the cpm produced by a known concentration of ion (cpm μmol$^{-1}$) is determined at the start of each experiment by counting a sample from the hot reservoir.

To standardise for any effect due to quenching on the scintillation counting of the β disintegrations of $^{22}\text{Na}$ and $^{36}\text{Cl}$ all 100μl aliquots taken from the "hot" reservoir were diluted to 2 mls (the volume of Ringer removed from the "cold" reservoir) by the addition of 1.9 mls of Ringer.

In practice, the effect of adding increasing volumes of Ringers solution (1-5 ml) to the counts measured on a 100μl aliquot of solution taken from the "hot" reservoir was small and is probably within experimental tolerance. This is illustrated in table 9.1.
Table 9.1. Effect increasing volumes of ringer solution on the measured counts per minute of 100µl of “hot” solution in 10 ml of scintillant fluid

<table>
<thead>
<tr>
<th>Volume of hot solution</th>
<th>Volume of Ringer added</th>
<th>Measured cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl</td>
<td>0 ml</td>
<td>18261</td>
</tr>
<tr>
<td>100 µl</td>
<td>1 ml</td>
<td>17738</td>
</tr>
<tr>
<td>100 µl</td>
<td>2 ml</td>
<td>17562</td>
</tr>
<tr>
<td>100 µl</td>
<td>3 ml</td>
<td>17791</td>
</tr>
<tr>
<td>100 µl</td>
<td>4 ml</td>
<td>17741</td>
</tr>
<tr>
<td>100 µl</td>
<td>5 ml</td>
<td>17929</td>
</tr>
</tbody>
</table>

9.4.4.3 γ emission spectra for $^{22}$Na and $^{36}$Cl

$^{22}$Na is a β and γ emitter and $^{36}$Cl a β emitter. This was verified by counting 100µl of $^{22}$Na (18 µCi ml$^{-1}$) and 100µl of $^{36}$Cl (10µ Ci ml$^{-1}$) separately in a well type γ counter (Compugamma). $^{36}$Cl registered no γ counts above background. The $^{22}$Na spectrum demonstrated powerful emission in the 180-156 eV energy range. The γ counter was subsequently programmed to read all samples in this energy range.

9.4.4.4 Estimation of conversion factor for γ to β counts for $^{22}$Na

The contribution of $^{22}$Na to the total ($^{22}$Na & $^{36}$Cl) β counts measured in the scintillation counter is determined by knowing the number of γ and β counts a given quantity of $^{22}$Na will produce. One can then convert the measured $^{22}$Na γ counts into estimated $^{22}$Na β counts and by subtracting this value from the measured total β counts derive a value for estimated $^{36}$Cl β counts. A conversion factor is therefore required to convert measured $^{22}$Na γ counts to estimated $^{22}$Na β counts.

To derive and validate a conversion factor a 120µl aliquot of stock $^{22}$Na (18 µCi ml$^{-1}$) was diluted to 15 ml with Ringer solution [ie 2.2 µCi of $^{22}$Na in 15 mls, which was equivalent to the situation in the "hot" reservoir]. Aliquots of this solution (10 - 100 µl) were made up to 2 mls with Ringer and counted in a γ counter. 10 mls of scintillant was added and the β counts measured.

When β counts were plotted against γ counts the relationship was linear (figure 9.5). The β/γ ratio was 3.32. The β and γ counts measured in the flux experiments normally fell within this linear range.
9.5 Preparation of tissues for Ussing chamber experiments

After an overnight fast, during which time free access to water was allowed, animals were anaesthetised (60 mg/kg sodium pentobarbitone IP) and a 6 cm length of jejunum a distance 15 cm distal to the ligament of Treitz was quickly removed, flushed gently with ice cold gassed Ringer bicarbonate and transported to the laboratory in this solution (the period from achieving anaesthesia to mounting in the Ussing chamber never exceeded 10 minutes).

Unstripped tissues were carefully opened by cutting along the mesenteric border and mounted as a rectangular sheet of tissue between the pins of the Ussing chamber.

In stripped preparations, the external smooth muscle of the intestine was removed by sliding the excised intact intestinal segment onto a cold moist glass rod, carefully cutting along the antimesenteric border with a blunt scalpel blade and then gently peeling away the muscle layers with a finger. The preparation was then mounted as a rectangular piece of tissue as described above.

A fall in Isc usually occurred after mounting the tissues followed by a rise to a
stable plateau within 15 minutes of mounting at which time open circuit pd and Rt had also reached a stable plateau. These values were taken as the basal electrical values. At the end of each experiment addition of mucosal glucose (10 mmol l⁻¹) resulted in a rise in Isc indicating the continued viability of the tissue.
CHAPTER 10

Topographical distribution of vitamin E in the small intestine and the evaluation of ischaemia / reperfusion injury as a means to increase free radical fluxes

10.1 Introduction

There is comparatively little information in the literature about the distribution of vitamin E within the intestine. The most detailed study currently available is that by Manohar et al. who reported the topographical distribution of alpha tocopherol along the length of the rat intestinal tract. The analytical methods used were, however, crude when compared with current HPLC methods. These authors also expressed the results in terms of tissue wet weight or subcellular organelle protein content rather than in terms of lipid content. It was of interest, therefore, to document the topographical distribution of vitamin E in the rat intestine in the animal model used in the current studies. These findings are reported in this chapter.

It was also of interest to measure the topographical concentrations of TEARS along the small intestine in vitamin E sufficient and deficient animals and to investigate which portion of the small intestine was most vulnerable to an oxidising stress applied in vivo. In these preliminary studies ischaemia / reperfusion injury was used as a means of generating an oxidising stress. I was encouraged to evaluate this method of producing an oxidising stimulus in vivo following the observations of Latimer et al. (vide infra).

10.2 Theoretical aspects of ischaemia / reperfusion injury

Ischaemia leads, after a variable period of time (the length of which depends upon the tissue studied) to irreversible tissue injury. In the early 80's McCord and coworkers demonstrated that reperfusion could result in an injury additional to that produced by ischaemia alone (ie reoxygenation or reperfusion injury). Reperfusion injury within the small intestine is associated with a flux of free radicals and this phenomenon has been demonstrated by a number of elegant experiments performed by other groups. Similar observations have been made in a number of other tissues notably the heart, liver, kidney, and knee joint. The "McCord Hypothesis" (figure 10.1) proposed that during the
ischaemic phase xanthine dehydrogenase is converted to xanthine oxidase. Simultaneously with this, tissue levels of ATP are depleted with the generation of AMP which may be degraded to adenosine, inosine and hypoxanthine. On reperfusion the hypoxanthine is oxidised under the influence of xanthine oxidase to generate the superoxide radical.

There are, however, a number of problems with this hypothesis. For example, subsequent studies by this same group have demonstrated that the time course of conversion of xanthine dehydrogenase to oxidase in the small intestine is best described in terms of hours rather than minutes as was initially proposed. Other explanations have therefore been sought, although none offer a full explanation for the rapid rise in free radical fluxes which follows reperfusion after a short period of ischaemia.

Some of the work subsequently undertaken by Granger's group is worthy of discussion. This group went on to demonstrate that reperfusion of ischaemic intestine results in a dramatic increase in neutrophil infiltration into the intestine which is associated with a loss of reduced glutathione, and an increase in
myeloperoxidase activity\textsuperscript{293}, and that the neutrophil infiltration was at least in part dependent upon the activation of xanthine oxidase. They demonstrated that the infiltration of neutrophils was dependent upon the generation of the superoxide anion\textsuperscript{294,295,293} and that substances which either chelate iron or scavenge the hydroxyl radical inhibit the formation of the neutrophil chemoattractant\textsuperscript{296}. The identity of this substance remains to be ascertained. Aliphatic aldehydes, which are secondary products of lipid peroxidation, are potent chemoattractive molecules. The infiltration of activated neutrophils into a tissue may provide a potent source of free radicals. Other studies by this group\textsuperscript{287} have demonstrated that the infiltration of granulocytes into the intestine is responsible for much of the ensuing mucosal dysfunction (increased mucosal permeability), but have not demonstrated a role for free radicals in this process.

It is clear therefore that superoxide anions are generated within the intestine following reperfusion and that the reaction of this anion with iron (the source of which is unclear but could include breakdown of haemoglobin following haemorrhage into tissues or decompartmentalisation of iron accompanying loss of cell integrity) can result in the generation of the highly reactive hydroxyl radical. Cell death and lysis, as may occur with a prolonged period of ischaemia, might therefore be expected to result in even greater fluxes of free radicals because of the decompartmentalisation of transition metal ions. None of these observations adequately explain the rapid increase in free radical fluxes which follows a short period of ischaemia / reperfusion.

Ischaemia / reperfusion injury to the stomach in rats, following coeliac artery occlusion for 30 minutes, is associated with a fall in both serum and stomach concentrations of vitamin E, a rise in TBARS and the appearance of gastric mucosal erosions. If rats were made vitamin E deficient (by feeding a vitamin E free diet for 7 weeks), the area of mucosal injury produced by this same stimulus was significantly greater\textsuperscript{288}. It was this and other similar data\textsuperscript{299} together with the interesting observation of Latimer et al\textsuperscript{281} of higher concentrations of TBARS following ischaemia / reperfusion in vitamin E deficient rat small intestine than in vitamin E sufficient intestine, which led me to explore ischaemia - reperfusion injury as a means of generating increased fluxes of free radicals within the small intestine. These studies are also presented in this chapter.
10.3 Topographical concentrations of vitamin E and TBARS in the intestine

10.3.1 Methods
Mucosal scrapings were prepared from 5 vitamin E sufficient and deficient animals (lard based diets only) aged 9 months following an 18 hour fast. After anaesthesia with 60mg / kg sodium pentobarbitone IP, the entire small intestine of each animal was removed, irrigated with ice cold 0.9% saline, trimmed of excess fat, opened along its antimesenteric border, and mucosal scrapings prepared with two microscope slides. Scrapings were snap frozen on aluminium foil using solid CO₂. The scrapings were stored at -70° C until biochemical analysis. Intestine between the pylorus and the ligament of Treitz was labelled "duodenum", the first 25 cms distal to the ligament of Treitz as "jejunum", and the remaining small intestine divided into two equal portions labelled "proximal" and "distal ileum".

Vitamin E (α tocopherol) was measured by HPLC following extraction into hexane as described in chapter 4, and a total lipid extract prepared from the same homogenate was used in the hydroxamic acid assay of lipid esters. The vitamin E content of the tissues were expressed as nmol α tocopherol / mg lipid.
10.3.2 Results

10.3.2.1 Topographical distribution of vitamin E

Vitamin E was undetectable in the small intestinal tissues prepared from the vitamin E deficient group of animals. Vitamin E concentrations in the small intestine of vitamin E sufficient animals declined in an aboral direction. Levels in the distal ileum were significantly lower than in the duodenum (P<0.05, Mann Whitney U test). The data is shown table 10.1.

Table 10.1. Topographical distribution of vitamin E within the small intestinal mucosa of 9 month old vitamin E sufficient rats (n=5 for each region)

<table>
<thead>
<tr>
<th>Region</th>
<th>Median [vitamin E] nmol mg⁻¹ lipid</th>
<th>95% confidence interval for median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>50.1</td>
<td>47.1-55.1*</td>
</tr>
<tr>
<td>Jejunum</td>
<td>46.1</td>
<td>40.3-52.0</td>
</tr>
<tr>
<td>Proximal ileum</td>
<td>45.2</td>
<td>40.5-49.5</td>
</tr>
<tr>
<td>Distal ileum</td>
<td>40.6</td>
<td>35.7-43.3b</td>
</tr>
</tbody>
</table>

(a) > (b) P<0.05

10.3.2.2 Topographical distribution of TBARS

The topographical distribution of TBARS was determined in both vitamin E sufficient and vitamin E deficient animals in the four regions of small intestine. In the vitamin E sufficient group of animals there were no significant differences in the levels of TBARS in these four regions [Kruskal Wallis one way ANOVA ] (table 10.2.)

Table 10.2. Topographical distribution of TBARS in mucosal scrapings prepared from the small intestine of 9 month old vitamin E sufficient rats (n=5 in each group).

<table>
<thead>
<tr>
<th>Region</th>
<th>Median [TBARS] nmol mg⁻¹ protein</th>
<th>95% confidence interval for the median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0.49</td>
<td>0.44-0.52</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.48</td>
<td>0.46-0.49</td>
</tr>
<tr>
<td>Proximal ileum</td>
<td>0.52</td>
<td>0.50-0.54</td>
</tr>
<tr>
<td>Distal ileum</td>
<td>0.53</td>
<td>0.51-0.57</td>
</tr>
</tbody>
</table>
In the vitamin E deficient group of animals there were again no significant differences in the topographical distribution of TBARS along the length of the small intestine [Kruskal Wallis ANOVA] (table 10.3).

Table 10.3. Topographical distribution of TBARS within the small intestine in 9 month old vitamin E deficient rats (n=5 in for each region).

<table>
<thead>
<tr>
<th>Region</th>
<th>Median [TBARS] nmol mg⁻¹ protein</th>
<th>95% confidence interval for the median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0.74</td>
<td>0.68-0.79</td>
</tr>
<tr>
<td>Jejunum</td>
<td>0.79</td>
<td>0.75-0.83</td>
</tr>
<tr>
<td>Proximal ileum</td>
<td>0.73</td>
<td>0.71-0.82</td>
</tr>
<tr>
<td>Distal ileum</td>
<td>0.82</td>
<td>0.74-0.88</td>
</tr>
</tbody>
</table>

In all regions of the intestine concentrations of TBARS were significantly higher in the vitamin E deficient animals than in the corresponding region of small intestine in vitamin E sufficient animals. (Mann Whitney U test, P<0.01 for all levels). The TBARS are compared graphically in figures 10.2 and 10.3.

![Figure 10.2](image1.png)  ![Figure 10.3](image2.png)

**Figure 10.2** Topographical distribution of TBARS in the small intestine of vitamin E sufficient rats

**Figure 10.3** Topographical distribution of TBARS in the small intestine of vitamin E deficient rats
10.4 Effects of ischaemia / reperfusion injury on mucosal TBARS
The aim of these studies was firstly to determine if the susceptibilities of the vitamin E sufficient and deficient tissues were different when exposed to a potent oxidising stimulus, and secondly to see if there was any particular region of the small intestine which was more vulnerable to this stress.

10.4.1 Methods
Mesenteric ischaemia was produced in surgically anaesthetised animals by completely occluding the anterior mesenteric artery at its origin with a small non crushing clamp and then returning the abdominal contents to the abdominal cavity for the period of ischaemia (and reperfusion when performed). Surgical anaesthesia was induced with intraperitoneal sodium pentobarbitone (60 mg / kg body weight) and maintained by the inhalation of 1% halothane in 40% nitrous oxide / 60% oxygen. In a series of experiments, animals were subjected to various periods of mesenteric ischaemia ranging from 1 to 20 minutes followed by 10 minutes of reperfusion. Others underwent a period of ischaemia without a reperfusion phase and others a sham laparotomy and mesenteric dissection only. The total length of anaesthesia in all cases was standardised to 25 minutes (with the exception of the 20l / 10R group in which the total duration of anaesthesia was 35 minutes). TBARS were measured as described previously in mucosal scrapings taken from the jejunum.

10.4.2 Results
The concentrations of TBARS following various periods of ischaemia and reperfusion are shown in figure 10.4. The figure compares TBARS in jejuna from vitamin E deficient (upper boxes) with vitamin E sufficient (lower boxes) animals after varying periods of ischaemia +/- reperfusion. The single box [marked (e)] represents 20 minutes ischaemia / 10 minutes reperfusion in vitamin E sufficient jejunum. Vitamin E deficient jejunum were not subjected to this period of ischaemia / reperfusion. Each box in the plot represents the pooled results from 5 animals. Periods of ischaemia between 1 and 5 minutes followed by 10 minutes reperfusion had no influence on TBARS in either group (Data not shown).
Figure 10.4 Effects of ischaemia +/- reperfusion on TBARS in the jejunum of vitamin E sufficient and deficient rats.

Comparison of the concentrations of TBARS following the various periods of ischaemia / reperfusion using the Kruskal Wallace one way ANOVA suggested the presence of data from more than one distribution in both the E sufficient and deficient jejuna [P=0.013 in sufficient and P=0.011 in deficient animals]. Subsequent comparison of data within each of the E sufficient and deficient groups as shown in figure 10.4 (Mann Whitney U test) demonstrated that for sufficient animals (e)>(a), and (e)>(b), P<0.005. For deficient animals (d)>(a), (d)>(b) and (d)>(c), P<0.005.

This data demonstrates that in the deficient group of animals 10 minutes of ischaemia (with 10 minutes reperfusion) produced a significant elevation of TBARS, and yet in the vitamin E sufficient animals a longer (20 minute) period of ischaemia was required to elevate TBARS.

10.4.3 Topographical distribution of TBARS following ischaemia / reperfusion

A further group of experiments examined the effect of 10 minutes ischaemia and reperfusion upon the topographical levels of TBARS in four regions of the small intestine (in this instance jejunum, proximal, mid and distal ileum). In vitamin E sufficient animals there was no elevation of TBARS in any region of the small
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In vitamin E deficient animals, however, levels of TBARS were significantly higher in the jejunum than in either the mid- or distal ileum (P<0.01, n=5) following this stimulus. This data is shown in figure 10,5.

![Figure 10.5](image)

**Figure 10.5** Topographical effects of ischaemia/reperfusion injury on TBARS in the vitamin E deficient small intestine (n=5)

10.5 Effects of 10 minutes mesenteric ischaemia / 10 minutes reperfusion upon jejunal morphology.

Tissues were fixed in 4% formol saline or in 5% glutaraldehyde and studied by light and electron transmission microscopy. These comparative studies were performed by Dr V. V. Smith of the Department of Histochemistry, Hospital for Sick Children. There were no apparent morphological changes in either group following 10' ischaemia / 10' reperfusion.

10.6 Effects of 10 minutes total mesenteric ischaemia / reperfusion upon intestinal short circuit current

In these studies unstripped jejuna from 12 month old vitamin E sufficient and deficient animals were studied in an Ussing chamber following a period of 10 minutes mesenteric ischaemia / 10 minutes reperfusion in vivo as described above.
Tissues were difficult to mount in the Ussing chambers following ischaemia / reperfusion because of contraction of the external muscle layers, and after mounting they were universally unstable with a poorly maintained basal Isc. It was difficult to decide what the basal Isc value was as stable plateaus were rare. The quality of this data is, therefore, below that of any of the other Ussing chamber studies where stable plateaus of 40 minutes in unstripped jejunum were the norm. The basal electrical characteristics of the two groups of tissues 10 minutes after mounting following ischaemia / reperfusion are shown in table 10,4.

Table 10.4. Basal electrical characteristics of unstripped jejunum from 12 month old vitamin E deficient and sufficient rats after 10 minutes of total ischaemia / reperfusion injury compared with age matched jejunum in the absence of ischaemia / reperfusion.

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT n=40</th>
<th>DEFICIENT n=40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Isc / μA cm²</td>
<td>73 ± 11</td>
<td>91 ± 10</td>
</tr>
<tr>
<td>Basal pd / mV</td>
<td>5.6 ± 0.4</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td>Tissue resistance / Ω cm²</td>
<td>77 ± 11</td>
<td>72 ± 6</td>
</tr>
</tbody>
</table>

Comparison of the basal electrical data for unstripped jejunum with / without ischaemia / reperfusion injury shows a significant reduction in tissue resistance in both sufficient (E+) and deficient (E-) groups (P<0.005) following ischaemia / reperfusion. This is reflected in a significant reduction in transepithelial potential difference in each of the groups. Basal Isc is unaffected in the E+ group, but is significantly reduced (P<0.01) in the E- group. The difference in Isc between E+ and E- jejuna not subjected to ischaemia / reperfusion is discussed fully in chapter 11.

The electrogenic responses of the tissues, following ischaemia / reperfusion, to mucosal galactose or serosal bethanecol were poorly reproducible. This data is not, therefore, given. The poorly sustained basal Isc which decayed inexorably, the fall in tissue resistance, the poor reproducibility of results and the small responses
to mucosal galactose seen in the tissues subjected to ischaemia / reperfusion point
to a problem with tissue viability. These observations render the interpretation of
the data in terms of the effects of oxidative stress difficult and perhaps misleading.

10.7 Discussion

10.7.1 Topographical distribution of vitamin E
The topographical distribution of vitamin E within the small intestinal mucosa
demonstrates a cranio - caudal concentration gradient. A similar trend remains
apparent if the concentrations are expressed in terms of wet weight of mucosal
scrapings. These findings are in general agreement with those of Manohar et al\(^{280}\)
although the cranio - caudal gradient was greater in their (younger) animals. It is
hard to explain this gradient on teleological grounds or a need for enhanced
antioxidant defences in the upper small intestine.
It is noteworthy that there is a cranio caudal gradient of a number of other
important antioxidant defences along the gastrointestinal tract including superoxide
dismutase, catalase, glutathione peroxidase and glutathione S transferase\(^{300}\).

10.7.2 Other lipid soluble antioxidants within the small intestine
There is no direct evidence that vitamin E is the most important antioxidant in the
small intestine, and work by Balasubramanian and various coworkers have
demonstrated the presence of a further important lipid soluble inhibitor of
peroxidation within the small intestine\(^{322}\). This substance only functioned as an
antioxidant in metal ion catalysed lipid peroxidation (as opposed to radiolysis or
azo dye initiated lipid peroxidation)\(^{323}\), and it was subsequently identified as mixture
cis monounsaturated free fatty acids (oleic and palmitoleic acid)\(^{324}\). Further studies
indicated that these free fatty acids seemed to be able to chelate transition metal
ions and hence prevent their "catalytic" role in the generation of free radical
species. I have been able to verify that the small intestinal mucosa of the rats used
in these studies contained abundant non esterified free fatty acids, including
palmitoleic and oleic acid using a variety of extraction methods\(^{301,302}\). Comparative
The TBA test was the only assay of lipid peroxidation used in these preliminary studies. TBARS were consistently higher in the vitamin E deficient mucosal scrapings at all levels of the small intestine than in the vitamin E sufficient scrapings. On teleological grounds, one might have expected concentrations of TBARS to mirror (inversely) the concentration of vitamin E. There was no evidence of such a gradient in either group. A cranio-caudal gradient of concentrations of TBARS did, however, become apparent on subjecting the intestine to the oxidative stimulus of ischaemia/reperfusion with an elevation of TBARS in both the jejunum and proximal ileum in the vitamin E deficient mucosa after 10 minutes of ischaemia/10 minutes reperfusion. A cranio-caudal gradient of susceptibility to ischaemia/reperfusion injury has been observed by others\textsuperscript{303}. The explanation for this is not obvious.

10.7.4 Complete versus partial ischaemia
One hypothesis might be that the proximal small intestine is more vulnerable to the ischaemic stimulus by virtue of its blood supply being totally dependent upon the superior mesenteric artery whilst the distal small intestine might receive some residual perfusion via arterial arcades from the posterior mesenteric artery. If this is true then we might be comparing partial ischaemia with complete ischaemia. Such a proposal highlights the debate about the mechanism of the generation of free radicals following ischaemia and reperfusion. The villi of the small intestinal mucosa contain the highest concentration of xanthine dehydrogenase of any tissue\textsuperscript{304} and this also has a cranio-caudal distribution within the small intestine\textsuperscript{292}. However the proposal of a rapid and complete conversion of the dehydrogenase form of the enzyme to the oxidase form within a short period (as

\textsuperscript{4} Nonesterified free fatty acids were separated from esterified lipids in a total lipid extract by both liquid-liquid partitioning and solid phase separation techniques, and the resulting free fatty acid extract saponified and measured as fatty acid methyl esters by GC. The solid phase separation was used principally with the aim of separating phospholipids from other lipid classes but carry over between different lipid classes was found to be unacceptably high. The method and its validation is not therefore described in detail in this thesis and no data produced by these means is presented.
little as 10 seconds) of total ischaemia\textsuperscript{305,306} has not been substantiated and the much slower time course of this conversion (50% conversion after 2 hours of total ischaemia) eluded to on page 167 brings the significance of the McCord hypothesis into question. In completely ischaemic tissues cell death and lysis and haemorrhage into the tissues upon reperfusion will be associated with decompartmentalisation of transition metals and of free radical generating enzyme systems and hence lead to increased fluxes of free radicals. Prolonged complete ischaemia is, therefore, likely to result in intestinal injury both as a result of ischaemic damage to the intestine and as a consequence of increased fluxes of free radicals seen upon reperfusion. The effects of the prolonged ischaemic phase is likely to outweigh any damage due to increased fluxes of free radicals\textsuperscript{307} and explains why complete ischaemia results in a greater degree of intestinal dysfunction\textsuperscript{308}.

Many published studies of the effects of ischaemia / reperfusion upon small intestinal function and the effects of various "antioxidant" agents or oxidising stimuli on the severity of intestinal injury use prolonged periods of total ischaemia\textsuperscript{296,309,310,311,312}. Complete mucosal ischaemia of the small intestine is associated with detectable morphological change after periods in excess of 10 minutes in the rat\textsuperscript{313} and dog\textsuperscript{314}. In studies in this rat model using doppler methods to measure mesenteric blood flow I was unable to obtain reproducible reductions in mesenteric blood flow suitable for use in a model of partial mesenteric ischaemia. I therefore chose to use a short period of total ischaemia as a stimulus. Periods of ischaemia of less than 10 minutes were evaluated in the first instance as this did not result in any demonstrable ultrastructural changes to the small intestinal mucosa in these animals. Discrete macroscopic haemorrhagic areas were seen in the mucosa of vitamin E sufficient rats following reperfusion after 20 minutes of mesenteric ischaemia and this might have influenced the TBARS measured in mucosal scrapings from this subgroup of animals.

\textbf{10.7.5 Vitamin E deficiency and susceptibility to gastric erosions after ischaemia / reperfusion}

Studies by others have shown that the vitamin E status of rats (7 weeks on a vitamin E deficient diet) influences the susceptibility of gastric mucosa to ischaemia reperfusion injury\textsuperscript{298}. The authors used a 30 minute period of total ischaemia
followed by a 60 minute period of reperfusion and found that vitamin E deficient animals exhibited a greater area of gastric erosions after ischaemia / reperfusion than age matched vitamin E sufficient controls. In the control animals, vitamin E levels in the gastric mucosa fell throughout the reperfusion period as mucosal TBARS rose [data was not provided for the vitamin E deficient animals]. The time course of the rise in TBARS mirrored that of the increase in mucosal area of erosion. Antioxidant status may therefore have an influence on the reperfusion injury which exacerbates the injury produced following a prolonged ischaemic phase.
CHAPTER 11

Biochemical and physiological studies after 12 months of vitamin E deficiency on a lard based diet.

11.1 Introduction
The first full cohort of animals in which both biochemical and physiological studies were performed were fed diets based on 10% lard with (E+) or without (E-) vitamin E added to the feed as discussed in chapter 2. A number of E- animals were switched to the E+ diet (repleted - ER) aged 30 weeks (27 weeks after weaning).

11.2 Growth and physical characteristics
The longitudinal weight data of the three groups of animals are shown in fig 11,1.

Figure 11,1 Weight data in vitamin E sufficient, deficient, and repleted rats. Error bars = 95% confidence interval for the mean.

The rate of weight gain in the E- group lagged behind that of the E+ group from 20 weeks post weaning. Aged 52 weeks (49 weeks post weaning) the E- animals
were significantly lighter than the E+ (E+ mean 733g ± 33, vs E- 519g ± 30, n = 40 in each group, P<0.01).

From the external appearance of these animals it was clear that a significant contribution to this difference in weight resulted from a loss of muscle bulk (in addition to adipose tissue) which accompanies the well described neurological syndrome associated with vitamin E deficiency. Of interest was the brisk period of weight gain seen in the repleted group of animals (ER) in response to the introduction of a vitamin E containing diet. The weight of this group of animals aged 52 weeks was comparable with that of the E+ group. (ER 732g ±35 n = 12) [Fig 11,1].

Animals in all three groups were allowed free access to food at all times and were not pair fed. When dietary intake was measured in the animals (E+ and E- only) 46 weeks after weaning, E- rats ate significantly less diet than E+ (E+ 21.5 g kg⁻¹ 24h⁻¹ ± 1.8 vs E- 23.5 g kg⁻¹ 24h⁻¹ ± 1.2, n=12, P<0.05). This difference in intake was larger when expressed independently of the animals weight (E+ 17.2 ±2.6 g/24hrs, vs E- 11.2 ±4.0). Neither group of animals developed diarrhoea, and stool outputs / 24hrs were similar in both E+ and E- animals (E+ 1.48 ±0.24 g/24 hrs, vs E- 1.27 ±0.54 g/24 hrs, n=6, P=NS). A number of E- animals suffered an acute diarrhoeal episode aged 44 - 48 weeks. In all these cases (n=4) the rats appeared extremely malnourished, the diarrhoea was blood stained, and the animal died within 36 hours of the onset of diarrhoea.

At the time of laparotomy it was noted that the E- animals were more likely to have dilated small bowel and a distended caecum than E+ animals, although no formal measurements were made of intestinal diameter. E- animals did not exhibit an increased prevalence of perineal staining with faecal residue. Metabolic balance studies were not performed on repleted animals and no information is available on either the dietary intake or stool output of this group.

11.3 Plasma vitamin E concentration

Plasma vitamin E concentration declined rapidly after weaning in the E- group and rose to a stable plateau in the E+ group as shown in figure 11,2 (Data supplied by Dr M. A. Goss - Sampson).

Aged 16 weeks plasma concentrations of α tocopherol were 34.5 ±1.4 μmol l⁻¹ in E+ animals, and undetectable in E- animals. Plasma concentrations did not change
significantly thereafter. Concentrations were not measured in the repleted group.

Figure 11.2 Longitudinal plasma vitamin E (α tocopherol) concentrations [n=10 in each group]

11.4 Results of biochemical and electrophysiological studies aged 52 weeks

11.4.1 Ussing chamber studies
In this cohort of animals (E+, E-, and E_R), jejunum was mounted in the Ussing chambers as an *unstripped* sheet. Intestinal short circuit current and tissue resistance were measured by the manual open circuit method as described in chapter 9.

11.4.1.1 Basal electrical characteristics
Basal short circuit current (Isc) was significantly higher in the E- group than in either the E+ or the E_R groups (Table 11.1). This was reflected in a higher transepithelial potential difference in this group. The tissue resistances were comparable in all 3 groups.
Table 11.1. Basal electrical characteristics of unstripped jejunum in 52 week old vitamin E sufficient, deficient and repleted rats

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT n = 40</th>
<th>DEFICIENT n = 40</th>
<th>REPLETED n = 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal pd mV</td>
<td>5.62 [±0.46] g</td>
<td>6.55 [±0.75] h</td>
<td>4.76 [± 0.36] i</td>
</tr>
</tbody>
</table>

b>a, and b>c, P<0.005
d,e,f not significantly different
h>g, g>i, and h>i P<0.05

Application of 1.25μM tetrodotoxin to the serosal surface of the jejunum had no effect on Isc in any group.

11.4.1.2 Electrogenic responses to secretogogues and mucosal galactose

Electrogenic secretory and absorptive responses were only measured in the E+ and E- groups (table 11,2). 10mM mucosal and serosal aminophylline produced a greater increment in Isc in the E-animals (56μA cm⁻² [± 15] vs 34μA cm⁻²[±9], n=14, P<0.05). E coli heat stable toxin (STa) applied to the mucosal surface at a concentration of 60 mouse units ml⁻¹ produced a greater increment in Isc in E-jejuna (medians 55 μA cm⁻² vs 31 μA cm⁻², n=10 P<0.01). Both the initial decrease and the subsequent increase in Isc seen with 1mM serosal bethanecol were comparable in the two groups of animals. The initial decrease in Isc is (at least in the mouse) due to an effect of bethanecol on the muscarinic M₁ receptor found on the enteric nerves. Studies to confirm this were not performed and this portion of the data is not given.
Table 11.2. Electrogenic secretory responses (δIsc) to a number of secretagogues in unstripped jejunum from vitamin E deficient and sufficient rats

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM bethanecol (serosa)</td>
<td>32 [±9]</td>
<td>34 [±9]</td>
</tr>
<tr>
<td>10mM aminophylline (mucosa and serosa)</td>
<td>34 [±9]</td>
<td>56 [±15]</td>
</tr>
<tr>
<td>E coli STa 60 mouse U / ml</td>
<td>15 [5-55]*</td>
<td>32 [25-98]*</td>
</tr>
</tbody>
</table>

n=12
NS

n=14
P<0.05

n=10
P<0.05*

* data is non parametric and is given as the median with the 95% confidence interval for the median.

*b groups were compared using the Mann Whitney U test.

The effect of adding galactose to the mucosal surface (with an equimolar concentration of mannitol applied to the serosal surface) is shown in figure 11.3. A greater electrogenic response to mucosal galactose was apparent at all concentrations of galactose tested above 5 mM.

Figure 11.3 Electrogenic responses to mucosal galactose in E+ and E- jejunum. * P<0.05, ** P<0.01
n = 10 - 12 for all points.
The maximal delta Isc produced by the addition of 28mM galactose was E- 115μA cm⁻² [±30] vs E+ 65μA cm⁻² [± 26], n=12, P<0.01).

Apparent Km values for the glucose transporters were not calculated as lines could not easily be fitted to the data in Hofstee plots {V against V/[S]} or Woolf plots {[S]/V against [S]}. The Lineweaver - Burk plot {1/V against 1/[S]} was not used as this overemphasises the data points at low substrate concentrations.

11.4.2 Morphological characteristics of the jejunal mucosa

Light microscopic studies of stained sections of jejuna of E+ and E- groups revealed a number of differences in mucosal morphology including an increase in crypt depth, a reduction in mucosal thickness and a decrease in villus / crypt ratio in vitamin E deficient jejuna.

Table 11.3. Morphological characteristics of vitamin E sufficient and deficient jejunum (12 months)

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>villus height / μM</td>
<td>624 ±31</td>
<td>648 ±39</td>
<td>NS</td>
</tr>
<tr>
<td>crypt depth / μm</td>
<td>139 ±14</td>
<td>163 ±19</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>villus / crypt ratio</td>
<td>4.68 ±0.44</td>
<td>4.03 ±0.40</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

Representative histological sections are shown in plates (11.1) and (11.2) on page 186. Electron microscopy (performed by Dr V. Smith) revealed no consistent differences in mucosal ultrastructure in jejuna from the two groups of animals. Histochemical studies were performed on a number of sections by Dr Charles Hoyle from the Department of Anatomy, University College, London. PGP 9.5 was used to demonstrate all nerves, and calcitonin gene related polypeptide (CGRP), vasoactive intestinal polypeptide (VIP) and galanin immunoreactivity demonstrated with appropriate antibodies. These studies did not demonstrate any quantitative or qualitative differences in the patterns of staining between groups in either the myenteric or the submucous plexi. This data is not shown.
Plate 11,1. Light microscopic section of mid jejunum from a vitamin E sufficient animal. Stained with haematoxylin & eosin. Magnification X 16

Plate 11,2. Light microscopic section of mid jejunum from a vitamin E deficient animal. Stained with haematoxylin & eosin. Magnification X 16
11.4.3 Apical membrane hydrolase activity

Activities of the mucosal hydrolases, alkaline phosphatase sucrase and lactase were similar in the two groups (table 11.4).

Table 11.4 Mucosal hydrolase activities in proximal small intestine from 12 month vitamin E sufficient and deficient animals (n=6 in each group).

<table>
<thead>
<tr>
<th></th>
<th>Alkaline phosphatase (U g⁻¹ protein)</th>
<th>Sucrase (µmol min⁻¹ g⁻¹ protein)</th>
<th>Lactase (µmol min⁻¹ g⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sufficient</td>
<td>287±55</td>
<td>49.2±7.1</td>
<td>34.1±5.5</td>
</tr>
<tr>
<td>Deficient</td>
<td>356±61</td>
<td>44.5±6.3</td>
<td>29.8±6.5</td>
</tr>
</tbody>
</table>

Arrhenius plots of alkaline phosphatase activity in apical brush border membrane preparations (figure 11.4) demonstrated a temperature breakpoint equivalent to approximately 34°C in jejuna from both groups.

![Figure 11.4 Arrhenius plot of alkaline phosphatase activity in BBM preparations from 12 month E+ and E- animals (mean of 3)
11.4.4 Vitamin E and indices of lipid peroxidation

11.4.4.1 Concentrations of vitamins E and A
Vitamin E was undetectable in mucosal scrapings from the E- jejuna, but was abundant in the E+ jejuna (mean concentration 1.33 [± 0.09] μmol / g lipid, n=6). Vitamin A concentrations, measured by HPLC, were comparable in scrapings from both groups (E+ 8.3 [± 2.5] vs E- 9.1 [± 1.9] mmol / mol lipid, n=6, NS).

11.4.4.2 TBARS
Mucosal TBARS were significantly higher in the E- animals (E+ 0.67 [±0.09] vs E- 0.83 [±0.09] nmol / mg protein, n=10 P<0.05). A plot of basal Isc vs TBARS suggested that there might be a relationship between these two parameters (r=0.803, P<0.05 for 1st order regression) [figure 11,5]. There are obvious reservations (in statistical terms) about pooling of data and the calculation of such a correlation.

Figure 11,5 Plot of basal short circuit current against mucosal TBARS. 12 month vitamin E deficient (△) and sufficient (●) animals
11.4.4.3 Malondialdehyde

Both free and total malondialdehyde, measured by HPLC, were significantly higher in the E- jejuna. (Free MDA; E- 307pmol mg\(^{-1}\) protein [±96] vs E+ 100 [±20] pmol mg\(^{-1}\) protein, n=11, P<0.01: total MDA; E- 397 [±109] vs E+ 168 [±30.3]nmol / mg protein, P<0.05 n=6).

![Figure 11.6](image)

Figure 11.6 Plot of mucosal free MDA against basal Isc in jejuna from 12 month vitamin E sufficient (○) and deficient (▲) animals.

A plot of free MDA against basal Isc is shown in figure 11.6. The data in this instance shows more clearly two populations of data points (E+ and E-) with different slopes. Correlation of Isc and MDA using pooled data would therefore be incorrect.

11.4.5 Biophysical characteristics of the apical brush border membrane.

Steady state anisotropy measurements with DPH and 12 AS using brush border membrane enriched fractions prepared from the proximal small intestine demonstrated significantly higher anisotropy values in E- BBM with both probes.
(DPH E+ 0.209 [±0.008 - 95% CI] vs E- 0.231 [0.0049] n=6, P<0.01; 12AS E+ 0.090 [±0.005 95%CI] vs E- 0.097 [±0.006] n=6, P<0.01).

Figure 11.7 Semi log plot of anisotropy parameter 1/[(R_q/R)-1] versus 1/ temperature (Kelvin)

Semi log reciprocal plots of the anisotropy parameter 1/[(R_q/R)-1] vs 1/temperature (Kelvin) were constructed (figure 11,7). The anisotropy parameter 1/[(R_q/R)-1] is used in these plots because it is traditionally related to microviscosity (the reciprocal of membrane fluidity)\(^\text{317,318}\) [see chapter 8]. These plots revealed a phase transition temperature equivalent to approximately 26.5°C in the E+ BBM. A temperature break point could not be determined with any confidence in BBM from E- animals.

11.4.6 Fatty acid composition of the apical brush border membrane
Measurement of the total fatty acid composition (expressed as a mol%) of BBM from the two groups (table 11,5) revealed no statistically significant differences in fatty acid profiles. However the ratio of lipid / protein of the BBM (μmol/mg) was significantly higher in the sufficient jejuna (E+ 0.62 [±0.03] μmol / mg, E- 0.54 [±0.04], n=12, P<0.05).
Table 11.5 Fatty acid composition (mol %) of total lipid extracts of proximal small intestine brush border membranes prepared from 12 month sufficient and deficient animals [n = 6 in each group, mean ± 95% confidence interval].

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.17 [±0.51]</td>
<td>0.91 [±0.33]</td>
</tr>
<tr>
<td>16:1</td>
<td>1.39 [±0.56]</td>
<td>1.11 [±0.51]</td>
</tr>
<tr>
<td>18:2</td>
<td>6.23 [±1.45]</td>
<td>5.27 [±0.64]</td>
</tr>
<tr>
<td>20:4</td>
<td>23.58 [±4.00]</td>
<td>25.74 [±3.70]</td>
</tr>
<tr>
<td>20:0</td>
<td>0.82 [±0.17]</td>
<td>0.58 [±0.31]</td>
</tr>
<tr>
<td>22:6</td>
<td>0.87 [±0.29]</td>
<td>1.01 [±0.33]</td>
</tr>
<tr>
<td>22:4</td>
<td>0.56 [±0.16]</td>
<td>0.69 [±0.28]</td>
</tr>
<tr>
<td>22:0</td>
<td>0.72 [±0.09]</td>
<td>0.59 [±0.32]</td>
</tr>
</tbody>
</table>

11.5 Discussion

The above data documents an increase in basal and secretogogue induced electrogenic secretion and electrogenic galactose absorption in jejunum from rats fed a vitamin E deficient diet for approximately 12 months when compared with age matched (vitamin E sufficient) controls. These changes in secretion and absorption were associated with increased levels of lipid peroxidation in small intestinal mucosal scrapings and a biophysical change in the enterocyte apical brush border membrane. A simple hypothesis which draws these observations together might be that vitamin E deficiency is associated with oxidative damage to the lipid and protein components of the enterocyte membrane which leads to an alteration in the functioning of the membrane. No direct evidence for this has been provided, and the mechanism(s) whereby the changes in electrogenic currents arise have not been determined. For example, no attempt has been made to perform detailed studies of the protein biochemistry of the apical brush border or basolateral membranes (to study the ion channels involved in the secretory process), or of the intracellular messengers / regulators of intestinal secretion, such as cAMP, cGMP, G proteins, inositol phosphate(s) and diacylglycerols. Perturbations of membrane
fluidity may be associated with changes in the functioning of membrane proteins and these issues are discussed in section 11.5.4.

11.5.1 Animal model
There are a number of reasons why the vitamin E deficient animals might not have developed diarrhoea despite the increase in basal and secretogogue induced electrogenic secretion. Firstly, the findings in the jejunum do not necessarily reflect what is happening in other regions of the small and large bowels, as has been made clear in other studies of electrogenic secretion in the rat\textsuperscript{319,320} and secondly, the enormous capacity of the rat caecum renders this species highly resistant to diarrhoea. It was noticeable that the E- animals were more likely to have a distended caecum than E+. Performing a caecectomy on the animals at an earlier age might have allowed the development of clinical diarrhoea.

11.5.1.1 Use of vitamin E deficiency as a model of increased free radical fluxes within the intestine
Vitamin E deficiency was used as a means to increase free radical fluxes because this seemed appropriate to a real life situation which may occur in malnourished infants. Vitamin E, as discussed in previous chapters, is an important \textit{structural} element of the cell membrane in addition to being the most important lipid soluble antioxidant. Hence, this animal model is best regarded as a model of both vitamin E depletion and increased free radical fluxes. Ionising radiation has been used by other groups to provide an oxidising stimulus within the intestines\textsuperscript{321}, but this is somewhat unphysiological and it was considered an inappropriate stimulus for the purposes of the present studies. The small intestine is, infact, more susceptible to radiolysis induced oxidative injury than to other types of oxidative stress\textsuperscript{322,323,324}, and this observation discouraged me further from its use. Other options which were available as a means of increasing oxidative stress included ischaemia / reperfusion injury\textsuperscript{284} and the addition of exogenous oxidising stimuli\textsuperscript{325}. Reservations about the use of ischaemia / reperfusion injury and the technical problems encountered with this stimulus in the rat have been discussed in chapter 10 and will not be discussed further.

The addition of an oxidising stress in vitro in the Ussing chamber has been used by a number of other groups. Don Powell's group demonstrated an increase in
electrogenic Cl secretion and a reduction in electroneutral Na and Cl absorption in rat colon following the generation of hydrogen peroxide on the serosal surface of the tissue using xanthine / xanthine oxidase. These alterations in ion transport were largely (~ 70%) due to the release of prostaglandins from within the lamina propria. No measurements were made of peroxidative injury to the intestinal mucosa. Interestingly, Desjeux's group in Paris have described an electrogenic secretory response to serosal H$_2$O$_2$ in rat small intestine which became greater when rats were fed a protein deficient diet. These studies did not provide any evidence that these responses were due to oxidative damage within the small intestinal mucosa. The electrogenic responses to H$_2$O$_2$ were small in the fed group of rats (~7 μA cm$^{-2}$). I was unable to demonstrate any change in Isc following the application of 1mM serosal H$_2$O$_2$ to jejunum from the rats used in my studies.

11.5.1.2 Problems with the animal model
There are a number of potential problems with the animal model as used with this particular cohort of animals. These are discussed in turn below.

11.5.1.2.1 Nutritional status and dietary intake of the animals
The E- group ate significantly less food than the E+ group, suffering in effect chronic undernutrition in comparison with their age matched controls. This may have influenced both the electrophysiological and the biochemical measurements (vide infra). For the purposes of this preliminary study I chose not to pair feed the animals. If vitamin E deficiency induced a state of anorexia (as does vitamin A deficiency) then it seemed appropriate, at least in the first instance, to allow "nature to take its course". Had the animals been pair fed then the question would have arisen whether they should have been fed on the basis of food per rat, or food per kg of body weight. Indeed, it would have been interesting to have seen what would have happened to the weights of the deficient animals had they been pair fed (on a food per rat basis) to the intake of the sufficient group.

Acute undernutrition (33% of normal intake for 9 days) has no effect on basal Isc in stripped duodenum, jejunum or ileum, but is associated with an increased electrogenic secretory response in jejunum to the Ca$^{2+}$ mobilising agonists bethanecol, 5 hydroxytryptamine, and prostaglandin E$_2$, and to E coli STa (which activates secretion via the guanylate cyclase / cyclic GMP pathway) but
not to dibutyryl cAMP and forskolin (which cause secretion via cyclic AMP dependent mechanisms). These patterns of jejunal hypersecretion are different from those found in this cohort of vitamin E deficient rats. The effects of prolonged malnutrition on these parameters have not been reported. Before one can be sure that the changes in intestinal short circuit current documented above are due to vitamin E deficiency per se rather than due to starvation / undernutrition appropriate studies with pair fed animals need to be performed. Studies in animals with similar dietary intakes are described in a later chapter.

11.5.1.2.2 Vitamin A status

Purkins et al\textsuperscript{330} used a diet which was similar to that used in these studies [the "control" diet contained 100 mg \( \alpha \) tocopherol / kg feed], and noted a significant fall in plasma vitamin A concentrations in weanling rats fed the diet for six months. The reasons for the fall in plasma vitamin A were not sought. In a previous cohort of vitamin E deficient rats in our laboratory, plasma concentrations of vitamin A fell by a median of 58\% (95\% CI 5-78\%, n=5 P<0.05) in the E- group by 12 months of age when compared with age matched vitamin E sufficient rats\textsuperscript{8}. Nzegwu et al\textsuperscript{331,332} have studied the effects of chronic vitamin A deficiency on rat small and large intestinal secretion and absorption. They found that vitamin A deficiency did not alter basal Isc in the jejunum, and resulted in a fall in Isc in the distal ileum. Electrogenic glucose transfer was not affected by vitamin A deficiency although bethanecol produced a significantly larger increment in Isc in the jejunum of vitamin A deficient animals than in controls. It seems unlikely therefore from this data that the changes in electrogenic absorption and secretion in this vitamin E deficient cohort of animals could be attributed to vitamin A deficiency per se. Small intestinal concentrations of vitamin A were also similar in both E deficient and sufficient animals. Intestinal concentrations of vitamin A were not reported by Nzegwu et al\textsuperscript{331}.

\* Willy Cohn, Hoffmann la Roche, Basel - unpublished data
11.5.1.2.3 Immune regulation of secretion

Chronic vitamin E deficiency is associated with a modulation of the immune system, including a reduction in the number of circulating T helper cells in rats and in other animals. Products of lipid peroxidation, for example 4-hydroxynonenal, have a potent chemoattractant effect on granulocytes. The immune system has effects on epithelial ion transport and perturbations of the immune system may therefore lead to modulation of intestinal secretion. These effects have not been specifically studied in the current rat model. Histological sections of the small intestine from vitamin E deficient animals did not demonstrate a hypercellularity of the lamina propria as might be expected if there were an augmented inflammatory response in this group of animals.

11.5.1.2.4 Enteric neuropathy

Chronic vitamin E deficiency in both man and this animal model is associated with a characteristic neuropathy of the peripheral, central, and autonomic nervous systems. The enteric nervous system has a regulatory role in the control of intestinal ion transport and the contribution of an enteric neuropathy to the observed changes in electrogenic secretion remains undefined. A number of observations may be made which are pertinent to this matter. Firstly, basal intestinal short circuit current in the rat small intestine (in contrast to rat colon) is not normally influenced by neural blockade with serosal tetrodotoxin. The lack of change in Isc after neural blockade with TTX in the E deficient animals in the present studies provides evidence against a direct role of the enteric nervous system in the generation of these higher basal short circuit currents. Matters may, however, be more complex in undernourished animals. Rats starved for three days exhibit increased stores of tyramine releasable noradrenaline in the enteric nervous system. The effects of catecholamines in the neural regulation of small intestinal secretion are modulated in vitamin E deficiency and this is discussed further in chapter 12. The histochemical studies performed by Charles Hoyle failed to provide any structural evidence of a neuropathy in the vitamin E deficient jejuna.

A number of attempts were made to study the electrogenic secretory response to

---

1 Charles Hoyle, Department of Anatomy, University College, London
Unpublished data
electrical field stimulation in this group of animals, but consistent results were
difficult to obtain in jejunum. The enteric nervous system has a role in the
electrogenic secretory response to STa\textsuperscript{343} but this seems to relate more to the
duration of the response than to its initial magnitude. [Both these parameters will
be of importance because it is the area under the curve which relates to the size
of the secretory response.] The peak response to STa was increased in the E
deficient animals, but the duration of the responses were similar in the two groups
of animals. From the evidence presented above it seems unlikely that an alteration
in the level of neural tone in the E deficient jejunum was directly responsible for the
increase in basal Isc.

11.5.1.2.5 Tissue capacitance
It is not possible to entirely rule out an alteration in the capacitance of deficient
jejunum when compared with the sufficient animals. This has the potential of leading
to an underestimation of short circuit current in the "bulkier" E+ tissues. It would
be surprising however if the difference in capacitance were sufficiently large to
explain the differences between the two groups given the relatively small
differences in tissue resistance and in tissue thickness.

11.5.2 Lipid peroxidation
Oxidative injury to the small intestinal mucosa was measured both as TBARS and
as MDA. As discussed in chapter 3, these assays are complimentary in the
information they provide. The findings of increased concentration of TBARS and
of free and total malondialdehyde in the E- jejunum are consistent with a greater
degree of oxidative damage in E- jejunum than in E+. The concentration of TBARS
in the intestine are quite modest when compared with concentrations found in other
tissues such as liver\textsuperscript{322}. This may be due to both the increased resistance of the
small bowel to oxidative damage and also the relatively low amounts of PUFA in
the intestine when compared with other tissues\textsuperscript{344}.

It is well established that lipid peroxidation has effects on the protein conformation
of the brush border membrane\textsuperscript{345}, on adrenoreceptors\textsuperscript{346}, and on the physical
properties of the biological membranes\textsuperscript{347}. Changes in the lipid environment of
transporter proteins is also known to have an effect on the functioning of these
proteins\textsuperscript{348}. 
11.5.3 Membrane biophysical characteristics

This line of investigation appeared particularly relevant because of the important structural role of vitamin E within biological membranes and because peroxidation of lipids within the membrane should affect these parameters. There is increasing evidence that a modulation of the "fluidity" of a membrane may result in a modulation of the membrane enzyme and transporter kinetics. Meddings group in Calgary have demonstrated that "fluidising" apical brush border membrane vesicles reduces the maximum rate of glucose transfer.\cite{418}

The higher anisotropy values found in E- BBM with both DPH and 12 AS may be indicative of a more ordered membrane (DPH) with a higher \( \tau_e \) for 12AS. The interpretation of this fluorescence polarography data is complicated by the fact that \( \alpha \) tocopherol itself confers a restriction on rotation of DPH within lipid bilayers, and causes a reduced mobility of the fluorescent probe pyrene in porcine intestinal BBM.\cite{350} Hence vitamin E deficiency and lipid peroxidation may have opposing effects upon the behaviour of the fluorescent probes. Eichenberger\cite{351} has reported a close correlation between the production of malondialdehyde and the steady state fluorescence polarisation (\( r_s \)) of DPH in rat liver microsomes. Malondialdehyde, when added exogenously, had no effect on \( r_s \). Hence the increase in \( r_s \) was not due to crosslinking within the lipid domain by MDA but was almost certainly due to covalent bonding between neighbouring acyl side chains of membrane lipids.

The increase in the protein lipid ratio seen in the E- BBM might also serve to increase the anisotropy values in these membranes. This issue might be addressed by repeating the anisotropy measurements on a lipid extract prepared from both groups of BBM, so that any effects of protein on the measurement could be excluded. A full and informed interpretation of the anisotropy data requires a substantial amount of further work which should at the minimum include documentation of cholesterol / phospholipid ratios, and information on the prevalence of trans- and cis- fatty acid isomers (which could not be measured by the present GC methods).

Only the apical membrane was studied and it seems unlikely that changes in this membrane alone would account for the changes in Isc which have been noted. It would have been of interest to study the basolateral membrane. Na\(^+\)K\(^+\)ATPase, an integral membrane protein found on the basolateral membrane, is generally
considered to generate the electrochemical gradient to drive both Cl secretion and Na coupled glucose (or galactose) absorption, although this view is not universally accepted. Changing the lipid environment of this protein is known to alter its function and hence a study of basolateral membrane fluidity and the activity of Na\textsuperscript{+}K\textsuperscript{-}ATPase would have been of interest.

11.5.4 Fatty acid composition of the apical enterocyte membrane
One might have expected a depletion of PUFA in the BBM prepared from the E-group when compared with those from the E+ group. The higher levels of MDA in this group is certainly evidence in favour of increased peroxidation of unsaturated fatty acids with three or more double bonds. Failure to detect a depletion of PUFA in the E- group might have arisen because of the shortcomings of the methodology (see chapter 7 for a full discussion). It is important to appreciate, however, that the fatty acid profiles say nothing about the turnover of these fatty acids.

11.5.5 Other antioxidant defences
The effects of vitamin E deficiency on other antioxidant defence mechanisms within the bowel, such as catalase, glutathione peroxidase and superoxide dismutase or the tissue ratio of reduced to oxidised glutathione have not been measured in these studies. This would have been of interest.

11.6 Conclusions
It should be clear from these discussions that there are a number of shortcomings with this preliminary study of the effects of a chronic and severe vitamin E deficiency upon small intestinal structure and function. The data obtained formed the basis for a series of better designed and more detailed experiments which are reported in chapters 12 - 15.

The data, as it stands, does however support the hypothesis that malnourished children with depleted antioxidant defences may be predisposed to increased upper small intestinal secretion. In the presence of compromised small intestinal function due to other causes this may provide one mechanism whereby malnutrition could predispose to the perpetuation of protracted diarrhoeal disease.
CHAPTER 12

Biochemical and physiological studies in stripped jejunum after 6 months of vitamin E deficiency.

In this cohort of animals, diets were based on 10% lard with (E+) or without (E-) vitamin E as described previously. An earlier time point at which to study the animals was chosen to ensure that the weights and dietary intakes of the two groups were comparable (so minimising any differences between the groups which were not a direct consequence of vitamin E deficiency) and because of my intention of comparing these cohorts with animals fed a high PUFA vitamin E deficient diet which were known to stop gaining weight at about this time⁹.

12.1 Further methodological details

12.1.1 Protocol for steady state radioisotope fluxes

Transmucosal fluxes of $^{22}$Na and $^{36}$Cl were measured on paired tissues taken from adjacent segments of jejunum as described in chapter 9. 15 minutes after mounting, 2.2 µCi of $^{22}$Na and 2.2 µCi of $^{36}$Cl were added to the mucosal surface of one tissue and to the serosal surface of the paired tissue under short circuit conditions. After a further 15 minute equilibration period a 2 ml sample was removed from each unlabelled bathing solution and a 100 µl sample from each labelled solution. Samples removed from the unlabelled solutions were replaced with an equal volume of fresh unlabelled Ringer bicarbonate. Duplicate samples were taken 20 minutes later in order to determine baseline flux rates. At the end of this period, isobutylmethylxanthine (IBMX), final concentration 200µM l¹, was added to both mucosal and serosal bathing solutions of each tissue pair. A 15 minute period under short circuit conditions was allowed for equilibration, and fluxes measured a second time as described above over a 20 minute period.

⁹D.P.R.Muller - unpublished data
12.1.2 Construction of cumulative concentration response curves for secretogogues
Cumulative concentration response curves for secretogogues were constructed by
the sequential addition of secretogogue (concentrations increasing by a factor of
≥10 with successive doses) timed so that each successive electrogenic response
was elicited before the previous response had started to wane. This approach was
validated by comparison of these curves with non cumulative concentration
response curves for acetylcholine (ACh). Cumulative and non cumulative curves
for ACh (in the presence of serosal neostigmine and tetrodotoxin (TTX) - vide infra)
were comparable. With each of the other secretogogues examined the dose of
agonist achieving the maximal increment in Isc (δIsc max) was applied to
preparations which had not previously been exposed to a secretogogue and the
responses found not to differ from those achieved with cumulative increments in
dose. The maximal increment in Isc and the concentration of secretogogue
required for 50% of the maximal electrogenic secretory response (EC_{50}) were
derived from the concentration - response curves. No tissue was exposed to more
than one secretogogue. The secretogogues acetylcholine (ACh), dibutyryl cyclic
adenosine monophosphate (db cAMP) and serotonin (5HT) were applied to the
serosal surface of the preparation. Acetylcholine was added in the presence of 10^{-4}
M serosal neostigmine to inhibit cholinesterase activity and in the presence of 1.25
µM serosal TTX (added 10 minutes before starting the concentration response
curves) to block the enteric nerves. IBMX was applied to both mucosal and serosal
surfaces simultaneously, and E coli heat stable toxin (STa) was applied to the
mucosal surface.

12.2 Results

12.2.1 General characteristics of the animals
The weights and dietary intakes of animals in both groups were comparable at all
times (figure 12,1).
Aged 24 weeks weights were E+ 523±56g, n=35; E- 564±57g, n=35, NS. Dietary
intakes for each group determined aged 22 - 24 weeks were E+ 29.8±3.0 g 24hr^{-1},
E- 31.6±1.8 g 24hr^{-1} (n=12 in each group).
12.2.2 Ussing chamber studies

12.2.2.1 Basal electrical characteristics and the effect of a 72 hour fast

Basal Isc, pd and Rt were similar in both groups [Isc; E+ 69±4 μA cm⁻², E- 71±4 μA cm⁻², n=55; pd; E+ 1.21± 0.10 mV, E- 1.14± 0.10 mV; Rt; E+ 17.2±1.0 Ωcm⁻², E- 16.6±0.8 Ωcm⁻²]. The addition of serosal TTX (final concentration of 1.25μM) had no influence on basal Isc, pd or Rt in preparations from either group of animals. Values of these basal electrical parameters 40 minutes after mounting of the tissues (during the "basal" period of the steady state flux experiments) were also similar [Table 12,1; page 202].

Fasting animals for 72 hours resulted in a significantly higher Isc in E- animals than in E+ (E+ 91±14 μA cm⁻², E- 122±12 μA cm⁻², n=8, P<0.05). This response to fasting is likely to involve a modulation of adrenergic neural tone in the rat small intestine. It seemed appropriate, therefore, to examine by pharmacological means the levels of noradrenergic tone and the response to noradrenergic agonists in jejunum. These data are discussed in section 12.2.2.7.
Table 12.1 Steady state unidirectional and net ion fluxes in stripped jejunum from 6 month old vitamin E sufficient and deficient rats (lard based diets). n=8 paired tissues for each group.

\( J_m^s \): mucosa to serosa flux; \( J_s^m \): serosa to mucosa flux; \( J_n^s \): net flux; \( I_{sc} \): short circuit current; \( J_{R_{net}} \): residual ion flux; \( R_t \): electrical resistance; \( \Delta P \): transmucosal potential difference; +: net absorption; -: net secretion; NS: not significant; IBMX 200\( \mu \)M serosal and mucosal isobutylmethylxanthine. Values are means ±1 sem.

<table>
<thead>
<tr>
<th></th>
<th>( J_{Na_{ms}} )</th>
<th>( J_{Na_{sm}} )</th>
<th>( J_{Na_{net}} )</th>
<th>( J_{Cl_{ms}} )</th>
<th>( J_{Cl_{sm}} )</th>
<th>( J_{Cl_{net}} )</th>
<th>( J_{R_{net}} )</th>
<th>( I_{sc} )</th>
<th>( \Delta P )</th>
<th>( R_t )</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>( \text{Na} / \mu \text{mol hr}^{-1} \text{ cm}^{-2} )</td>
<td>( \text{Cl} / \mu \text{mol hr}^{-1} \text{ cm}^{-2} )</td>
<td>( \mu \text{mol hr}^{-1} \text{ cm}^{-2} )</td>
<td>( \text{mV} )</td>
<td>( \Omega \text{ cm}^{-2} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BASAL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUFFICIENT</td>
<td>34.69 ±1.18</td>
<td>30.62 ±1.74</td>
<td>4.07 ±1.08</td>
<td>31.29 ±1.48</td>
<td>29.43 ±1.65</td>
<td>1.86 ±1.13</td>
<td>-0.10 ±0.70</td>
<td>2.11 ±0.09</td>
<td>0.75 ±0.07</td>
<td>13.27 ±1.24</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>29.71 ±0.42</td>
<td>31.98 ±0.83</td>
<td>-2.27 ±1.02</td>
<td>26.41 ±1.49</td>
<td>30.17 ±1.57</td>
<td>-3.76 ±1.07</td>
<td>0.55 ±0.62</td>
<td>2.04 ±0.10</td>
<td>0.81 ±0.08</td>
<td>14.84 ±1.44</td>
</tr>
<tr>
<td>IBMX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUFFICIENT</td>
<td>33.52 ±1.51</td>
<td>36.42 ±1.65</td>
<td>-2.9 ±1.74</td>
<td>29.40 ±2.31</td>
<td>39.89 ±3.47</td>
<td>-10.94 ±2.56</td>
<td>-4.52 ±2.37</td>
<td>3.52 ±0.18</td>
<td>2.88 ±0.27</td>
<td>13.66 ±1.72</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>28.45 ±0.83</td>
<td>32.50 ±1.26</td>
<td>-4.05 ±1.19</td>
<td>25.16 ±1.49</td>
<td>37.55 ±2.40</td>
<td>-12.39 ±3.43</td>
<td>-4.41 ±2.61</td>
<td>3.93 ±0.19</td>
<td>3.94 ±0.41</td>
<td>20.84 ±2.18</td>
</tr>
</tbody>
</table>
12.2.2.2 Steady state transmucosal fluxes of $^{22}\text{Na}$ and $^{36}\text{Cl}$

The steady state transmucosal fluxes of $^{22}\text{Na}$ and $^{36}\text{Cl}$ are shown in table 12.1. There was net Na and Cl absorption in E+ jejunum and Na and Cl secretion in E- jejunum. Basal Isc was comparable in the two groups as was $J_{R\text{net}}$. 200 µM mucosal and serosal IBMX resulted in Na and Cl secretion in both E+ and E- jejunum. Following IBMX Isc became significantly greater in E- animals. $J_{\text{Cl net}}$ also increased, consistent with an increase in electrogenic Cl secretion, although the difference in $J_{\text{Cl net}}$ between the tissues did not reach statistical significance.

12.2.2.3 Cumulative concentration response curves for ACh

Cumulative concentration response curves for ACh ($10^{-9}\text{M} - 10^{-4}\text{M}$) after serosal pretreatment with TTX and neostigmine produced a greater δIsc max in E- jejunum [Fig 12,2]. There was no significant difference in the EC$_{50}$ for this response [δIsc max; E+119±24 μA cm$^{-2}$ n=8, E- 167±42 μA cm$^{-2}$ n=8 P<0.05: EC$_{50}$; E+ 5.6x10$^{-7}$M (95% confidence interval 3.49-9.13), E- 4.4x10$^{-7}$ (95% confidence interval 3.11-6.24)].

![Cumulative concentration response curves for acetylcholine in stripped jejunum](image-url)
12.2.2.4 Cumulative concentration response curves for IBMX

The δIsc max for the electrogenic secretory response to the specific phosphodiesterase inhibitor IBMX was also higher in E- jejuna [δIsc max E+ 189±18 μA cm⁻², E- 211±20 μA cm⁻², n=8, P<0.05]. The EC₅₀ for responses to IBMX were comparable [EC₅₀, E+ 8.1x10⁻⁶M (95% confidence interval 4.4-15.0 x 10⁻⁶M), E- 1.1x10⁻⁵M (95% confidence interval 0.75-1.9 x 10⁻⁵M), n=8, P=ns]. This data is shown in figure 12,3.

![Figure 12,3 Cumulative concentration response curves for mucosal and serosal isobutylmethylxanthine (IBMX).](image)

12.2.2.5 Responses to STa and db cAMP

The electrogenic secretory response to 350μM dbcAMP was significantly higher in E- jejuna [median δIsc E+ 30 (95% CI 20-52) μA cm⁻², E- 52 (36-65) μA cm⁻², n=8, P<0.05]. E coli STa (35 mouse units ml⁻¹) produced a similar increment in Isc in both groups (median δIsc E+ 31 (17-42) μA cm⁻², E- 28 (18-38) μA cm⁻², n=7, P=ns). The data are shown in figure 12,4.
12.2.2.6 Concentration response curves for serotonin
Cumulative concentration response curves for the electrogenic response to 5HT [Fig 12.5] revealed a greater δIsc max in E+ animals [E+ 48±16 μA cm⁻² E- 21±6 μA cm⁻², n=7, P<0.01], and a lower EC₅₀ in E+ jejunum [E+ 1.6x10⁻⁶M (95% CI 1.0-2.5x10⁻⁶M), E- 4.9x10⁻⁶ (95% CI 2.3-10.4 x 10⁻⁶M), n=7, P<0.05].

Pretreatment of the serosal surface with the 5HT₃ antagonist ondansetron (1μM) resulted in a shift of the concentration response curve to the right in E+ jejunum with no change in δIsc max, yet a 10 fold increase in EC₅₀ (P<0.05) [Fig 12.6]. In E-jejunum there were no significant changes in δIsc max or EC₅₀ following pretreatment with ondansetron (δIsc max, E+ 41±12 μA cm⁻², E- 20±6 μA cm⁻²; EC₅₀ E+ 1.02x10⁻⁶M (95% CI 0.6-1.57 x 10⁻⁶M), E- 0.93x10⁻⁶M (95% CI 0.62-1.39 x 10⁻⁶M), n=8). The EC₅₀'s were comparable in E+ and E- after ondansetron pretreatment.
Figure 12.5 Cumulative concentration response curves for 5HT in stripped jejunum

Figure 12.6 Concentration response curves for 5HT before and after 1µM ondansetron

In figure 12.6 the concentration response curve for 5HT is shifted to the right post ondansetron in the sufficient jejunum, but is not shifted in the deficient jejunum.
12.2.2.7 Responses to noradrenaline

These studies were performed in animals following the "usual" 18 hour fast and not after a 72 hour fast. In this set of experiments, tissues were maximally stimulated with 200μM IBMX and then sequentially exposed to serosal pargyline (a monoamine oxidase inhibitor), tyramine (a false neurotransmitter which releases endogenous noradrenaline stores), noradrenaline (NA) and yohimbine (a selective α₂ adrenergic antagonist). Yohimbine was used to verify that the inhibitory response of NA on Isc was mediated through α₂ adrenergic receptors. In all cases the addition of 50μM serosal yohimbine brought about a ≥90% reversal of the NA effect on Isc. An example of the protocol is illustrated in figure 12.7 below.

![Figure 12.7 Protocol for investigating the inhibitory response to α₂ adrenergic stimulation](image)

250μM serosal pargyline produced a greater decrement in Isc in E+ than E- jejuna. (median δIsc, E+ 47 [33-55] μA cm⁻², E- 25 [23-33] μA cm⁻², n=6, P<0.01). 100 μM tyramine did not significantly alter Isc in either group. Subsequent addition of 100μM serosal noradrenaline brought about a greater decrement in Isc in E+ jejuna (median E+ 28 [18-40] μA cm⁻², E- 12 [9-14] μA cm⁻², n=6, P<0.01). This data is
summarised graphically in figure 12.8.

![Box plot showing change in Isc produced by serosal pargyline and noradrenaline](image)

Figure 12.8 Change in Isc produced by serosal pargyline and noradrenaline

### 12.2.2.8 Electrogenic response to mucosal galactose

Electrogenic galactose absorption was studied by applying one of a number of concentrations of galactose to the mucosal surface of the jejunal preparation coupled with the simultaneous application of an equimolar amount of mannitol to the serosal surface. These studies were carried out after a tissue had been subjected to a secretogogue and Isc had returned back to basal levels. Tissues subjected to a low concentration of mucosal galactose (which elicited a small response) were subsequently exposed to a high concentration (30mM) as a check of tissue viability. Making these measurements towards the end of an experiment in this manner is clearly not as desirable as making them at the start of an experiment. The quality of the measurements were, therefore, such that apparent Michaelis Menten kinetic parameters could not be reliably estimated for the glucose transporter (see chapter 11).

The increase in Isc generated by mucosal galactose measured in this manner was significantly higher in E- jejunum at all concentrations of ≥5mmol. This is shown graphically in figure 12.9 and in tabular form in table 12.2.
Table 12.2. Increment in Isc (µA cm⁻²) produced by mucosal galactose in stripped rat jejunum. n=6-7 for all concentrations.

<table>
<thead>
<tr>
<th></th>
<th>5 mmol</th>
<th>10 mmol</th>
<th>20 mmol</th>
<th>30 mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUFFICIENT</td>
<td>10.5 (±1.5)</td>
<td>34.5 (±11.0)</td>
<td>61.0 (±18.5)</td>
<td>75.5 (±18.5)</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>19.0 (±6.5)</td>
<td>58.0 (±19.0)</td>
<td>91.5 (±29.0)</td>
<td>112 (±39.5)</td>
</tr>
</tbody>
</table>

P<0.05

12.3 Morphological studies

A segment of jejunum adjacent (distal) to that used in the Ussing chamber experiment was fixed in 4% (v/v) formaldehyde in phosphate buffer. Paraffin sections were cut and stained with haematoxylin and eosin. Computerised morphometric analysis of the sections was performed using an Imagan video system and image analysis software supplied by Kompira. 10 villi / crypts were
measured on each of 4 representative sections from each group of animals. Villus height and mucosal thickness were significantly greater in E- jejuna (table 12.3).

Table 12.3. Morphological characteristics of distal jejunum from 6 month old vitamin E sufficient and deficient rats (lard based diets).

<table>
<thead>
<tr>
<th></th>
<th>E sufficient</th>
<th>E deficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus height / μM</td>
<td>587 ± 72</td>
<td>684 ± 64</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>Crypt depth / μM</td>
<td>167 ± 28</td>
<td>182 ± 24</td>
<td>NS</td>
</tr>
<tr>
<td>Villus / crypt ratio</td>
<td>3.54 ± 0.78</td>
<td>3.80 ± 0.68</td>
<td>NS</td>
</tr>
<tr>
<td>Mucosal thickness / μM</td>
<td>755 ± 78</td>
<td>867 ± 68</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

12.4 Biochemical studies

12.4.1 Apical membrane hydrolases
The activities of the mucosal hydrolases lactase, sucrase and alkaline phosphatase were similar in the two groups (table 12.4).

Table 12.4. Apical membrane hydrolase activities in mucosal homogenates from the proximal small intestine (n=8 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Lactase (μmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Sucrase (μmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Alkaline phosphatase (U g⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUFFICIENT</td>
<td>25.1 (± 8.1)</td>
<td>55.3 (± 15.9)</td>
<td>396 (± 77)</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>21.1 (± 6.6)</td>
<td>45.5 (± 13.0)</td>
<td>449 (± 86)</td>
</tr>
</tbody>
</table>

12.4.2 Fatty acid composition of the apical membrane
Fatty acid analysis of a total lipid extract prepared from the apical brush border membrane demonstrated a small but significant reduction in the content of docosahexaenoic acid in the E- membranes but no other differences in the fatty acid profiles (table 12.5).
Table 12.5. Fatty acid composition (mol %) of a total lipid extract from the apical brush border membrane (n=8 in each group)

<table>
<thead>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SUFFICIENT</td>
<td>1.19</td>
<td>22.73</td>
<td>26.76</td>
<td>0.50</td>
<td>0.39</td>
<td>11.14</td>
<td>0.37</td>
<td>7.23</td>
<td>27.43</td>
<td>0.53</td>
<td>1.64</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>0.97</td>
<td>21.21</td>
<td>27.01</td>
<td>0.85</td>
<td>0.31</td>
<td>12.20</td>
<td>0.42</td>
<td>6.12</td>
<td>28.40</td>
<td>0.85</td>
<td>1.22</td>
</tr>
</tbody>
</table>

P value NS NS NS NS NS NS NS NS NS NS <0.05

The lipid content and the lipid / protein ratios of the apical membrane preparations from the two groups of animals were similar.

12.4.3 Indices of lipid peroxidation
Small intestinal mucosal TBARS were significantly greater in the deficient animals than the sufficient animals [SUFFICIENT 0.78 (±0.10) nmol / mg protein, DEFICIENT 1.22 (±0.11) nmol / mg protein, n=12, P<0.01]. Mucosal concentrations of free MDA were also greater in the vitamin E deficient jejuna than in the vitamin E sufficient counterparts [SUFFICIENT 246 ±46 pmol / mg protein, DEFICIENT 459 (±49) pmol / mg protein, n=12 in each group. P<0.01].

12.4.4 Apical membrane biophysical characteristics
Steady state fluorescence anisotropies measured in apical brush border membranes with both DPH and 12 AS were significantly greater in the vitamin E deficient animals (table 12.6).

Table 12.6. Steady state fluorescence anisotropy of apical brush border membranes. Diphenylhexatriene (DPH) and 12 (anthroloxy) stearic acid (12 AS).

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>0.221 (±0.007)</td>
<td>0.231 (±0.007)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>12AS</td>
<td>0.106 (±0.007)</td>
<td>0.120 (±0.0010)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
12.5 Discussion

12.5.1 Ussing chamber studies
This data provides evidence of a number of changes in ion secretion and absorption in rat jejunum in chronic vitamin E deficiency.

12.5.1.1 Steady state flux studies
The steady state isotopic flux studies demonstrate net secretion of Na and Cl in E- jejuna in the basal (unstimulated) state as compared to net Na and Cl absorption in E+ jejuna. This difference arises through a reduction in the mucosal to serosal flux rates of Na and Cl in E- jejuna (as opposed to an increase in serosal to mucosal flux in E- jejuna) when compared with E+ jejuna. Whilst at first sight this may appear to demonstrate a reduction in absorption in E- jejuna, comparison in this manner may be misleading. The jejunum is a "leaky" epithelium (as opposed to a "tight" epithelium such as colon), and this is reflected in the high unidirectional flux rates across the tissues (for comparison one might expect to find unidirectional fluxes of the order of 10 μmol hr⁻¹ cm⁻² in stripped colon). The lower mucosal to serosal fluxes in the E- jejuna may be a manifestation of a difference in paracellular permeability to these ions as this is most likely the major contributor to the high flux rates. Given that mucosal thickness is significantly higher in the E- tissues it would not be surprising if the passive permeability of the E- tissues to small charged molecules was altered (although the major contributor to this will be the density and size of the epithelial tight junctions). If this were the case then the difference in net ion fluxes could be explained by an increase in secretion in E- jejuna. This issue could be resolved by studies of passive permeability using metabolically inert radiolabelled ions which are not actively transported by a specific carrier protein in the epithelium.

12.5.1.2 Electrogenic responses
The electrogenic secretory responses to dbcAMP, phosphodiesterase inhibition and to non-neuronal cholinergic [muscarinic M3] stimulation were enhanced in E-jejuna. These changes, together with the poor electrogenic response of E- jejuna to the proabsorptive α₂ adrenergic influence of noradrenaline would be maladaptive in individuals with PDD and might serve to enhance secretion. Only jejunum has
been studied here and the effects of vitamin E deficiency upon secretion and absorption in the ileum, a site of great functional small intestinal reserve, will be important in this context. Experience has demonstrated that different parts of the small intestine react in different ways to nutritional deficiencies.\textsuperscript{331} In vivo perfusion studies would also be important to conduct as these would provide complimentary data. From the limited information provided here it would be surprising if vitamin E deficiency per se resulted in diarrhoeal disease. It seems more likely that vitamin E deficiency might serve to enhance a secretory state. In support of this, evidence is provided that vitamin E deficiency enhances the secretory response to fasting in jejunum. Hence, these observations are in keeping with the hypothesis that a deficiency of vitamin E could promote diarrhoea and predispose to the perpetuation of PDD.

The basis for the increase in jejunal secretion as described above remains undefined. It is tempting to speculate that the increase in intestinal secretion in the E-jejuna could be explained on the basis of the morphological changes in the mucosa. Recent evidence suggests that muscarinic secretion (which is \textit{Ca}^{2+} mediated) may occur in the villus\textsuperscript{357} and not in the crypt whilst the cystic fibrosis transmembrane conductance regulator (CFTR), which is a chloride conductance providing a major final common pathway for cyclic nucleotide mediated chloride secretion\textsuperscript{358,359}, is located predominantly in the intestinal crypts\textsuperscript{360}. Other membrane transporters are also asymmetrically distributed along the crypt-villus axis\textsuperscript{361,362}.

Despite this well described "polarisation" of secretory and absorptive events along the crypt-villus axis generalisations about morphology and function are likely to be misleading.

Vitamin E is both an important structural element and an antioxidant in biological membranes. Depletion of vitamin E will therefore render the lipids and proteins of the intestinal mucosa susceptible to damage by oxidative stimuli and this could modify intestinal secretion through a number of mechanisms. Peroxidation of membrane lipids in the intestine in vitamin E deficiency has an effect on the biophysical characteristics of the enterocyte apical membrane. Such an alteration in membrane dynamics in either the apical or the basolateral membrane, might give rise to a change in the functioning of membrane transporter proteins perhaps as a result of altered lipid-protein interactions. This matter has been discussed in chapter 11. Vitamin E also protects membrane proteins from direct oxidative
damage the consequences of which could also modulate epithelial function. Other mechanisms by which oxidative stress might modulate intestinal secretion include release of prostaglandins which act on enterocytes and enteric nerves to cause secretion, an increase in cytosolic calcium, an increase in paracellular permeability, and activation of guanylate cyclase. Observations pertinent to some of these mechanisms have been made during the course of the studies described above.

(i) Addition of either TTX or indomethacin (10⁻⁵M) [prostaglandin synthase inhibitor] to the serosal surface of E+ and E- jejuna had no effect on basal Isc (data not shown).

(ii) The isotopic flux data suggest, if anything, a reduction in paracellular permeability in E- jejuna.

(iii) Previous studies in this animal model failed to demonstrate any differences in the concentrations of cAMP and cGMP in the small intestine in vitamin E deficiency.

12.5.1.3 Electrogenic responses to serosal serotonin

The poor response of E- jejuna to 5HT is of interest. Serotonin is found in high concentrations throughout the small intestine particularly in the enterochromaffin cells. 5HT has a role in modulating intestinal secretion through its influences within the submucosal nerve plexi and both the 5HT₂ and the 5HT₃ receptor are abundant within the enteric nervous system. The pro secretory action of exogenous 5HT involves both cholinergic and non cholinergic neurons and both these receptor subtypes have been implicated in this secretory response. A consensus view seems to be that 5HT has little to do with "intrinsic transport tone" in the muscle stripped rat small intestine but that it is released postprandially from within the enteric nerves to bring about a coordinated increase in intestinal motility, secretion and blood flow.

Chronic vitamin E deficiency causes a characteristic neuropathy in both humans and the rat which may also effect the gastrointestinal tract. Hence an enteric neuropathy in the E- jejuna might explain, at least in part, the diminished responsiveness of these jejuna to 5HT. The studies with the selective 5HT₃ antagonist, ondansetron, provide some tentative evidence that this receptor is not involved in the electrogenic secretory response to 5HT in E- jejuna whereas it is...
involved in E+ jejuna. Does this imply a selective problem with the 5HT₃ receptor in vitamin E deficiency? There is emerging evidence that vitamin E deficiency selectively affects aminergic neurotransmitters at an early stage in the central nervous system⁴ and this could be a manifestation of the same phenomenon. It would be informative to perform 5HT₃ receptor binding studies to evaluate this idea further. The secretory response to STa in perfusion studies in vivo is mediated in part via the serotinergic 5HT₃ receptor. Vitamin E deficiency had no effects upon the electrogenic secretory response to STa in the present studies. The evidence, at present suggests, however, that the enteric nervous system in which the 5HT₃ receptors are found is only involved in the electrogenic response to STa following a fast.³⁴³

Serotonin is clearly an important regulator of small intestinal secretion, and activation of the serotinergic nervous system is generally believed to tip the balance between absorption and secretion in favour of secretion. The evidence available here suggests that there may be a perturbation of this system within the small intestine in vitamin E deficiency. A clarification of the many issues raised in this section will, however, require further studies of secretion and absorption both in vivo and in vitro together with complimentary biochemical, histochemical and receptor binding studies.

12.5.1.4 Electrogenic responses to noradrenaline

Fasting animals for 72 hours is known to increase small intestinal Isc although the origins of this change remain incompletely understood. Part of this increase may arise through reduced release of NA from sympathetic afferent fibres within the enteric nervous system. The secretory response to fasting was increased in the E- jejuna. Hence the interest in studying the proabsorptive actions of exogenous noradrenaline in this group of animals.

It is interesting to note that in this same animal model after a period of 9 months vitamin E deficiency the release of [³H] NA from nerve terminals within the caecum muscularis is decreased and that this is associated with an increase in the NA content of caecal muscle. The observations here of reduced responsiveness to

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³⁴³ Dexter D.T. - personal communication

³⁴³ Charles Hoyle, Department of Anatomy, University College, London. Unpublished data.
exogenous NA and of a larger increment in Isc on fasting in E-jejuna may therefore be different manifestations of an alteration in catecholamine turnover within the enteric nervous system in vitamin E deficiency. The smaller response to pargyline in E-jejuna is difficult to interpret as this may reflect in part a reduced responsiveness of these tissues to NA. The observations reported above of a predilection of the aminergic neurotransmitter system to the effects of vitamin E deficiency\textsuperscript{374} may also be of relevance.

### 12.5.1.5 Electrogenic galactose transport

The increase in the electrogenic transfer of mucosal galactose compares well with the increase documented in the preliminary studies after 12 months of vitamin E deficiency. The reasons for this have been discussed in chapter 11. The increase in villus height seen in this cohort of animals may also be of relevance to this phenomenon. A recent report\textsuperscript{375} in which guinea pig brush border membrane vesicles were subjected to ferrous sulphate (200\textmu M) / ascorbic acid (2 mM) induced peroxidation in vitro has indicated that this stimulus may bring about a reduction in Vmax for glucose transport in the presence of a proton gradient associated with a (nearly significant) reduction in Km for the transporter. Approximately 30% of the decline in Vmax could be reversed by "fluidisation" of the membrane vesicles with A\textsubscript{2}C [2-(2-methoxyethoxy)ethyl 8-(cis-2-n-octyl-cyclopropyl) octanoate ] i.e by reversing the change in membrane biophysical characteristics which accompanied the peroxidation of the membrane vesicles. The levels of peroxidation produced in these tissue preparations were, however, much higher than those found in the present studies of vitamin E deficiency [i.e TBARS = approximately 5 nmol MDA mg\textsuperscript{-1} protein as compared to 1.2 nmol mg\textsuperscript{-1} in the E-mucosal homogenates]. It is also difficult to extrapolate from vesicle studies to studies of whole epithelia in which the generation of the sodium gradient to drive sodium coupled glucose transport may also be affected by the oxidative stress imposed on the system.

### 12.5.2 Morphological studies

The increase in villus height and mucosal thickness seen in the E-jejuna are not easily explained. It is of interest that the jejunal morphology in the 12 month E-animals did not demonstrate villus lengthening. 12 month old rats are "old" rats and...
the differences between the 6 and 12 month old rats may arise because of senescence. Alternatively, the 12 month E- rats were significantly malnourished when compared with both the 6 month E+/E- and the 12 month E+ rats. This will also influence mucosal morphology.

12.5.3 Biochemical studies
The findings of increased indices of lipid peroxidation and of an increase in the values for fluorescence anisotropy with both 12 AS and DPH are perhaps what one might expect in the light of the data presented in chapter 11 and these issues have therefore already been discussed. It is worth noting that in the studies of 6 month old rats described in this chapter the differences in the anisotropy values were much smaller than the changes described in chapter 11. This is, perhaps, what one might expect given the relatively small changes in anisotropy found in the studies of Jourd'heuil in the face of considerable oxidative damage. The larger differences seen in the older cohort of animals may have related to the altered lipid / protein ratio seen in the E- group, or perhaps due to the effects of ageing or malnutrition on these parameters rather than to higher levels of peroxidative damage.

12.6 Closing remarks
This chapter provides evidence that vitamin E deficiency is associated with abnormalities of intestinal secretion and absorption and that muscarinic M3, serotonergic 5HT3, and cAMP mediated secretory mechanisms and α2 adrenergic proabsorptive mechanisms are affected. Whatever the origins of the vitamin E deficiency in infants and children with protracted diarrhoea a strong case can be made for early and aggressive replacement therapy with vitamin E and other vitamins / trace metals which help provide defences against oxidative stress.
CHAPTER 13

Biochemical and physiological studies in stripped jejunum after 6 months on lard based or corn oil based diets (vitamin E sufficient animals)

13.1 Introduction

A recent Government White Paper, "The Health of the Nation", and published DHSS guidelines in the United Kingdom\textsuperscript{377} make specific recommendations about both the quantity and the composition of fat in the diet. In short, these entail a reduction in the total number of fat derived calories, a reduction in the amount of saturated fat in the diet to no more than 10\% and an increase in the amounts of monounsaturated and polyunsaturated fats in the diet. The rationale for these recommendations relies to a large extent on epidemiological data relating to morbidity and mortality from cardiovascular disease and cancer\textsuperscript{377}.

In addition to providing an important energy source, lipids are an important structural component of plasma and intracellular organelle membranes. The effect of these specific dietary recommendations upon the structure and functions of the cell membrane are less clearly defined. It is well established that the quantity and quality of dietary fat can influence the composition of the enterocyte plasma membrane\textsuperscript{378} as well as small intestinal morphology and gut regulatory peptides (somatostatin and substance P)\textsuperscript{379}. Manipulation of the fat composition and content of the diet has previously been shown to alter the transport of monosaccharides\textsuperscript{380} and amino acids\textsuperscript{378} and to modify the secretory response to cholera toxin\textsuperscript{381} in the small intestine. Dietary fat may also influence the activity of alkaline phosphatase\textsuperscript{382} and modulate membrane fluidity\textsuperscript{353}. It seemed appropriate, therefore, to study the effects of an increased dietary intake of polyunsaturated fats upon small intestinal structure and secretory function.

13.2 Animal model and diet

Weanling male Wistar rats were fed isocaloric diets containing approximately 10\% fat as described in chapter 2. In one group the fat source was tocopherol stripped lard ("LARD") and in the other tocopherol stripped corn oil ("CORN"). Vitamin E (\(\alpha\) tocopheryl acetate) was added to both feeds (40mg / kg of feed).
The fatty composition of the diets is shown in table 13.1.

Table 13.1. Fatty composition of diets (mol %)

<table>
<thead>
<tr>
<th></th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>16:1</th>
<th>18:1</th>
<th>20:1</th>
<th>18:2</th>
<th>18:3</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td>1.6</td>
<td>26.8</td>
<td>15.6</td>
<td>2.5</td>
<td>40.7</td>
<td>0.8</td>
<td>8.7</td>
<td>0.1</td>
</tr>
<tr>
<td>CORN OIL</td>
<td>0.3</td>
<td>8.5</td>
<td>1.7</td>
<td>0.4</td>
<td>29.8</td>
<td>0.6</td>
<td>59.6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

The corn oil based diet contained larger amounts of linoleic acid (18:2) and the lard based diet larger amounts of palmitic (16:0), stearic (18:0) and oleic (18:1) acids. The lard based diet also contained approximately 0.1% cholesterol, and the corn oil based diet none (manufacturers data). The diets were not, therefore, isocholesterolic. The corn oil based diets were originally set up to study the effects of vitamin E deficiency in membranes containing an increased amount of peroxidisable (unsaturated) lipid. The cohort was not set up with the intention of studying the effects of dietary fat manipulation per se, although this was inevitable and proved to be interesting.

Animals were fed these diets ad libitum as described previously. They were subjected to metabolic balance studies after approximately 20 weeks on the diet and jejunum was studied in vitro after 21-23 weeks on the diet [ie aged 24-26 weeks]. All animals were fasted for 18 hours before anaesthesia and study to ensure that measurement of the fatty composition of the apical membrane was not influenced by unabsorbed fatty acids free in the gut lumen. The procedures for stripping and mounting of tissues in the Ussing chambers and of constructing concentration response curves for secretogogues have been described in earlier chapters.
13.3 Results

13.3.1 Growth, dietary intake and stool output

Weight gain (figure 13.1), dietary intake and stool output of the two groups of animals were similar.

After 21 weeks on the respective diet the weights of the two groups were: LARD 599g (95% confidence interval ±28), CORN 614g ±38 (n = 24 in each group, P = NS). Dietary intakes in the two groups were [ LARD 29.8 ± 3.0, CORN 31.8 ± 1.5 g 24hr⁻¹].

![Figure 13.1 Longitudinal weights of animals fed lard or corn oil based diets.](image)

13.3.2 Ussing chamber studies

13.3.2.1 Basal electrical characteristics

Basal short circuit current (Isc), tissue resistance (Rt) and transepithelial potential (Vt) were similar in the two groups (table 13.2).
13.3.2.2 Cumulative concentration response curves for secretogogues

Cumulative concentration response curves were constructed for acetylcholine (ACh), bethanecol (BCh), and isobutylmethylxanthine as described in chapter 12. The ACh concentration response curves were carried out in tissues pretreated with serosal TTX (1.25 μM) and neostigmine (10⁻⁴ M), and the IBMX and BCh curves in tissues pretreated with serosal TTX as described in chapter 12. The EC₅₀ for all three of these secretogogues was significantly lower in jejunum from the CORN fed animals yet the maximal electrogenic responses (δIsc_max) for all three agents were similar in both groups (table 13,3 and figures 13,2, 13,3 and 13,4).

Table 13,3. Electrogenic secretory responses and EC₅₀

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>LARD</th>
<th>CORN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (n=8)</td>
<td>EC₅₀</td>
<td>5.60 x 10⁻⁷ M (3.49-9.13)</td>
</tr>
<tr>
<td></td>
<td>δIsc_max</td>
<td>119 (±24) μA cm²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>BCh (n=7)</td>
<td>EC₅₀</td>
<td>2.21 x 10⁻⁸ M (1.41-3.47)</td>
</tr>
<tr>
<td></td>
<td>δIsc_max</td>
<td>90 (±16) μA cm²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P&lt;0.02</td>
</tr>
<tr>
<td>IBMX (n=7)</td>
<td>EC₅₀</td>
<td>8.04 x 10⁻⁸ M (5.96-10.80)</td>
</tr>
<tr>
<td></td>
<td>δIsc_max</td>
<td>159 (±21) μA cm²</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>
Figure 13.2 Cumulative concentration response curves for acetylcholine

Figure 13.3 Cumulative concentration response curves for bethanecol

Figure 13.4 Cumulative concentration response curves for IBMX
13.3.2.3 Concentration response curves for serotonin

Cumulative concentration response curves for 5HT were constructed in tissues which had not been pretreated with any agent (figure 13.5).

![Graph showing concentration response curves for serotonin](image)

**Figure 13.5** Cumulative concentration response curves for 5HT

In this instance $\delta I_{sc, max}$ was greater in the LARD group than the CORN group and the $EC_{50}$ was significantly lower in the LARD group ($\delta I_{sc, max}$ LARD 48±16 $\mu$A cm$^{-2}$, CORN 33±8 $\mu$A cm$^{-2}$, n = 8, P<0.05: $EC_{50}$ LARD 1.6 x $10^{-6}$ M (95% CI 1.0 - 2.5 x$10^{-6}$ M), CORN 1.69 x $10^{-5}$ M (95% CI 0.75 - 3.8 x $10^{-5}$ M), P<0.05).

13.3.2.4 Responses to dbcAMP, STa, pargyline and noradrenaline.

The electrogenic secretory responses to mucosal E coli STa (60 mouse units ml$^{-1}$) were similar in the two groups of jejuna (Figure 13.6), as were the responses to 350$\mu$M serosal db cAMP (Figure 13.7).

The median responses to STa were LARD 31 $\mu$A cm$^{-2}$ (95% CI 17-42), CORN 28$\mu$A cm$^{-2}$ (95% 10-35), P>0.05, n = 8 in each group (Mann Whitney U test) and the median responses to dbcAMP LARD 30$\mu$A cm$^{-2}$ (95% 20-52), CORN 32 $\mu$A cm$^{-2}$ (95% 24-53) P>0.05, n = 7 in each group. The responses of tissues maximally stimulated with IBMX to pargyline and 100$\mu$M serosal noradrenaline (as outlined
in chapter 12) were also similar. [Pargyline; LARD median 48 (33-55) μA cm⁻², CORN 38 (28-53) μA cm⁻², n = 7-8, P>0.05: NA LARD 28 (15-40) μA cm⁻², CORN 28 (18-40) μA cm⁻², n = 8 in each group, P>0.05].

Figure 13.6 Response to E coli STa (60 mouse Figure 13.7 Response to 350 μM db cAMP units ml⁻¹)

13.3.2.5 Electrogenic responses to mucosal galactose
The electrogenic responses to mucosal galactose are shown in table 13.4 and figure 13.8. The electrogenic responses to 2.5, 5 and 10 mM mucosal galactose were significantly higher in the CORN group.

Table 13.4. Electrogenic responses to various concentrations of mucosal galactose.

<table>
<thead>
<tr>
<th>Galactose</th>
<th>LARD</th>
<th>CORN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 mM</td>
<td>-</td>
<td>6 (±4) μA cm⁻²</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>2.5 (±2) μA cm⁻²</td>
<td>13 (±9) μA cm⁻²</td>
</tr>
<tr>
<td>5 mM</td>
<td>10.5 (±1.5) μA cm⁻²</td>
<td>32 (±12) μA cm⁻²</td>
</tr>
<tr>
<td>10 mM</td>
<td>34.5 (±11) μA cm⁻²</td>
<td>55 (±32) μA cm⁻²</td>
</tr>
<tr>
<td>20 mM</td>
<td>61.0 (±18) μA cm⁻²</td>
<td>71 (±36) μA cm⁻²</td>
</tr>
<tr>
<td>30 mM</td>
<td>75.5 (±18) μA cm⁻²</td>
<td>83 (±39) μA cm⁻²</td>
</tr>
</tbody>
</table>
Figure 13.8 Electrogenic response to mucosal galactose in stripped jejunum

13.3.3 Morphological studies
These were performed as described in chapter 12. There were no differences between the two groups of jejunum (table 13.5).

Table 13.5. Morphological characteristics of distal jejunum from 6 month old vitamin E sufficient rats fed lard or corn oil based diets.

<table>
<thead>
<tr>
<th></th>
<th>LARD</th>
<th>CORN</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Villus height / μM</td>
<td>587 ± 72</td>
<td>605 ± 72</td>
<td></td>
</tr>
<tr>
<td>Crypt depth / μM</td>
<td>167 ± 28</td>
<td>161 ± 28</td>
<td></td>
</tr>
<tr>
<td>Villus / crypt ratio</td>
<td>3.54 ± 0.78</td>
<td>3.86 ± 0.68</td>
<td></td>
</tr>
<tr>
<td>Mucosal thickness / μM</td>
<td>755 ± 78</td>
<td>766 ± 78</td>
<td></td>
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</tbody>
</table>

13.3.4 Biochemical studies

13.3.4.1 Apical membrane hydrolase activities
The activities of the mucosal hydrolases lactase, sucrase and alkaline phosphatase were similar in mucosal scrapings prepared from both groups of jejunum (table 13.6).
Table 13.6. Apical membrane hydrolase activities in mucosal homogenates from the proximal small intestine

<table>
<thead>
<tr>
<th></th>
<th>Lactase (µmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Sucrase (µmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Alkaline phosphatase (U g⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td>25.1 (± 8.1)</td>
<td>55.3 (± 15.9)</td>
<td>396 (± 77)</td>
</tr>
<tr>
<td>CORN</td>
<td>21.5 (± 4.6)</td>
<td>42.0 (± 5.4)</td>
<td>438 (± 78)</td>
</tr>
</tbody>
</table>

13.3.4.2 Fatty acid composition of the apical membrane

Fatty acid analysis of the total lipid extract from brush border membranes prepared from the proximal small intestine demonstrated increased levels (mol %) of linoleic acid (18:2) and docosatetraenoic acid (22:4) and decreased levels of docosahexaenoic acid (22:6) and oleic acid (18:1) in animals fed the corn oil based diet (table 13.7).

Table 13.7. Fatty acid composition (mol %) of a total lipid extract from the apical brush border membrane

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td>1.19</td>
<td>22.73</td>
<td>26.76</td>
<td>0.50</td>
<td>0.39</td>
<td>11.14</td>
<td>0.37</td>
<td>7.23</td>
<td>27.43</td>
<td>0.53</td>
<td>1.64</td>
</tr>
<tr>
<td>±0.79</td>
<td>±3.18</td>
<td>±2.26</td>
<td>±0.05</td>
<td>±0.24</td>
<td>±1.94</td>
<td>±0.14</td>
<td>±1.47</td>
<td>±3.48</td>
<td>±0.19</td>
<td>±0.35</td>
<td></td>
</tr>
<tr>
<td>CORN</td>
<td>1.38</td>
<td>20.40</td>
<td>24.29</td>
<td>0.59</td>
<td>0.43</td>
<td>8.80</td>
<td>0.29</td>
<td>16.91</td>
<td>24.65</td>
<td>1.30</td>
<td>0.76</td>
</tr>
<tr>
<td>±0.65</td>
<td>±4.34</td>
<td>±3.52</td>
<td>±0.13</td>
<td>±0.22</td>
<td>±2.09</td>
<td>±0.26</td>
<td>±2.38</td>
<td>±3.96</td>
<td>±0.59</td>
<td>±0.18</td>
<td></td>
</tr>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

13.3.4.3 Apical membrane biophysical characteristics

Steady state fluorescence anisotropies were measured with the two probes DPH and 12-AS as discussed previously. Anisotropy values were lower with both probes in the CORN group of animals although this difference did not achieve statistical significance with DPH (table 13.8).
Table 13.8. Steady state fluorescence anisotropy of apical brush border membranes. Diphenylhexatriene (DPH) and 12 (anthroloxy) stearic acid (12 AS).

<table>
<thead>
<tr>
<th></th>
<th>LARD</th>
<th>CORN</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>0.221 (±0.007)</td>
<td>0.212 (±0.012)</td>
<td>NS</td>
</tr>
<tr>
<td>12AS</td>
<td>0.106 (±0.007)</td>
<td>0.097 (±0.010)</td>
<td>P&lt;0.05</td>
</tr>
</tbody>
</table>

13.3.4.4 Indices of lipid peroxidation

Mucosal TBARS were significantly higher in the LARD jejunum (LARD 0.78 ± 0.10 nmol / mg protein, CORN 0.58 ± 0.09 nmol / mg protein, n=12 in each group, P<0.05). Free MDA measured by HPLC was also lower in the CORN group than in the LARD group (LARD 246 ± 46 pmol / mg protein, CORN 126 ± 22 pmol / mg, n = 12 in each group, P<0.05).

13.4 Discussion

13.4.1 General observations and summary of data

It is well established that manipulating the lipid composition of the diet can lead to a change in the lipid composition of cell membranes within numerous tissues of the body. Changing the lipid composition of cell membranes may have effects upon the physiology of these tissues and membranes both in vivo and in vitro. This study has demonstrated that a qualitative modification of the fatty acid composition of the diet leads to an alteration in the fatty acid composition of the enterocyte apical membrane. This dietary manipulation is associated with a change in the biophysical characteristics of the apical membrane and a change in electrogenic secretion and absorption. When jejunal epithelium was studied as a muscle stripped preparation under conditions of neural blockade (with TTX) the electrogenic secretory response to a number of agents was increased at submaximal levels of stimulation, yet the maximal electrogenic secretory responses were unchanged. Contrasting effects were apparent when studying the electrogenic secretory response to serotonin, which is dependent upon the release of neurotransmitters (acetylcholine) from the enteric nerves. In this instance the
maximum secretory response is reduced and the EC_{50} increased. The two indices of lipid peroxidation used in this study were not elevated but rather depressed by the high PUFA intake. The effects of a high PUFA intake in the presence of compromised vitamin E status will be discussed in chapter 14.

13.4.2 Relevance of the study in the context of nutritional rehabilitation

The nutritional rehabilitation of infants with protracted diarrhoea often involves the administration of infant formula feeds in which the protein (eg pepdite, prejomin, peptijunior, wysoy, pregestamil), the carbohydrate (eg wysoy, pregestamil) or the lipid (eg pregestamil, nutramigen) component have been highly modified when compared with a standard term infant formula feed or naturally occurring breast milk which the latter is trying to emulate. For example, the fat source used in nutramigen is corn oil, and the fat source in pregestamil is a mixture of medium chain triglyceride and corn oil. This is evident by the increased amounts of linoleic acid found in these feeds as shown in figure 13.9 (manufacturers data).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Human milk</th>
<th>Cow and Gate Premium</th>
<th>Pregestamil</th>
<th>Nutramigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0</td>
<td>-</td>
<td>-</td>
<td>25.8</td>
<td>-</td>
</tr>
<tr>
<td>10:0</td>
<td>1.4</td>
<td>1.5</td>
<td>13.0</td>
<td>-</td>
</tr>
<tr>
<td>12:0</td>
<td>5.4</td>
<td>9.8</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>14:0</td>
<td>7.4</td>
<td>5.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16:0</td>
<td>26.5</td>
<td>20.5</td>
<td>6.9</td>
<td>11.5</td>
</tr>
<tr>
<td>18:0</td>
<td>9.5</td>
<td>4.2</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>16:1</td>
<td>4.0</td>
<td>0.5</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>18:1</td>
<td>35.5</td>
<td>38.5</td>
<td>16.9</td>
<td>28.3</td>
</tr>
<tr>
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<td>7.2</td>
<td>17.0</td>
<td>33.8</td>
<td>56.3</td>
</tr>
<tr>
<td>18:3</td>
<td>0.8</td>
<td>0.3</td>
<td>0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The fatty composition (mol %) of the LARD and CORN oil based diets are similar to that of breast milk and nutramigen respectively\textsuperscript{390,391}. It was, therefore, of interest
to compare the effects of a LARD versus a CORN oil based diet upon gastrointestinal secretion and absorption. Whilst the duration that rats were fed their respective diet is long in the lifetime of a laboratory rat, the period of time used reflects the mean time of hospitalisation of infants undergoing nutritional rehabilitation for protracted diarrhoea at the hospital for Sick Children. There is evidence to suggest that the change in fatty composition of the enterocyte membrane will occur within 8 weeks of manipulating the diet, and that the time course of the change is probably much quicker than this. The longer time chosen in the present study was to allow the group of animals fed corn oil based diets which were deficient in vitamin E to become chronically vitamin E deficient (see chapter 14). This time scale was used for all the studies reported in chapters 12-14.

13.4.3 Electrogenic secretion and absorption

13.4.3.1 Secretory responses to cholinomimetics and cyclic nucleotides
The decrease in EC$_{50}$ in the CORN group with ACh, BCh, and IBMX represents an increased propensity to secretion in these jejuna in response to these stimuli. This decrease in EC$_{50}$ was not associated with a change in the maximal electrogenic secretory response. ACh and BCh cause secretion through a different mechanism to IBMX. Under these conditions (with the enteric nerves blocked with TTX), ACh and BCh cause secretion via actions on muscarinic M$_3$ receptors which are located principally on the lower two thirds of the villus within the small intestine, whereas IBMX raises tissue concentrations of cAMP by the specific inhibition of phosphodiesterase. The cystic fibrosis transmembrane conductance regulator (CFTR), which is located in the intestinal crypts, is the final membrane associated pathway for a major portion, although probably not all, of the cAMP mediated chloride secretion. The increased propensity to secretion in the CORN fed animals could therefore arise through the effects of the dietary manipulation upon cells in both villus and crypt.

The similar Isc in the two groups in response to the stable cAMP analogue dbcAMP may relate to the relatively low concentration of this secretogogue used in these studies and the position of this concentration on the concentration response curve. Concentration - response curves were not constructed for
dbcAMP. Similar observations may apply to the comparable responses to STa in the two tissues. STa causes secretion through the activation of the endogenous guanylin receptor and activation of guanylate cyclase. It is not possible to explain the decrease in EC\textsubscript{50} for ACh and BCh in the CORN group in terms of an increase in muscarinic receptor affinity without performing receptor binding studies with an appropriate ligand. IBMX does not work via a cell surface receptor and hence the decrease in EC\textsubscript{50} in this instance will not be due to a cell surface receptor mediated event. A unifying explanation for the decrease in EC\textsubscript{50} might be an increased driving force for secretion in the CORN group. The electrochemical gradient for apical chloride secretion in the cells of the small intestine is generated by the joint actions of basolateral Na\textsuperscript{+}K\textsuperscript{+}ATPase and the Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} co transporter. There has been a traditional view that Na\textsuperscript{+}K\textsuperscript{+}ATPase provides the main driving force behind secretion by virtue of the fact that it is both electrogenic and consumes energy, and as such has a pivotal role in generating the electrochemical gradient for Cl\textsuperscript{−} secretion. Feeding rats a diet supplemented with polyunsaturated fats has previously been shown to increase the activity of Na\textsuperscript{+}K\textsuperscript{+}ATPase and this might offer an explanation for the heightened secretion (and increased sodium coupled glucose absorption) in the CORN group of animals. The activity of Na\textsuperscript{+}K\textsuperscript{+}ATPase is known to change along the crypt - villus axis and an alteration in small intestinal morphology, however subtle, might also result in an alteration in this electrochemical driving force for Cl\textsuperscript{−} secretion.

The recent observation that the basolateral Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} co transporter is a regulated channel adds a further perspective to the observation that an alteration in the activity of membrane transporters and pumps may follow manipulation of membrane lipid composition by dietary means. Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} cotransport can be stimulated either directly by cAMP or else as a secondary event following G - protein activation. An upregulation of Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} transport could heighten the electrochemical gradient for chloride secretion via both cAMP and Ca\textsuperscript{2+} activated Cl\textsuperscript{−} channels and so lead to an increase in secretion. The activity of adenylate cyclase, which regulates the Na\textsuperscript{+}K\textsuperscript{+}2Cl\textsuperscript{−} cotransporter may also be modulated by its lipid environment.

On teleological grounds, however, one would expect that an increased electrochemical gradient for Cl secretion would be associated with increased secretory maxima which have not been observed in these studies.
13.4.3.2 Proabsorptive action of noradrenaline
The electrogenic response to noradrenaline was similar in the two groups of jejuna. This response is likely to involve a number of different ion channels and intracellular mediators. The proabsorptive $\alpha_2$ adrenergic effects of noradrenaline involve a reduction in net $\text{HCO}_3^-$ secretion and an increase in Na and Cl absorption and are manifest (in part) as a reduction in intestinal Isc. Recent evidence suggests that $\alpha_2$-adrenergic stimulation activates $\text{HCO}_3^-$ absorption in the villus ($\text{HCO}_3^-$ being secreted in the crypts) to bring about the reduction in net $\text{HCO}_3^-$ secretion. The intracellular events which bring about these changes in secretion and absorption remain unclear but may involve an inhibition of adenylate cyclase through the activation of $G_i$ (a transduction unit of a membrane associated GTP binding protein heterotrimer) or an inhibition of a $\text{Ca}^{2+}$ dependent mechanism, although several other mechanisms have been suggested.

13.4.3.3 Electrogenic secretory responses to serotonin
This data demonstrates a reduction in the maximal secretory response and an increase in the EC_{50} for the electrogenic response to serotonin. This data is fundamentally different from the remainder of the data shown in that the enteric nervous system is pharmacologically intact in these preparations and the electrogenic response is being mediated through the enteric nerves (see chapter 12 for a fuller discussion). Given that the final common pathway for 5HT mediated secretion will involve the release of ACh from the submucosal plexus the finding of this attenuated response is puzzling.
Serotonergic nerves are involved in the secretory response to cholera toxin, and a modulation of serotonergic transmission might explain the diminished secretory response to this toxin found in perfusion studies by Saggers group.

13.4.3.3 Electrogenic galactose absorption
The higher rates of electrogenic galactose absorption in the CORN fed group could arise through a variety of mechanisms. Thompson et al fed 250g rats diets containing 20% fat which were either high in polyunsaturated or saturated fats for 3 weeks and then measured glucose uptake in the jejunum using a rapid uptake apparatus. They found higher rates of glucose uptake in jejuna from rats fed the high polyunsaturated fatty acid diet at glucose concentrations of 2.5, 5, and 20mM.
Similar studies in the ileum of the same animals, however, demonstrated no differences in glucose uptake.

The electrochemical gradient for sodium which drives sodium coupled hexose absorption by the specific apical membrane transporter protein SGLT1 is provided by the basolateral enzyme Na⁺K⁺ATPase\(^{413}\). Changes in the activity of Na⁺K⁺ATPase have been described in animals fed diets high in polyunsaturated fats. An increase in the activity of this transporter may increase intestinal sugar absorption. There is a gradient of SGLT1 activity along the crypt - villus axis\(^{414,415}\). This is due to a gradient of expression of the protein at the cell surface\(^{416,417}\) and also to a change in the kinetic properties (Vmax and Km) of the transporter along the villus axis\(^{418,419}\). The change in these kinetic properties has been attributed to a modulation of the lipid environment of the transporter protein along the crypt villus axis rather than the existence of two different types of sugar transporter\(^{418}\), although this view has been challenged\(^{419}\).

The kinetics of the sugar transporter in brush border membrane vesicles prepared from along the crypt villus axis have not been studied here but this would clearly be of great interest. Recent evidence also suggests that the intracellular metabolism of absorbed glucose has an effect on the Vmax of SGLT1 (but not on the Km of the transporter)\(^{420}\).

### 13.4.3.5 Further observations

#### 13.4.3.5.1 Perfusion studies

Sagher et al\(^{581}\) fed weanling rats for 8 weeks on isocaloric diets containing 20% fat which was either derived from corn oil or butter and performed steady state perfusions of the jejunum (20 cm segment starting at the DJ flexure). On this high fat regimen the animals exhibited net jejunal water, Na and Cl secretion in the saturated fat group but net absorption in the corn oil fed group with no change in glucose absorption (1.6 mM). Two hours after the instillation of 75 μg of cholera toxin the animals fed corn oil exhibited reduced secretion of sodium but not chloride when compared with the animals fed on the butter fed diet. These perfusion studies are not easy to reconcile with the data presented in this chapter. It should be emphasised that Saghers diets were quite different from those used here.
Despite the fact that perfusion studies measure a different variable to Ussing chamber studies, one would have hoped that electrogenic chloride secretion in vitro might bear some relationship to total chloride secretion in vivo. The responses to cholera toxin are also interesting because it is clear from the data presented by these authors (although raw data is not provided) that a number of the animals fed on corn oil must have exhibited net sodium absorption in response to the toxin! The data of glucose absorption is difficult to interpret because of the low concentrations used in these perfusion studies.

**13.4.3.5.2 Ussing chamber studies**

Further studies by this same group\textsuperscript{392} using the same animal model and diet for 11 weeks from weaning in which unstripped jejunum was studied in vitro in an Ussing chamber demonstrated a significant increase in basal intestinal short circuit current in the corn oil fed group when compared with the butter fed group. The responses of these tissues to secretogogues is difficult to evaluate because of problems with the design of their experiments and in presentation of results. It is clear, however, that the corn oil fed group exhibited a greater electrogenic secretory response to 1mM serosal acetylcholine. They did not report the electrogenic response to mucosal hexose sugars.

**13.4.3.5.3 Peptide neurotransmitters**

Sagher et al\textsuperscript{379} have also compared the effects of the 20% butter and corn oil diets upon small intestinal morphology and peptide neurotransmitters. The corn oil fed group had longer villi and longer crypts than the butter fed group although the villus / crypt ratios were similar in the two groups [3.2 vs 3.8]. No morphological differences were apparent in studies by other groups of rats fed similar diets to those in Saghers studies\textsuperscript{378}. Sagher also noted that the corn oil group had higher levels of substance P in the proximal small intestine. This finding is interesting because the serosal addition of substance P to rat small intestine in vitro has a prosecretory action\textsuperscript{421,422,423}.

**13.4.3.5.4 Eicosanoid synthesis**

Eicosanoids, which are synthesised from long chain polyunsaturated fatty acids are important modulators of intestinal secretion and are in general prosecretory through
actions both directly on the enterocyte and also via the enteric nerves. Modulating dietary lipid intake can lead to alterations in the gastrointestinal mucosal content and composition of lipids, and this can have an effect upon eicosanoid production. Sagher and colleagues were unable to demonstrate any effect of dietary lipid (corn vs butter) on the luminal production rate of PGE$_2$ and PGF$_{2\alpha}$ in their perfused rat jejuna. In the present study, feeding the corn oil based diet resulted in a twofold increase in apical enterocyte membrane linoleic acid but this was not associated with a change in membrane associated arachidonic acid, the substrate for cyclo oxygenase and hence prostaglandin production. The significance of this is, however, unclear as the majority of eicosanoids in the small intestine are derived from cells within the subepithelium and not from the enterocytes themselves.

13.4.3.5.5 Regulation of secretion
The effect of diet on the numerous intracellular regulators of intestinal secretion and absorption is largely unknown. A number of these, for example diacyl glycerol and the inositol phosphates, are derived from the hydrolysis of membrane phospholipids by specific phospholipases some of which are activated by receptor coupled G proteins. Inositol triphosphates are involved in the mobilisation of intracellular calcium stores and diacyl glycerols in the activation of protein kinase C and hence both of these substances may modulate intestinal secretion. Recent evidence has demonstrated that manipulation of dietary lipid intake may lead to a change in the production of inositol phosphates and diacylglycerols in neutrophils in response to various stimuli. There is little literature concerning changes in the levels of these regulatory substances within the enterocytes in response to dietary manipulation. It has recently been noted that protein kinase C, which has a major role in the control of cell proliferation as well as intestinal secretion, can be induced by dietary cis - unsaturated fatty acids. Animals fed the LARD based diet demonstrated higher levels of oleic acid in the apical membrane than those fed the CORN diet. It is not known whether this has any effect on the intracellular signalling cascades. Fatty acids are also important regulators of cell surface charge and this may have an influence upon intestinal secretion.
13.4.3.5.6 Membrane trafficking
Modulation of the fluidity of the endoplasmic reticulum and Golgi apparatus may lead to an alteration in the post translational modification of proteins-supplied by Golgi apparatus, and many of the transmembrane proteins involved in secretion by the cell are known to undergo post translational modification, although this is not so with SGLT1-supplied by SGLT1. The occurrence and influence of such changes in the rat model described here have not been evaluated.

13.4.3.5.7 Programming
The early feeding experience of animals and humans may have a lifelong influence upon growth, development, and epithelial function-supplied by growth factors, development factors, and epithelial factors. This phenomenon has been referred to as "critical period programming". Thompson's group-supplied by Critical Period Programming observed that feeding weanling rats a diet high in polyunsaturated fat for 2 weeks before switching to a saturated fat diet led to a reduction in small intestinal D-glucose uptake which persisted (for at least 8 weeks) after switching to the lard based diet when compared with animals who were fed the lard based diet from weaning. This phenomenon of programming has not been controlled for in the studies described in this chapter. Likewise the cholesterol content of the lard based diet used in the present studies has not been controlled for and this may also exert a programming effect upon intestinal sugar transport-supplied by Intestinal Sugar Transport. These factors should be addressed in any subsequent studies.

13.4.4 Mucosal hydrolase activities and membrane fluidity
The activities of the mucosal hydrolases were similar in the two groups of jejuna. These observations are at variance with those made by Brasitas' group-supplied by Brasitas in which rats fed a corn oil based diet exhibited an increased alkaline phosphatase (ALP) activity when compared with rats fed a butter based diet. The increase in overall fatty acid unsaturation and increase in microvillus membrane fluidity observed by these authors in the corn oil fed group is comparable with that noted in the present studies. Thompson-supplied by Thompson, however, did not find an increase in ALP activity in rats fed a high polyunsaturated fat diet. Studies in which the fatty composition of the apical microvillus membrane have been altered by fish oil supplementation-supplied by Fish Oil Supplementation have also shown an increase in ALP activity (an increase in the specific activity of the enzyme rather than an increase in the amount of enzyme) but without a detectable
change in membrane fluidity measured by steady state fluorescence anisotropy. This suggests that the effects of altering membrane lipid composition on the functioning of this membrane protein result from changes in the local lipid microenvironment rather than from changes in the "macroscopic" biophysical characteristics of the membrane. Changing the cholesterol content of the apical membrane may also have an influence upon alkaline phosphatase activity\(^1\), with a decrease in cholesterol / phospholipid ratio being associated with an increase in ALP activity. The latter changes do not appear to be dependent upon "membrane fluidity" as fluidisation of the microvillus membranes with benzyl alcohol does not result in a change in ALP activity. Other studies have demonstrated that sucrase lactase and maltase activity is not affected by changing the fatty acid or cholesterol content of the microvillus membranes\(^2,3\). These observations are not entirely surprising because these enzymes are class I (peripheral) membrane proteins. Alkaline phosphatase is a class II (partially embedded) membrane protein and so might be less consistently affected by changes in membrane fluidity than a class IV transmembrane protein with a number of membrane spanning domains such as Na\(^+\)K\(^-\)ATPase, SGLT1, CFTR, the Na\(^+\)K\(^-\)2Cl cotransporter or indeed any other ion channel / membrane spanning ion channel or pump. There is good evidence for this with Na\(^+\)K\(^-\)ATPase\(^\text{\textsuperscript{353}}\) although the evidence for SGLT1 is ambiguous\(^4,5\). The transport of glucose across the small intestine may also be influenced by supplementing the diet with fish oil\(^\text{\textsuperscript{380}}\). Many receptors are class III proteins with a single membrane spanning domain and hence it would not be entirely surprising if the functions of these proteins were modulated by a change in the adjacent lipid milieu of the cell membrane. G proteins, which are involved in the regulation of many cellular processes including secretion are also membrane associated although in this instance the G protein \(\alpha\) subunit is peripheral to the membrane and is attached to the membrane via a thioester bond to myristic acid. The changes in apical membrane fluidity in the CORN group are small, although substantial changes in membrane function may occur with little or no alteration in "macroscopic" membrane fluidity\(^\text{\textsuperscript{435}}\). The energy for chloride secretion, as discussed previously, is derived from the activity of basolateral pumps and channels\(^\text{\textsuperscript{401}}\) and it would therefore have been informative to have studied the fatty acid composition and the biophysical characteristics of the basolateral membrane. It would be surprising if the changes
in apical membrane composition and fluidity in isolation accounted for the observed changes in secretion.

13.4.5 Indices of lipid peroxidation
Both TBARS and MDA were lower in the CORN fed group of animals despite the presence of higher amounts of unsaturated fatty acids in the apical membrane (and presumably the other cell membranes) and hence, presumably, a lower vitamin E / PUFA ratio in these membranes. This could be explained by the notion that the PUFA were acting as a free radical "sink". Dormandy has proposed that, on a high PUFA diet, stores of intracellular PUFA, as opposed to membrane phospholipid associated PUFA, may be increased and function as protective antioxidants\textsuperscript{440}. There is certainly a wealth of evidence that feeding high PUFA diets to young animals can be protective against the effects of oxygen toxicity\textsuperscript{441,442,443}. In the light of these observations it may be less surprising that the higher PUFA content of the enterocytes in the CORN group was not associated with an increase in TBARS.

13.5 Closing remarks
The work described in this chapter eludes to a modulation of small intestinal secretion by dietary lipid. Whilst this is not the first demonstration of this phenomenon the data presented adds to the information already available. It is worth noting that the fatty composition of the diets used is similar to that which may be used in the nutritional rehabilitation of infants with protracted diarrhoea. Failure of regrading of this group of infants back onto oral feeds is commonly associated with an increased responsiveness to secretory stimuli and hence clinical relapse of the diarrhoea. The enhanced responsiveness to secretory stimuli noted here may, therefore, have clinical implications for the choice of formula feed used in the nutritional rehabilitation of these infants.
CHAPTER 14

Biochemical and physiological studies in stripped jejunum after 6 months on a vitamin E sufficient or deficient corn oil based diet.

14.1 Introduction
Polyunsaturated lipids (ie lipids that contain more than two carbon - carbon double covalent bonds) provide a substrate for lipid peroxidation due to the comparative ease of hydrogen abstraction from a carbon adjacent to a double bond. The rationale behind the limb of the experimental protocol described in this chapter was to see if increasing the amount of peroxidisable substrate within the gastrointestinal mucosa would result in a heightened flux of free radicals in the presence and absence of compromised lipid soluble antioxidant defences (ie vitamin E deficiency). It is well established that a high polyunsaturated fatty acid (PUFA) content in the diet is reflected in the fatty acid composition of the small intestinal mucosal membranes. Studies of microsomal membranes from animals fed a high PUFA diet have demonstrated an increased rate of in vitro peroxidation. Similar studies with apical or basolateral membrane fractions are lacking in the literature. Studies in which rats were fed either a conventional (saturated fat based) feed or a corn oil based feed have also shown levels of TBARS to be elevated in the livers of the corn oil fed group.

14.2 Animal model and diet
The diets used are as outlined in chapter 2. Both diets contained 10% tocopherol stripped corn oil as a fat source. The vitamin E sufficient diet contained 40mg α-tocopheryl acetate / Kg of feed. The α- tocopherol / PUFA ratio of the vitamin E sufficient feed was, of course, lower than that in the animals fed the 10% lard based diet containing the same amount of α-tocopherol.

Animals were again allowed free access to diet and water at all times except for the 18 hours before anaesthesia and removal of tissues for study. Metabolic balance studies were performed after 20, 24 and 28 weeks on each of the respective diets (ie aged 23, 27, and 31 weeks) and tissues taken for biochemical and physiological studies at 24-26 weeks of age as described in chapter 13. The stripping and mounting of jejunum and the protocols for producing the dose response
curves were as described in preceding chapters.

14.3 Results

14.3.1 Growth, dietary intake and stool output

After 21 weeks on each diet (ie aged 24 weeks) the vitamin E sufficient animals were heavier than their vitamin E deficient counterparts (figure 14.1) despite an identical dietary intake (vide infra). Weights were E+ 564g ±28 (95% confidence interval), E- 439g ±19 (n=24 in each group, P<0.01).

![Figure 14.1 Weight gain of vitamin E sufficient and deficient animals from weaning](image)

This difference arose following a plateauing of weight in the vitamin E deficient group. A number of animals were not studied in vitro within the allocated time window and the weights of the E deficient animals in this group subsequently fell (by 28 weeks of age) as illustrated in figure 14.1. The weights of the animals aged 28 weeks (which were not studied in vitro) were E sufficient 614g ±35, E deficient 394g ±31, n=5 in each group, P<0.01. The dietary intakes at three ages are shown in table 14.1.
Table 14.1. Dietary intakes of vitamin E deficient and sufficient (high PUFA) rats at 23, 27 and 31 weeks of age.

<table>
<thead>
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<th>Age / weeks</th>
<th>23</th>
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<th>31</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30.9</td>
</tr>
<tr>
<td></td>
<td>95% CI ±1.5</td>
<td>±1.5</td>
<td>±2.3</td>
</tr>
<tr>
<td>E deficient</td>
<td>mean (g/24 hrs)</td>
<td>30.8</td>
<td>30.1</td>
</tr>
<tr>
<td></td>
<td>95% CI ±1.4</td>
<td>±1.4</td>
<td>±1.1</td>
</tr>
<tr>
<td>n</td>
<td>6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>P value (E+ vs E-)</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

It can be seen that the dietary intakes of the two groups of animals were similar at age 23 and 27 weeks, but at 31 weeks the vitamin E deficient animals ate significantly less food than their vitamin E sufficient counterparts. Animals were all studied aged 24-27 weeks (21-24 weeks on the diet) at a time when intakes were comparable. The differences in weight aged 24 weeks are not due to a decreased intake of diet.

14.3.2 Ussing chamber studies

14.3.2.1 Basal electrical characteristics

Basal short circuit current was significantly greater in the vitamin E deficient group of animals. This is reflected in a greater transepithelial potential difference. The difference in tissue resistance did not reach statistical significance (table 14.2).

Table 14.2. Basal electrical characteristics of stripped jejunum from vitamin E sufficient and deficient animals fed a corn oil based diet.

<table>
<thead>
<tr>
<th></th>
<th>Isc (µA cm⁻²)</th>
<th>Rt (Ω cm²)</th>
<th>Vt (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E sufficient (n= 30)</td>
<td>70 ±7</td>
<td>16.6 ±0.9</td>
<td>1.1 ±0.1</td>
</tr>
<tr>
<td>E deficient (n= 28)</td>
<td>77 ±6</td>
<td>16.3 ±0.8</td>
<td>1.25 ±0.1</td>
</tr>
<tr>
<td>P&lt;0.05</td>
<td>NS</td>
<td>P&lt;0.05</td>
<td></td>
</tr>
</tbody>
</table>
14.3.2.2 Electrogenic secretory response to ACh, BCh, and IBMX
Cumulative concentration response curves were constructed for acetylcholine, bethanecol, IBMX, and serotonin as described in chapters 12 and 13. The EC$_{50}$ values for ACh, BCh, and IBMX were lower in the vitamin E sufficient animals. The maximal electrogenic secretory responses ($\delta$isc max) to ACh and BCh were significantly greater in deficient jejuna. The maximal response to IBMX was similar in the two groups (table 14.3 and figures 14.2, 14.3 and 14.4).

Table 14.3. Maximal electrogenic secretory responses and EC$_{50}$ values for these responses in stripped jejuna from vitamin E sufficient and deficient animals fed corn oil based diets.

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>EC$_{50}$ 2.70 x 10$^{-7}$M (1.60-4.61)</td>
<td>6.22 x 10$^{-7}$M (4.30-9.01)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>$\delta$isc$_{max}$ 116 (±9) µA cm$^2$</td>
<td>193 (±27) µA cm$^2$</td>
<td></td>
</tr>
<tr>
<td>BCh</td>
<td>EC$_{50}$ 1.06 x 10$^{-4}$M (0.62-1.81)</td>
<td>2.28 x 10$^{-4}$M (1.29-4.02)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>$\delta$isc$_{max}$ 90 (±19) µA cm$^2$</td>
<td>119 (±27) µA cm$^2$</td>
<td></td>
</tr>
<tr>
<td>IBMX</td>
<td>EC$_{50}$ 4.55 x 10$^{-9}$M (3.54-5.85)</td>
<td>6.94 x 10$^{-9}$M (5.92-8.88)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>$\delta$isc$_{max}$ 185 (±33) µA cm$^2$</td>
<td>172 (±26) µA cm$^2$</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 14.2 Cumulative concentration response curves for acetylcholine

Figure 14.3 Cumulative concentration response curves for bethanecol

Figure 14.4 Cumulative concentration response curves for IBMX
14.3.2.3 Electrogenic secretory responses to serotonin

Cumulative concentration response curves for 5HT were constructed in tissues which had not been pretreated with any agent (figure 14,5).

![Graph](image)

Figure 14,5 Cumulative concentration response curves for 5HT

In this instance $\delta I_{sc \text{ max}}$ was significantly greater in the sufficient group when compared with the deficient group, and the $EC_{50}$ was significantly higher in the deficient group [$\delta I_{sc \text{ max}}$ E+ 33±8 $\mu$A cm$^{-2}$, E- 20±7 $\mu$A cm$^{-2}$, n=8 in each group, $P<0.05$; $EC_{50}$ E+ 1.69 x $10^{-6}$M (0.74-3.81), E- 5.13 x $10^{-6}$M (1.85 - 15.9), n=8, $P<0.05$].
14.3.2.4 Electrogenic secretory responses to STa, dbcAMP, pargyline and noradrenaline

The electrogenic secretory responses to mucosal E coli STa (60 mouse units ml⁻¹) and to 350μM serosal db cAMP were significantly greater in the deficient group of animals [STa: E+ median 28 (95% CI 10-35) μA cm⁻², E- 63 (35-90) μA cm⁻², n=7, P<0.05; dbcAMP: E+ 32 (24-53) μA cm⁻², E- 54 (38-76) μA cm⁻², n=8, P<0.05] (figures 14.6 and 14.7).

![Figure 14.6 Electrogenic response to STa (60 mouse units ml⁻¹)](image1)

![Figure 14.7 Electrogenic secretory response to db cAMP (350μM)](image2)
The responses of the tissues to 250 μM serosal pargyline and 100 μM serosal noradrenaline after maximal stimulation with IBMX were studied as outlined in chapter 12. The responses to pargyline (figure 14,8) were similar in the two groups [E+ median 37μA cm⁻² (95% CI 28-53), S- 36μA cm⁻² (30-60), n=8, NS]. The decrease in isc following the addition of serosal noradrenaline (figure 14,9) was, however, significantly greater in the vitamin E deficient jejuna [E+ median 28 μA cm⁻² (18-40), E- 70 μA cm⁻² (50-95), n=8, P<0.01].

Figure 14,8 Response to 250μM serosal pargyline

Figure 14,9 Response to 100μM serosal noradrenaline
14.3.2.5 Electrogenic responses to mucosal galactose

The electrogenic responses to mucosal galactose are shown in figure 14,10 and table 14,4.

Figure 14,10 Electrogenic responses to mucosal galactose in vitamin E deficient and sufficient jejuna.

2.5, 10, 20 and 30 mM mucosal galactose produced significantly greater electrogenic responses in vitamin E deficient jejuna.

Table 14, 4. Electrogenic responses to various concentrations of mucosal galactose.

<table>
<thead>
<tr>
<th>Galactose</th>
<th>Sufficient</th>
<th>Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 mM</td>
<td>6 (±4) μA cm(^{-2})</td>
<td>11 (±10) μA cm(^{-2})</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>13 (±9) μA cm(^{-2})</td>
<td>26 (±14) μA cm(^{-2})</td>
</tr>
<tr>
<td>5 mM</td>
<td>32 (±12) μA cm(^{-2})</td>
<td>52 (±24) μA cm(^{-2})</td>
</tr>
<tr>
<td>10 mM</td>
<td>55 (±32) μA cm(^{-2})</td>
<td>101 (±45) μA cm(^{-2})</td>
</tr>
<tr>
<td>20 mM</td>
<td>71 (±36) μA cm(^{-2})</td>
<td>138 (±48) μA cm(^{-2})</td>
</tr>
<tr>
<td>30 mM</td>
<td>83 (±39) μA cm(^{-2})</td>
<td>166 (±49) μA cm(^{-2})</td>
</tr>
</tbody>
</table>
14.3.3 Jejunal morphology

There were clear morphological differences between jejuna from the two groups of animals when studied by light microscopy (Table 14.5). Measurements were made using computerised morphometric analysis as described earlier. 10 villi / crypts were measured in a representative section from each of 4 animals from each group.

Table 14.5. Morphological characteristics of vitamin E sufficient and deficient jejuna (high PUFA diet).

<table>
<thead>
<tr>
<th></th>
<th>sufficient (n = 40)</th>
<th>deficient (n=40)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>villus height / μM</td>
<td>605 ±35</td>
<td>434 ±32</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>crypt depth / μM</td>
<td>161 ±14</td>
<td>131 ±12</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>villus / crypt ratio</td>
<td>3.86 ±0.39</td>
<td>3.32 ±0.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>mucosal thickness / μM</td>
<td>766 ±38</td>
<td>566 ±34</td>
<td>P&lt;0.005</td>
</tr>
</tbody>
</table>

14.3.4 Apical membrane hydrolases

The activities of the mucosal hydrolases lactase, sucrase and alkaline phosphatase were significantly depressed in vitamin E deficient animals (table 14.6).

Table 14.6. Apical membrane hydrolase activities in mucosal homogenates from the proximal small intestine (n=6 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Lactase (μmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Sucrase (μmol glucose min⁻¹ g⁻¹ protein)</th>
<th>Alkaline phosphatase (U g⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUFFICIENT</td>
<td>21.5 (± 4.6)</td>
<td>42.0 (± 5.4)</td>
<td>438 (± 78)</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>13.1 (±2.9)</td>
<td>25.2 (±6.7)</td>
<td>157 (±34)</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

14.3.5 Fatty acids composition of the apical enterocyte membrane

Fatty acid analysis of a total lipid extract from an apical membrane enriched fraction prepared from the proximal small intestine demonstrated significantly decreased levels (mol%) of linoleic acid in the vitamin E deficient group (table 14.7).
Table 14.7. Fatty acid composition (mo%) of a total lipid extract from the apical brush border membrane (n=6 in each group).

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SUFFICIENT</td>
<td>1.38</td>
<td>20.40</td>
<td>24.29</td>
<td>0.59</td>
<td>0.43</td>
<td>8.80</td>
<td>0.29</td>
<td>16.91</td>
<td>24.65</td>
<td>1.30</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>±0.85</td>
<td>±4.34</td>
<td>±3.52</td>
<td>±0.13</td>
<td>±0.22</td>
<td>±2.09</td>
<td>±0.26</td>
<td>±2.38</td>
<td>±3.96</td>
<td>±0.59</td>
<td>±0.18</td>
</tr>
<tr>
<td>DEFICIENT</td>
<td>0.89</td>
<td>24.56</td>
<td>23.24</td>
<td>0.55</td>
<td>0.34</td>
<td>8.31</td>
<td>0.19</td>
<td>13.62</td>
<td>22.34</td>
<td>1.40</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>±0.17</td>
<td>±3.90</td>
<td>±1.35</td>
<td>±0.14</td>
<td>±0.18</td>
<td>±1.31</td>
<td>±0.11</td>
<td>±2.05</td>
<td>±1.17</td>
<td>±0.59</td>
<td>±0.15</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

14.3.6 Apical membrane biophysical characteristics

Steady state fluorescence anisotropies measured in apical brush border membranes with both DPH and 12 AS were significantly higher in the vitamin E deficient animals (table 14.8).

Table 14.8. Steady state fluorescence anisotropy of apical brush border membranes measured with diphenylhexatriene and 12 anthroyloxy stearic acid.

<table>
<thead>
<tr>
<th></th>
<th>SUFFICIENT</th>
<th>DEFICIENT</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPH</td>
<td>0.212 (±0.012)</td>
<td>0.225 (±0.007)</td>
<td></td>
</tr>
<tr>
<td>12 AS</td>
<td>0.097 (±0.010)</td>
<td>0.108 (±0.009)</td>
<td></td>
</tr>
</tbody>
</table>

14.3.7 Indices of lipid peroxidation

Small intestinal mucosal TBARS were significantly higher in deficient than in sufficient animals [ E+ 0.56 (±0.09) nmol / mg protein, E- 1.38 (±0.28) nmol / mg protein, n=12 in each group, P<0.01]. Similar findings were made with mucosal concentrations of free MDA [ E+ 126 (±22) pmol / mg protein, E- 517 (±61) pmol / mg protein, n=12, P<0.01].

14.4 Discussion

14.4.1 General comments

The combination of a diet high in PUFA with vitamin E deficiency resulted in a failure of growth and ultimately weight loss, anorexia, a severe neurological syndrome and cachexia. It would have been fascinating to have studied the E deficient animals in this group aged 31 weeks or greater when they were loosing
weight, but interpretation of the results would have been difficult because of the dramatic fall off in dietary intake in this group. After 40 weeks of age the progression of the neurological syndrome was so dramatic that all 6 remaining animals in the high PUFA vitamin E deficient group had to be killed. At no time during this inexorable decline did any of the animals develop clinical diarrhoea. The resistance of rats to diarrhoea has been discussed in chapter 11.

14.4.2 Development of an enteropathy
The histological appearance of the vitamin E deficient high PUFA jejunum following H&E staining was the most dramatic seen in any of the 4 groups of rats studied, with a diminution in villus height, crypt depth, mucosal thickness and villus - crypt ratio when compared with the E sufficient high PUFA group. Vitamin E deficiency in animals fed a low PUFA (lard based) diet resulted in an increase in villus height and mucosal thickness (chapter 12) and a high PUFA (corn oil) based diet without vitamin E deficiency did not alter mucosal morphology (chapter 13). These striking changes resulted, therefore, from the combined effect of vitamin E deficiency and a high PUFA intake.

At the time of study the rate of weight gain in the vitamin E deficient animals had fallen off dramatically when compared with the vitamin E sufficient animals despite a maintenance of their dietary intake. The animals were, therefore, malnourished and either not absorbing sufficient nutrients from the diet or else the "normal" dietary intake was insufficient for an increased calorie requirement. Malnutrition on its own may affect mucosal morphology\(^{448,449}\) and the thinning of the mucosa with preservation of mucosal morphology is reminiscent of the findings in marasmus\(^{450,451}\). The mitotic index within the crypts is typically depressed in marasmus\(^{451}\) and there is good evidence that a decrease in the availability of energy will slow the mitotic cycle\(^{452}\). This issue has not been specifically addressed in these animals. The advent of a confocal microscopic method\(^{453}\) for estimating crypt cell production rate in routinely fixed tissue will provide information on this matter in the future. \(^{[6]H}\) thymidine labelling studies to estimate the rate of DNA synthesis would also have been interesting and enlightening in this group of animals. Under normal circumstances the thickness of the intestinal mucosa and the villus / crypt ratio are kept comparatively constant in any one part of the intestine, and the processes of cell production in the crypts and cell desquamation
at the villus tip will be in a dynamic equilibrium. Hormonal influences (systemic and enteroendocrine / paracrine) upon the gastrointestinal mucosa in malnourished individuals may both alter the crypt cell production rate and influence the process of differentiation along the crypt villus axis. Other trophic influences on the small intestine, for example pancreatic and biliary secretions may also be reduced in malnutrition. No efforts were made to study these in the present study. Ultrastructural studies of the small intestine in marasmus have demonstrated the presence of short, sparse and often branched microvilli on the enterocyte lumenal surface as well as a number of abnormalities within the enterocyte interior. The brush border of these tissues has not been studied by electron microscopy in the current study.

The degree of malnutrition in the E deficient animals at the time of study was not severe yet within 1 month of the study period (by 31 weeks of age) the remaining animals had a body weight 35% lower than the E sufficient group. It is therefore, not surprising that the E deficient group exhibited such a florid change in mucosal morphology akin to that found in severe malnutrition. If the morphological appearances in the rats are so similar to those of malnutrition then are the abnormalities of transport similar to those of marasmus? There is very little information about transport abnormalities in marasmus although impaired absorption of D-xylose and an alteration in gut permeability have been described. The junctional complexes (tight junctions) between the enterocytes appear to be intact in malnutrition and such a change cannot account for the increased gut permeability. Studies in animal models of "malnutrition" have described both an increase and a decrease in absorptive capacity and a number of complex changes in secretion depending upon the experimental protocol used. It is not possible, therefore, to relate the transport data presented in this chapter easily and directly to the malnourished (marasmic) child.

14.4.3 Electrogenic secretion

14.4.3.1 General comments

The changes in electrogenic transport in this part of the study are complex. The E deficient high PUFA animals were the only animals in the 4 groups studied to manifest a raised basal intestinal Isc. This is reminiscent of the changes seen in
rat jejunum following fasting but not "undernutrition".

The results provide evidence of an increase in basal secretion and an "enhanced" secretory response to a number of Ca\(^{2+}\), cAMP, and cGMP mediated secretagogues. The electrogenic secretory responses to 5HT were smaller in the E deficient group as manifest by a smaller \(\delta r\) max and a higher EC\(_{50}\). As discussed in previous chapters the enteric nerves were not blocked with TTX for the serotonin concentration response curves and so the serotonin data is not strictly comparable with the remainder of the data. It is likely that the principal actions of exogenous serotonin are on the enteric nerves of the submucosal plexus and that the final common pathway for secretion at the level of the enterocyte is via the release of ACh from enteric nerves which acts on the enterocyte muscarinic M\(_3\) receptor. Evidence has been provided here that muscarinic M\(_3\) receptor mediated secretion is enhanced. This then provides indirect evidence that the enteric nervous system might be affected by the vitamin E deficiency in a way analogous to that described in the lard fed group of E deficient animals (chapter 12). It is well established in the central nervous system that dopaminergic and serotonergic nerves are particularly vulnerable to vitamin E deficiency. Histochemical and functional studies of the enteric nervous system in a subsequent cohort of these animals would be of great interest. Some preliminary data in which the levels of serotonin, 5 hydroxyindole acetic acid, dopamine and noradrenaline were measured in mucosal scrapings from these animals revealed differences in the endogenous concentrations of these substances between the two groups of animals. While, in quantitative terms, the highest amounts of these amines will be found in the enterochromafin cells of the submucosa and not in the enteric nerves\(^{367}\), the differences in the concentrations of these substances raises the possibility of an alteration in the neural control and regulation of secretion in the vitamin E deficient group of animals.

**14.4.3.2 Hypotheses to explain increased secretion**

The reasons why secretion might be enhanced in vitamin E deficient jejunum when the enteric nerves are blocked have been discussed in chapters 11 and 12. A number of additional observations may be made about secretion in the E deficient

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\(^1\) Brant M., Heales S.J. (unpublished data)
tissues in which the levels of oxidative damage within the mucosa are increased. There is increasing evidence that oxidants and reactive oxygen species have a prosecretory effect within the intestine\textsuperscript{466,467,325} and that some of these reactive oxygen metabolites selectively enhance Ca\textsuperscript{2+} mediated chloride secretion\textsuperscript{468} without altering phosphatidylinositol metabolism or cyclic nucleotide levels. Evidence was presented in these studies\textsuperscript{468} that an increase in Ca\textsuperscript{2+} permeability of the cell membrane and an increase in intracellular Ca\textsuperscript{2+} was responsible for the enhanced chloride secretion in the cell line studied. Elevations of intracellular Ca\textsuperscript{2+} appear to promote apical Cl\textsuperscript{-} secretion by increasing basolateral K\textsuperscript{+} conductance (leading to K\textsuperscript{+} efflux) which depolarises the cell interior and increases the electrochemical driving force for apical Cl\textsuperscript{-} secretion\textsuperscript{469}. It is well established that oxidative damage to cell membranes increases the Ca\textsuperscript{2+} permeability of these membranes\textsuperscript{470} and one might speculate that this is one mechanism which might account for a number of the changes in secretion seen in E deficient jejuna. Oxidants also affect the intracellular sequestration of Ca\textsuperscript{2+} in intracellular Ca\textsuperscript{2+} stores\textsuperscript{471} and this may also perturb the secretory response.

Hydrogen peroxide has been shown to increase the paracellular permeability in cultured MDCK monolayers, as indicated by a decrease in transepithelial resistance, which was associated with a disruption of the actin cytoskeleton\textsuperscript{365}. Changing the organisation of the cytoskeleton is associated with alterations in epithelial function\textsuperscript{472}. There was no change in tissue resistance in the vitamin E deficient jejuna described here and hence no evidence of an increase in paracellular permeability as assessed by this technique. Studies with horseradish peroxidase or by measuring lactulose / mannitol absorption might have provided additional information about this matter, as would have electron transmission microscopy with immunostaining of microfilaments.

The morphological changes in the E deficient animals might explain some of the observations. The state of differentiation of a cell line is known to alter the secretory response to a number of agents. In particular, poorly differentiated cells express CFTR at the cell surface in a fashion analogous to that by differentiated cells, but are unable to mount a cAMP mediated Cl\textsuperscript{-} secretory response through the protein\textsuperscript{473}. The "maturity" of an epithelium will also influence its susceptibility to E coli STa\textsuperscript{474,475} and there is evidence that peptidases at the cell surface may change the secondary structure of STa and that this altered form has less
biological activity than the parent molecule\textsuperscript{476}. Definitive experimental evidence for this is still required but if the activity of mucosal endopeptidases is important in regulating the secretory response to STa, then the enhanced response to STa in the E deficient animals could also be related to a decrease in apical brush border membrane peptidase activity.

14.4.3.3 Hypotheses for increased absorption

The greater decrease in I\textsubscript{sc} in E deficient jejuna in response to noradrenaline was amongst the most dramatic of differences found in any of the experiments conducted. This presumably represents an increase in HCO\textsubscript{3} absorption (see chapter 13). Explanation is difficult. If, as one might expect, endogenous levels of noradrenaline are low in these animals (vide supra) then one has to question whether this represents a phenomenon akin to receptor supersensitivity. The contribution of malnutrition to this greatly enhanced response may also be important, particularly as the vitamin E deficient "LARD" fed animals had a diminished response to noradrenaline.

The increased electrogenic transfer of mucosal galactose is also striking in the vitamin E deficient group and explanations for this have been discussed in chapters 11, 12, and 13. The galactose data could not easily be fitted to Michaelis Menten type kinetics and apparent Km and Vmax values have not been quoted for the electrogenic transfer of galactose.

14.4.3.5 Nitric oxide

A topical point for discussion is the effect of vitamin E deficiency and increased free radical fluxes on nitric oxide and the biological consequences of a perturbation of this system. Nitric oxide (endothelium derived relaxing factor) is an important regulator of vascular tone in biological systems\textsuperscript{477} and an important non cholinergic non adrenergic neurotransmitter in the gut\textsuperscript{478}. The superoxide radical, however, effectively inactivates nitric oxide\textsuperscript{479,480} and the effects of this in the intestine have not been completely evaluated. It is known that nitric oxide is a regulator of epithelial permeability within the small intestine and it has been suggested that an increased rate of breakdown of nitric oxide, for example by reaction with superoxide, might lead to an increase in epithelial permeability\textsuperscript{481}. Studies of the mesenteric vasculature of these rats at University College have provided evidence
of deranged vascular control in the vitamin E deficient animals (both LARD and CORN groups). The effects of such a derangement of non cholinergic non adrenergic neurotransmission on small intestinal secretion are not known. Preliminary data by Rolphe et al. has demonstrated in the rat that nitric oxide is prosecretory and, in addition to producing a small increase in short circuit current in its own right, it potentiates the secretory response to E coli STa. It is difficult to imagine, therefore, how a diminution in nitric oxide in association with increased superoxide could account for the increased secretory responses observed in vitamin E deficiency in these studies. The complexities of this biological signal transduction system are such that this area is worthy of further study.

14.4.3.5 Tissue capacitance

It is theoretically possible that the superior performance of the E deficient tissues in most of the measurements made in the Ussing chambers could result from the decreased mucosal thickness in this group. This could result in these thinner tissues being more efficiently short circuited because of a lower tissue capacitance and improved short circuiting of the crypts. This parameter is difficult to quantify because of unbulit damping in the clamp circuitry and because of difficulties in applying a square wave current pulse. It is worth noting, however, that tissue resistances were similar in the two groups which makes a large difference in tissue capacitance less likely.

14.4.4 Mucosal hydrolases

The activities of the mucosal hydrolases lactase, sucrase and alkaline phosphatase were reduced in the E deficient group. Specific activities of these enzymes (i.e. the hydrolase activity per mg of enzyme) were not measured here. For the same reasons as discussed in chapter 13, it would be surprising if the changes in activities were due directly to a change in the lipid milieu of the enzymes as they comprise class I and II peripheral membrane proteins. Peroxidation of lipid within a biological membrane may, however, lead to the cross linking of lipids with proteins, to direct oxidative damage to membrane proteins, and also to the

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k Charles Hoyle, Department of Anatomy, University College, London
Unpublished data
production of aliphatic aldehydes which may affect enzyme function\textsuperscript{483}. The diminution in villus size might also be responsible for reduced hydrolase activities as is seen in enteropathies\textsuperscript{206}. The post translational modification of the apical membrane hydrolases is complex\textsuperscript{484} and there is little information available on how this process may be affected by oxidative damage to the cell membranes, changes in membrane fluidity, and malnutrition.

14.4.5 Lipid peroxidation
The increase in indices of lipid peroxidation in mucosal scrapings is large in the E deficient group (4 fold increase in free MDA), and is associated with a decrease in apical membrane linoleic acid (18:2), but not of docosatetraenoic (22:4) or docosahexaenoic (22:6) acids. The combination of vitamin E deficiency and increased PUFA intake has been shown to lead to a dramatic increase in TBARS in a number of tissues\textsuperscript{447, 484} (serum, liver mitochondria and microsomes, adipose tissue). Such a rise has never been described in the intestinal mucosa, and the magnitude of this is impressive considering the innate resistance of the small intestine to the peroxidative process (see chapters 10, 11, 12). Iritani et al\textsuperscript{447, 484} also noted a rise in TBARS in thoracic lymph from animals fed the corn oil based diet and suggested that this was due to breakdown of the corn oil diet in vitro before it was ingested by the rats. No special measures were taken with the diets used in these studies to prevent this other than storage in the dark at 4°C and the presence of a standard amount of BHT (0.05% w/w lipid). Fresh diet was also manufactured every two months in an effort to minimise this phenomenon. TBARS were not measured on the diets during storage. Iritani et al\textsuperscript{447, 484} only measured TBARS and it would be surprising if TBARS did not go up in thoracic lymph following a high PUFA meal as there will be an increased substrate to react with TBA (in the inevitable presence of iron !) during the heating phase of the assay. This matter remains open to question in the current experiments.

14.5 Closing remarks
In conclusion, vitamin E deficiency in association with an increased peroxidisable substrate of polyunsaturated fat within the enterocyte leads to a number of dramatic alterations in intestinal morphology and secretion, a change in apical membrane composition and biophysical characteristics and an increase in indices
of oxidative damage within the enterocyte. These observations provide further evidence that increased fluxes of free radicals might be of importance in the vicious cycle of malabsorption and malnutrition seen in association with protracted diarrhoeal disease in infancy.
This study has examined the effects of a severe and chronic deficiency of vitamin E in the presence and absence of a high dietary polyunsaturated fatty acid intake upon indices of small intestinal secretion and absorption, lipid peroxidation, apical brush border membrane composition and apical membrane biophysical characteristics. The study was established to answer two questions. Firstly, does vitamin E deficiency result in increased fluxes of free radicals within the small intestine and if so are these associated with an alteration in secretion and absorption. Secondly, does a diet high in polyunsaturated fats, in the presence of vitamin E deficiency, lead to a greater flux of free radicals and is this associated with a further change in secretion and absorption. This is the first study of its kind to use the combination of a high polyunsaturated fat diet and vitamin E deficiency and study the effects upon gastrointestinal function.

The changes in secretion and absorption following a severe and chronic deficiency of vitamin E are complex, and the studies presented here have not provided a simple explanation for the changes. These studies have, for example, done little to distinguish between a structural effect at the cell surface of the enterocyte membrane, from an alteration in cell surface expression of transporters, or from effects upon the enteric nerves. Given the susceptibility of the nervous system to vitamin E deficiency, a study of this aspect would be particularly interesting. The studies described here have also not distinguished the effects of vitamin E deficiency per se from those due to increased fluxes of free radicals.

These studies have demonstrated that the fatty composition of the diet is reflected in the fatty composition of the enterocyte apical membrane, and that a manipulation of dietary fat may bring about a number of changes in gastrointestinal absorption and secretion. The documentation of effects of dietary polyunsaturated fat upon gastrointestinal function is important, both because of recent Government guidelines on the content and composition of dietary fat, and because of recent initiatives in infant nutrition which add long chain polyunsaturated fats to infant formula feeds.

The finding that a higher content of PUFA in the intestine was not associated with elevated indices of lipid peroxidation in the presence of an adequate vitamin E intake is of great importance, particularly in the light of the recent DHSS dietary
guidelines.

Extrapolation of this data to the in vivo situation is difficult, and hence the significance of vitamin E deficiency in malnourished children with PDD in terms of the natural history of the diarrhoeal state is unclear. Increasing awareness of the importance of vitamin E in clinical practice has led to the observation that a severe deficiency is not restricted to children with abeta- or hypobetalipoproteinemia but also occurs in children with PDD. This has modified clinical practice and has led to early and aggressive replacement therapy with fat soluble vitamins and micronutrients in infants with PDD presenting to the Hospital for Sick Children, Great Ormond Street.

The observations reported here might perhaps explain, at least in part, the success of the age old practice of nutritional rehabilitation with comminuted chicken feeds, in which fats, usually in the form of Calogen™, which is low in polyunsaturates, are added to the feed comparatively late in the course of the rehabilitation.
APPENDIX 1

Statistical methods and presentation adopted in the thesis

This appendix sets out the standardised format used to present results and provides a background and rationale for the choice of these methods of presentation of data.

General notes on presentation
Throughout the thesis a standardised format of presentation of results has been adopted.
MEANS are followed by the 95% confidence interval for the mean in parenthesis ie (± 95% CI). In the case of geometric means (for example with EC₆₀ data) the limits of the 95% confidence interval are quoted as these will not be symmetrical about the mean in non transformed data. Where wider confidence intervals are used this is clearly stated in the text.
MEDIANS are followed by the 95% confidence interval for the median in parenthesis.
ERROR BARS on graphs are always the standard error of the mean (sem) as this improves the clarity of these figures.
In BOX PLOTS the horizontal line in the middle of the box is the MEDIAN, the upper and lower limits ("error bars") are the 95% confidence interval for the median and the distance between the upper and lower edges of the box represents the interquartile range.
Any data which is not presented in this fashion is accompanied by a full explanation of the method of presentation in the text or figure legend.

1. PARAMETRIC DATA

Confidence intervals
Undue emphasis on hypothesis testing when comparing two samples and hence a preoccupation with levels of "significance" provides valuable information about the probability of a difference arising by chance but provides little information about the magnitude of this difference⁴⁸⁵. It is more useful to present results from
measurements made on a "sample" of a population as an estimate of the results that would be obtained if the whole population were studied. The confidence interval represents such a measurement by providing a range of possibilities for the population value although in theory the population mean is most likely to reside nearer the centre of the confidence interval. A confidence interval for the mean extends either side of the mean by a multiple of the standard error. Throughout this document the format of mean (± 95% confidence interval) has been adopted for the sake of clarity in the tables. It would have been statistically more correct to present the data in the form; mean (95% confidence interval "a" to "b")

The confidence interval for the mean indicates, therefore, the precision of the sample mean as an estimate of the population mean.

Confidence intervals for the mean are constructed using the t distribution. When two samples are compared, and differences between the means sought, the data should have similar standard deviations. For the sake of clarity in the text I have chosen not to indicate the confidence intervals for the differences between two means when two samples are being compared. In statistical terms it would have been correct to have done so.

Confidence intervals for single samples and for the differences between two samples (unpaired case) were calculated using an IBM computer programme "Confidence Interval Analysis" published by the British Medical Journal in conjunction with the book "Statistics with Confidence". The mathematical formulae used were as follows;

(a) Single sample
The confidence interval for a population mean is derived using the mean (x) and its standard error from a sample size n.
For this case standard error (SE) = standard deviation (SD)/√n. Thus the confidence interval is given by the equation;

\[
\bar{x} - (t_{1 - \alpha/2} \times SE) \text{ to } \bar{x} + (t_{1 - \alpha/2} \times SE)
\]

where \( t_{1 - \alpha/2} \) is the appropriate value from the t distribution with \( n - 1 \) degrees of freedom associated with a "confidence" of \( [100 \ (1 - \alpha)]\% \). Thus, for a 95% confidence interval \( \alpha \) is 0.05.
(b) Two samples (unpaired)

The confidence interval for the difference between two population means is derived in a similar fashion. Assume for example that \(x_1\) and \(x_2\) are the two sample means, \(s_1\) and \(s_2\) the corresponding standard deviations and \(n_1\) and \(n_2\) the sample sizes. An estimate of the "pooled" standard deviation is given by:

\[
S = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}}
\]

From this the standard error of the difference between the two means is:

\[
SE_{diff} = S \times \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}
\]

The 100(1-\(\alpha\))% confidence interval for the difference in the two population means is then:

\[
\bar{x}_1 - \bar{x}_2 - (t_{1-\alpha/2} \times SE_{diff}) \text{ to } \bar{x}_1 - \bar{x}_2 + (t_{1-\alpha/2} \times SE_{diff})
\]

where \(t_{1-\alpha/2}\) is taken from the t distribution with \(n_1 + n_2 - 2\) degrees of freedom.

In cases where the standard deviations of the two groups differed considerably the use of a pooled estimate of SD is not appropriate and in these instances comparisons were made using more complex methods [Welch test] as found in the STATGRAPHICS computer programme.

**Multiple comparisons**

Where comparisons were made (as in appendix 2) between more than one normally distributed group (with similar variances) these were performed using a one way ANOVA to reduce the type 1 error (ie false positive results) of multiple t tests. The Scheffe multiple range test is one such method of reducing type 1 error in comparing more than 2 groups, and this method has been used here. The analysis was performed on a microcomputer using the STATGRAPHICS programme.
Logarithmic transformation of non parametric data
Mean EC$_{50}$ values were calculated by estimating this value for each concentration response curve and then taking the geometric mean of these values. Confidence intervals for this mean were calculated by the methods described above after logarithmic transformation of the data. The antilog of the intervals was taken before quoting the data in its tabulated form. Differences between the means of logarithmically transformed data were estimated using the Confidence Interval Analysis microcomputer programme.

2. NON PARAMETRIC DATA

Confidence Interval for the median
The median value is the preferred measure of location of a sample of non-normal continuous data, and this was used where appropriate. The calculation of confidence intervals for the median was performed using the Confidence Interval Analysis computer software as described by Hill\textsuperscript{489}. In defining confidence intervals for the difference between medians it is assumed that the data come from distributions which are identical and differ only in location. The method is outlined in Gardner and Altman\textsuperscript{490}.

Non Parametric one way ANOVA - Kruskal - Wallis test
Just as the one way ANOVA may be considered a more general form of the t test, so may the Kruskal - Wallis test be considered a more general form of the Mann Whitney U test (vide infra).

In essence the complete set of N observations are ranked from 1-N regardless of which group they are in and for each group a sum of ranks is calculated and the test statistic, H, calculated. When the null hypothesis is true, this statistic follows the Chi squared distribution. A statistically significant result implies that the hypothesis that the groups come from populations with the same median should be rejected. Two sample Mann Whitney U tests are then used to find where these differences lie.

This test was performed using the STATGRAPHICS statistical software package.
Mann Whitney U test
As with the Kruskall Wallis test, the Mann Whitney U test requires that all the observations are ranked as if they were a single sample. The U statistic was calculated on a microcomputer (STATGRAPHICS software). The method depends on the calculation of rank sums and the probability of obtaining any particular rank sum can be calculated.
APPENDIX 2

Overall comparison of jejunal structure and function in vitamin E sufficient and deficient rats fed on lard or corn oil based diets.

A.1 Introduction

This appendix provides data side by side to allow a comparison of the four groups of rats described in chapters 12 - 14. The data shown in not exhaustive and the reader is referred to the individual chapters for more information and a discussion of specific comparisons.

A.2 Morphology

Table A.1. Morphological measurements made on jejuna from the 4 groups of animals (n=10 observations from each of 4 sections from each group)

<table>
<thead>
<tr>
<th></th>
<th>Lard</th>
<th>Corn oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>Villus height / µM</td>
<td>587 ±36 a</td>
<td>684 ±36 b</td>
</tr>
<tr>
<td>Crypt depth / µM</td>
<td>167 ±14 e</td>
<td>182 ±14 f</td>
</tr>
<tr>
<td>Villus / crypt ratio</td>
<td>3.54 ±0.39 i</td>
<td>3.80±0.39 j</td>
</tr>
<tr>
<td>Mucosal thickness / µM</td>
<td>755±39 m</td>
<td>867 ±39 n</td>
</tr>
</tbody>
</table>

Scheffe multiple range test:
(a) vs (d), (b) vs (d), (c) vs (d). P<0.01
(a) vs (b), (b) vs (c). P<0.05
(e) vs (f), (e) vs (h), (f) vs (g), (f) vs (h), (g) vs (h). P<0.01
(k) vs (l). P<0.05
(m) vs (n), (m) vs (p), (n) vs (o), (n) vs (p), (o) vs (p). P<0.01
**Figure A.1** Villus / crypt ratio of jejunum

**Figure A.2** Mucosal thickness of jejunum

**Figure A.3** Villus height of jejunum

**Figure A.4** Crypt depth of jejunum
Plate A.1. Light microscopic sections of mid jejunum from an E+ LARD animal. Stained with haematoxylin & eosin. Magnification X 16

Plate A.2. Light microscopic sections of mid jejunum from an E- LARD animal. Stained with haematoxylin & eosin. Magnification X 16
Plate A,3. Light microscopic sections of mid jejunum from an E+ CORN animal. Stained with haematoxylin & eosin. Magnification X 16

Plate A,4. Light microscopic sections of mid jejunum from an E- CORN animal. Stained with haematoxylin & eosin. Magnification X 16
A.3 Mucosal hydrolase activities

Pooled data for mucosal hydrolase activities are shown in table A.2.

Table A.2. Apical membrane hydrolase activities in mucosal homogenates from the proximal small intestine (n = 6-8 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Lactase (umol glucose min⁻¹ g⁻¹ protein)</th>
<th>Sucrase (umol glucose min⁻¹ g⁻¹ protein)</th>
<th>Alkaline phosphatase (U g⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD E+</td>
<td>25.1 (± 8.1) (a)</td>
<td>55.3 (± 15.9) (e)</td>
<td>396 (± 77) (l)</td>
</tr>
<tr>
<td>LARD E-</td>
<td>21.1 (± 6.6) (b)</td>
<td>45.5 (± 13.0) (f)</td>
<td>449 (±86) (j)</td>
</tr>
<tr>
<td>CORN E+</td>
<td>21.5 (± 4.6) (c)</td>
<td>42.0 (± 5.4) (g)</td>
<td>438 (± 78) (k)</td>
</tr>
<tr>
<td>CORN E-</td>
<td>13.1 (± 2.9) (d)</td>
<td>25.2 (± 6.7) (h)</td>
<td>157 (±34) (l)</td>
</tr>
</tbody>
</table>

Scheffe multiple range test;
(a) vs (d), (b) vs (d), (c) vs (d) P<0.01
(e) vs (h), (f) vs (h), (g) vs (h) P<0.01
(i) vs (l), (j) vs (l), (k) vs (l) P<0.01

A.4 Mucosal TBARS and MDA

Table A.3. Unstimulated levels of TBARS and MDA in jejunal mucosal scrapings (n=12 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Lard</th>
<th>Corn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+</td>
<td>E-</td>
</tr>
<tr>
<td>TBARS / nmol /</td>
<td>0.78 ±0.12 (a)</td>
<td>1.23 ±0.12 (b)</td>
</tr>
<tr>
<td>mg protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA / pmol /</td>
<td>246 ±77 (e)</td>
<td>440 ±77 (f)</td>
</tr>
<tr>
<td>mg protein</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Scheffe multiple range test;
(a) vs (b), (a) vs (c), (a) vs (d), (b) vs (d), (b) vs (c) P<0.05; (c) vs (d) P<0.01
(e) vs (f), (e) vs (g), (e) vs (h), (f) vs (h), (f) vs (g) P<0.05; (g) vs (h) P<0.01
A.5 Membrane biophysical characteristics

Table A.4. Steady state anisotropy data for brush border membrane enriched fractions from the proximal small intestine of the four groups of rats (n= 6 in each group)

<table>
<thead>
<tr>
<th></th>
<th>Lard</th>
<th>Corn</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+ (a)</td>
<td>E- (b)</td>
<td>E+ (c)</td>
<td>E- (d)</td>
</tr>
<tr>
<td>DPH</td>
<td>0.221 ±0.007</td>
<td>0.231 ±0.007</td>
<td>0.212 ±0.012</td>
<td>0.225 ±0.007</td>
</tr>
<tr>
<td>12 AS</td>
<td>0.106 ±0.007</td>
<td>0.120 ±0.010</td>
<td>0.097 ±0.010</td>
<td>0.108 ±0.009</td>
</tr>
</tbody>
</table>

Scheffe multiple range test:
(b) vs (a), (b) vs (c), (d) vs (c) P<0.05
(f) vs (e), (e) vs (g), (f) vs (g), (h) vs (g) P<0.05

A.5 Ussing chamber studies

Basal intestinal electrical parameters

Table A.5. Basal electrical parameters of stripped jejunum

<table>
<thead>
<tr>
<th></th>
<th>Lard</th>
<th>Corn</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E+ (n=55)</td>
<td>E- (n=55)</td>
</tr>
<tr>
<td>Isc / μA cm²</td>
<td>69 ±4 (a)</td>
<td>71 ±4 (b)</td>
</tr>
<tr>
<td>pd / mV</td>
<td>1.21 ±0.1 (e)</td>
<td>1.14 ±0.1 (f)</td>
</tr>
<tr>
<td>Rt / Ωcm²</td>
<td>17.2 ±0.5</td>
<td>16.6 ±0.4</td>
</tr>
</tbody>
</table>

Scheffe multiple range test:
(a) vs (d), (c) vs (d) P<0.05
(f) vs (h), (g) vs (h) P<0.05
Response to secretagogues

(i) Acetylcholine

Figure A.5 Cumulative concentration response curves for ACh in stripped jejunum

Table A.5. Concentration response data for acetylcholine in stripped jejunum (n=8 in each group)

<table>
<thead>
<tr>
<th></th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>δIsc max</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µA cm&lt;sup&gt;-2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>LARD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>5.60 x 10&lt;sup&gt;-7&lt;/sup&gt;M (3.49-9.13)</td>
<td>119 (±24)</td>
</tr>
<tr>
<td>E-</td>
<td>4.4 x 10&lt;sup&gt;-7&lt;/sup&gt;M (3.1-6.2)</td>
<td>167 (±42)</td>
</tr>
<tr>
<td>CORN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>2.70 x 10&lt;sup&gt;-7&lt;/sup&gt;M (1.60-4.61)</td>
<td>116 (±9)</td>
</tr>
<tr>
<td>E-</td>
<td>6.22 x 10&lt;sup&gt;-7&lt;/sup&gt;M (4.30-9.01)</td>
<td>193 (±27)</td>
</tr>
</tbody>
</table>

Scheffe multiple range test on log transformed data:
(c)<(d), (c)<(a), (b)<(d) P<0.05

Scheffe multiple range test on non transformed data:
(e)<(f), P<0.05
(g)<(h), (e)<(h) P<0.01
(ii) Serotonin

Figure A.6 Cumulative concentration response curves for 5HT in stripped jejunum

Table A.6. Concentration response data for 5HT in stripped jejunum

<table>
<thead>
<tr>
<th></th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>δIsc max</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>1.6 x 10&lt;sup&gt;-9&lt;/sup&gt;M (1.0-2.5)</td>
<td>(a) 48 (±8) μA cm&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>E-</td>
<td>4.9 x 10&lt;sup&gt;-9&lt;/sup&gt;M (2.3-10.4)</td>
<td>(b) 21 (±3) μA cm&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>CORN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>1.7 x 10&lt;sup&gt;-9&lt;/sup&gt;M (0.7-3.8)</td>
<td>(c) 38 (±8) μA cm&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>E-</td>
<td>5.1 x 10&lt;sup&gt;-9&lt;/sup&gt;M (1.9-15.9)</td>
<td>(d) 20 (±7) μA cm&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Scheffe multiple range test (log transformed data)
(a)<(b), (a)<(c), (b)<(c), (c)<(d) P<0.05
(a)<(d) P<0.01

Scheffe multiple range test
(f)<(g), (h)<(g), (g)<(e) P<0.05
(f)<(e) P<0.01
(iii) Responses to STa and dbcAMP

Figure A.7 Responses of stripped jejunum to E coli STa (60 mouse units ml⁻¹)

Figure A.8 Responses of stripped jejunum to 300 μM dbcAMP

Table A.7. Median response to STa (60 mouse units ml⁻¹) and 300 μM dbcAMP in stripped jejunum (n= 7-8 for all)

<table>
<thead>
<tr>
<th></th>
<th>STa</th>
<th>dbcAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>31 (17-42) (a)</td>
<td>30 (20-52) (e)</td>
</tr>
<tr>
<td>E-</td>
<td>28 (18-38) (b)</td>
<td>52 (38-65) (f)</td>
</tr>
<tr>
<td>CORN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E+</td>
<td>28 (10-35) (c)</td>
<td>32 (24-53) (g)</td>
</tr>
<tr>
<td>E-</td>
<td>63 (35-90) (d)</td>
<td>54 (38-76) (h)</td>
</tr>
</tbody>
</table>

Kruskal Wallis P=0.02; Mann Whitney U; (a)<(d), (b)<(d) P<0.05 (c)<(a) P<0.01

Kruskal Wallis P=0.02; Mann Whitney U; (e)<(f), (e)<(h) P<0.05 (e)<(f), (g)<(h) P=0.05
(iv) Response to serosal noradrenaline

![Graph showing the decrease in Isc produced by 100µM serosal noradrenaline in stripped jejunum.]

**Figure A.9** Decrease in Isc produced by 100µM serosal noradrenaline in stripped jejunum.

**Table A.9.** Median decrease in Isc produced by 100µM noradrenaline

<table>
<thead>
<tr>
<th></th>
<th>δIsc / µA cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>E+ LARD</td>
<td>28 (15-40)</td>
</tr>
<tr>
<td>E- LARD</td>
<td>12 (9-14)</td>
</tr>
<tr>
<td>E+ CORN</td>
<td>28 (18-40)</td>
</tr>
<tr>
<td>E- CORN</td>
<td>70 (50-95)</td>
</tr>
</tbody>
</table>

*Kruskal Wallis one way ANOVA P<5x10⁻⁵*
*Mann Whitney U:*

(b)<(a), (b)<(c) P<0.01
(b)<(d), (a)<(d), (c)<(d) P<0.001
(v) Response to mucosal galactose

![Graph showing electrogenic responses to mucosal galactose in stripped jejuna.](image)

**Figure A.10** Electrogenic responses to mucosal galactose in stripped jejunum.

<table>
<thead>
<tr>
<th>Concentration (mmol)</th>
<th>E+ LARD</th>
<th>E- LARD</th>
<th>E+ CORN</th>
<th>E- CORN</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>11 (±2)</td>
<td>19 (±7)</td>
<td>32 (±12)</td>
<td>52 (±24)</td>
</tr>
<tr>
<td>10</td>
<td>35 (±11)</td>
<td>58 (±19)</td>
<td>55 (±32)</td>
<td>101 (±45)</td>
</tr>
<tr>
<td>20</td>
<td>61 (±19)</td>
<td>92 (±29)</td>
<td>71 (±36)</td>
<td>138 (±48)</td>
</tr>
<tr>
<td>30</td>
<td>76 (±19)</td>
<td>112 (±40)</td>
<td>83 (±39)</td>
<td>166 (±49)</td>
</tr>
</tbody>
</table>

Table A.10 δisc (μA cm⁻²) in response to mucosal galactose in stripped jejunum. (n= 6-8 for all concentrations)

*Scheffe multiple range test;*

\( n>j, o>k, p>l, i>a, j>b, e>a, f>b, g>c, h>d \) \( P<0.05 \)

\( m>a, n>b, o>c, p>d \) \( P<0.01 \)
Table A.11 Fatty acid composition (mol%) of apical brush border membrane preparations. n=6-8 for all.

<table>
<thead>
<tr>
<th></th>
<th>E+ Lard</th>
<th>E- Lard</th>
<th>E+ PUFA</th>
<th>E- PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>22.73 ±3.18</td>
<td>21.21 ±3.30</td>
<td>20.40 ±4.34</td>
<td>24.56 ±3.90</td>
</tr>
<tr>
<td>18:0</td>
<td>26.76 ±2.26</td>
<td>27.01 ±3.31</td>
<td>24.29 ±3.52</td>
<td>23.24 ±1.35</td>
</tr>
<tr>
<td>18:1</td>
<td>11.14 ±1.94</td>
<td>12.20 ±0.85</td>
<td>8.80 ±2.09</td>
<td>8.31 ±1.31</td>
</tr>
<tr>
<td>18:2</td>
<td>7.23 ±1.47</td>
<td>6.12 ±1.15</td>
<td>16.91 ±2.38</td>
<td>13.62 ±2.05</td>
</tr>
<tr>
<td>20:4</td>
<td>27.43 ±3.48</td>
<td>28.40 ±1.15</td>
<td>24.65 ±3.96</td>
<td>22.34 ±1.17</td>
</tr>
<tr>
<td>22:4</td>
<td>0.53 ±0.19</td>
<td>0.85 ±0.59</td>
<td>1.30 ±0.59</td>
<td>1.40 ±0.59</td>
</tr>
<tr>
<td>22:6</td>
<td>1.64 ±0.35</td>
<td>1.22 ±0.36</td>
<td>0.76 ±0.18</td>
<td>0.93 ±0.15</td>
</tr>
</tbody>
</table>
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