Human red cell calcium pump: plasma lipids and diabetes

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To my parents

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ABBREVIATIONS

ACh	acetylcholine
ADP	adenosine 5'-diphosphate
AGEs	advanced glycosylation end-products
Apo	apoprotein
ATP	adenosine 5'-triphosphate
ATPase	adenosine 5'-triphosphatase
BMI	body mass index
cAMP	cyclic adenosine 5'-monophosphate (or adenosine 3': 5'-cyclic monophosphate)
CETP	cholesterol ester transfer protein
cGMP	cyclic guanosine 5'-monophosphate (or guanosine 3': 5'-cyclic monophosphate)
CHD	coronary heart disease
CI	confidence intervals
DHA	docosahexaenoic acid
DPH	1,6-diphenyl-1,3,5-hexatriene
DSC	differential scanning calorimetry
ECL	enhanced chemiluminescence
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
e.s.r.	electron spin resonance
ET-1	endothelin-1
FCCP	carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone
G	grating factor
GTP	guanosine 5'-triphosphate
HbA_1	glycosylated haemoglobin A_1

HDL	high density lipoprotein
HMGCoA	3-hydroxy-3-methylglutaryl coenzyme A
IDDM	insulin-dependent diabetes mellitus
K_m	Michaelis constant
LCAT	lecithin cholesterol acyltransferase
LDL	low density lipoprotein
M_r	molecular weight
MRI	magnetic resonance imaging
NIDDM	non-insulin-dependent diabetes mellitus
P	fluorescence polarization
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PROCAM	prospective cardiovascular Munster
r	fluorescence anisotropy
r_S	Spearman's rank correlation coefficient
RDS	respiratory distress syndrome
SDS	sodium dodecyl sulphate
SHR	spontaneously hypertensive rat
SLC	sodium lithium countertransport
SM	sphingomyelin
™	trade mark
TMA-DPH	1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene
TMFS	trifluoromethane sulphonic acid
VLDL	very low density lipoprotein
V_{max}	maximum velocity
WKYN	Wistar-Kyoto normotensive rat

PREFACE

The existence of the red cell calcium pump was proposed some 28 years ago and subsequently confirmed 3 years later. In the red cell this enzyme is the main calcium extruding mechanism that maintains the low intracellular calcium concentrations necessary for normal cell function.

In 1972 the fluid mosaic model of biological membranes was propounded. In this theory a biological membrane was seen as a leaflet of phospholipids in which various proteins and other components were interspersed, the whole structure being arranged in the form of a bilayer. By the mid-1970s, when fairly sensitive techniques had been developed for assessing the movement of these components within the membrane bilayer, it became apparent that changes in the membrane lipid content, in particular the cholesterol to phospholipid ratio, affected the overall fluidity of the membrane.

By the late 1970s it was possible to isolate the calcium pump from its membrane environment and reconstitute it in liposomes. Changes in the cholesterol and phospholipid content of these liposomes affected not only their fluidity but also the activity of the reconstituted calcium pump. Although these were *in vitro* studies it seemed logical to assume that similar changes would occur in cell membranes *in vivo*. So was born the membrane hypothesis that serum and membrane lipids are in dynamic equilibrium and that the functions of the various membrane protein structures are influenced by their immediate environment within the membrane.

Recent studies in hypertension have indicated that membrane environment is important in determining the activity of the red cell calcium pump in this disorder, although there is no consensus on the relationship between membrane fluidity and hypertension. The work described in this thesis was an attempt to dissect further the membrane structure and function relationships in metabolic disorders that are expected to affect membrane environment (hypercholesterolaemia and combined hyperlipidaemia) and the physical properties of membrane proteins [type II (non-

insulin dependent) diabetes]. In chapter 7, the relationship between serum and red cell membrane cholesterol and the possibility that the physical properties of the membrane can be altered by changes in serum lipid levels are investigated. In chapter 8, the effects of serum lipids and type II diabetes on the stucture (represented here by membrane cholesterol content and membrane fluidity) and function (represented by calcium pump activity) of the red cell membrane are investigated. The possible effects of type II diabetes on the physical properties of the calcium pump protein itself are also investigated in chapter 8. Chapter 9 presents a discussion of the findings of this study as they relate to various disease processes.

S. I. M.

Leicester, August 1994

Chapter 1

HISTORICAL BACKGROUND

1.1. DISCOVERY OF THE RED CELL CALCIUM PUMP

In human cells *in vivo* there exists a calcium gradient of more than 10^4 from outside to inside. Passow (1961, 1963) found that calcium introduced into substrate-depleted cells was not extruded upon addition of adenosine or inosine and concluded that the calcium gradient observed in normal cells was due to an impermeable membrane. The impermeability of cell membranes to calcium was later confirmed by Schatzmann and Vincenzi (1969). It was from the earlier work of Dunham and Glynn (1961) that Schatzmann (1966) proposed an additional mechanism for the maintenance of the calcium gradient in normal cells. While working on the sodium pump (Na^+-K^+-ATPase), Dunham and Glynn (1961) discovered that ATPase activity of human red cells consisted of two components. The first component required the presence of magnesium ions (Mg^{2+}) but occurred in the absence of alkali metals and was not inhibited by cardiac glycosides. In the presence of magnesium its activity was greatly increased by small amounts of calcium but inhibited by larger amounts. The second component required the presence of magnesium and both sodium and potassium ions. It was completely inhibited by cardiac glycosides in concentrations sufficient to inhibit ion transport in intact red cells. Calcium ions inhibited this latter activity at both low and high concentrations. These they called 'glycoside-insensitive' and 'glycoside-sensitive' ATPase activities, respectively.

From Dunham and Glynn's findings that 100 μM calcium inhibited the Na^+-K^+-pump, Schatzmann reasoned that the Ca^{2+}-sensitive site must be located on the internal surface of the membrane, and therefore the intracellular calcium concentration in intact cells must be considerably lower than the calcium concentration in the plasma or else the Na^+-K^+-pump would be incapacitated. He considered the possibility that this low intracellular calcium concentration might be maintained by an active transport of Ca^{2+} ions out of the cell and suspected that the ATPase of Dunham and Glynn which was strongly activated by calcium might be

connected to active outward transport of Ca^{2+} ions. In an ingenious experiment he incubated red cell ghosts containing Mg-ATP, Ca^{2+}, and, as a major osmotic constituent, KCl in isotonic medium containing $CaCl_2$, NaCl and tris buffer, and measured the calcium concentration in the external medium over a 2.5 hour period. Calcium emerged from ATP-loaded ghosts at a high rate and the release proceeded to a higher external concentration than with control ghosts treated in the same way except for the addition of Mg-ATP (Figure 1.1). The ATP-dependent loss of calcium from the ghosts resulted in a reversal of the original gradient, the final internal calcium concentration being considerably lower than the concentration for equal distribution of calcium between inside and outside, this despite a negative internal membrane potential (Figure 1.2). He argued against a role for released inorganic phosphate (P_i), Na^+/K^+- gradients and the Na^+-K^+-pump in the observed calcium movements and concluded that human red cells were able to maintain low intracellular calcium concentration by aid of an active transport mechanism for this cation which derives its energy from ATP-hydrolysis and operates independently from the Na^+-K^+-pump mechanism.

Schatzmann and Vincenzi (1969) confirmed the existence of an active extrusion mechanism for calcium in human red blood cell membranes based on the following observations:

(1) Addition of glucose to intact cells accelerated calcium efflux by a factor of about 2.

(2) The rate of loss of calcium from resealed cells was dramatically increased if ATP was introduced into the cells.

(3) Calcium transport occurred against an electrochemical gradient.

(4) Additional ATP was hydrolysed during calcium movement by the same system which, in isolated membranes, appeared as a Ca^{2+}-Mg^{2+}-activated ATPase.

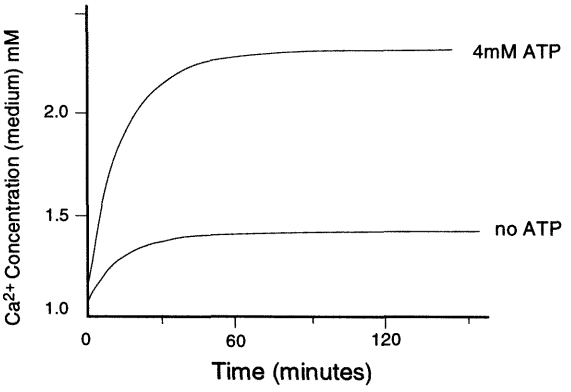

Figure 1.1. Appearance of calcium in the external fluid at 37°C. Average of 2 experiments.

Adapted from Schatzmann (1966), with permission.

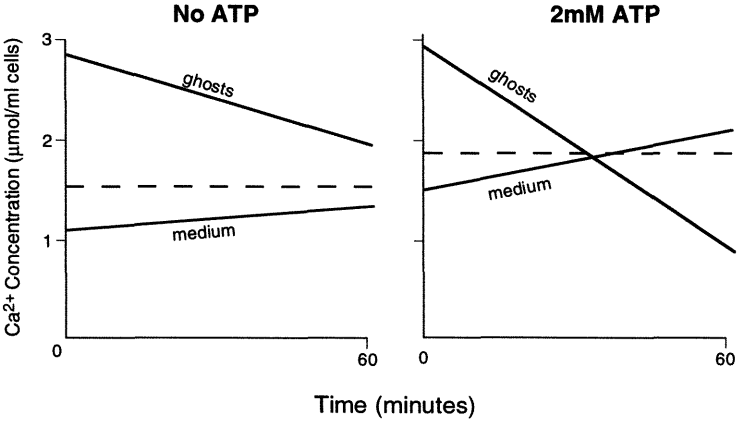

Figure 1.2. Calcium shifts with and without added ATP.

Dotted lines represent calcium concentrations calculated for equal distribution between ghosts and medium from initial concentrations and haematocrit. Straight lines joining the experimental points do not imply that the rates are constant.

Adapted from Schatzmann (1966), with permission.

(5) The activation of this ATPase system by calcium was restricted to the internal surface of the membrane only, the side from which calcium was actively transported.

(6) Magnesium was necessary, not only for the ATPase activity, but also for calcium transport.

(7) The high Q_{10} of 3.5 for calcium transport excluded the possibility of a diffusion process and favoured the idea of a chemically mediated transport.

They suggested that the Ca^{2+}-Mg^{2+}-activated membrane ATPase and the active transport of calcium were two manifestations of the same process.

For a calcium transporting system to be identified as the calcium pump it should transport calcium with high affinity and calcium transport should be coupled to ATP hydrolysis with an ample range of ATP concentrations in a pure plasma membrane preparation. Muallem and Karlish (1979) demonstrated such coupling in human red cells.

1.2. THE CALCIUM PUMP IN OTHER TISSUES

In addition to the plasma membrane, intracellular organelles possess calcium transporting systems and hydrolyse ATP. Demonstrating the existence of the calcium pump in the plasma membranes of cells with intracellular organelles is therefore more difficult than in red cells which lack intracellular organelles.

Before the discovery of the calcium pump in red cells it had been established in excitable cells that there is sodium-calcium exchange across their plasma membranes (Baker *et al.* 1967a, 1967b; Niedergerke 1963). Calcium efflux against its electrochemical gradient is driven by sodium influx down its electrochemical gradient, the sodium concentration gradient being generated by the sodium pump. Knowledge of the existence of this alternative calcium extrusion mechanism could explain why an ATP-driven calcium pump in these cells was not postulated until some ten years after its discovery in red cells.

Robinson (1976) reported Ca^{2+}-Mg^{2+}-stimulated ATPase activity and ATP-dependent calcium accumulation in rat brain microsomes. Although he was unable to demonstrate a direct relationship between the two he argued that the Ca^{2+}-Mg^{2+}-ATPase activity could represent a neural calcium transport system across the plasma membrane. Duncan (1976) demonstrated an active Ca^{2+}-Mg^{2+}-ATPase in rat, pig and sheep brain preparations. This ATPase had the typical properties associated with a high-affinity calcium transport system including K_m for the preparation of 4×10^{-7} M calcium, the pattern of activation and inhibition by calcium and the marked sensitivity to temperature. Like Robinson, he too offered no experimental evidence for the link between ATPase activity and calcium transport.

Cittadini and van Rossum (1978) showed that incubation of slices of rat liver in anaerobic conditions resulted in the net entry of calcium but during aerobiosis there was net extrusion of calcium from the slices. Calcium efflux required magnesium to attain its maximal rate and was not affected by ouabain or the absence of sodium from the medium. They concluded that the extrusion of calcium from liver cells was a metabolically dependent transport process that occurred independently of the exchange of sodium between tissue and medium. However, they, too, offered no conclusive evidence for the existence of the calcium pump.

DiPolo (1978), using the internally dialysed giant axon of the squid *Dorytheutis plei*, made the first direct demonstration of an ATP driven calcium pump in any system other than the red cell. Internal calcium was set at 0.45 μM using ethyleneglycol tetra-acetic acid (EGTA) and, to eliminate ionic gradients across the membrane, axons were perfused internally and externally with the same medium. At zero ionic gradient across the membrane in the absence of ATP, calcium influx and efflux were not significantly different in magnitude. In the presence of internal ATP calcium efflux increased 10-fold. Removal of external sodium (Na_o) reduced this ATP-activated calcium efflux by 36%. Subsequent exposure to 180 mM Na_o increased the calcium efflux to a value higher than that found at 90 mM Na_o. Thus, a fraction of this ATP-dependent calcium extrusion was dependent on external sodium.

Calcium, magnesium and ATP were without effect when applied externally suggesting that they exerted their effects from the cytosol. Although DiPolo (1978) demonstrated that calcium extrusion was ATP-dependent he offered no direct evidence of ATP hydrolysis during calcium transport - although only hydrolysable analogues of ATP (e.g. 2 deoxy-adenosine 5'-triphosphate) were able to activate calcium efflux.

Morcos and Drummond (1980) used enriched fractions of plasma membranes from dog heart. A calcium concentration of 5 μM was optimal at stimulating the enzyme. In addition, the solubilized and partially purified enzyme assumed a vesicular structure which had some ability to bind and accumulate calcium in an ATP-dependent manner, suggesting a role for the Ca^{2+}-Mg^{2+}-ATPase as a calcium pump extruding this cation to the exterior of the cell.

A direct link between Ca^{2+}-Mg^{2+}-ATPase activity, ATP hydrolysis and calcium transport was demonstrated by Caroni and Carafoli (1980) in studies on dog heart sarcolemma. The sarcolemmal vesicles hydrolysed ATP in the presence of calcium and magnesium. Concomitant with the hydrolysis of ATP the vesicles accumulated calcium. Neither oligomycin nor the uncoupler of oxidative phosphorylation, FCCP, had any effect on calcium transport ruling out any mitochondrial involvement. Oxalate had no effect and the accumulated calcium was released by sodium, thus excluding a role for the sarcoplasmic reticulum which has no calcium-sodium exchange mechanism.

1.3. DISCOVERY AND ROLE OF CALMODULIN

Cyclic adenosine monophosphate (cAMP) is an intracellular messenger whose levels rise in response to a variety of hormones. Cyclic 3',5'-nucleotide phosphodiesterase catalyses the hydrolysis of this nucleotide to 5'-AMP. Cheung (1970) found that the activity of a mixture of purified and crude bovine brain cyclic 3',5'-nucleotide phosphodiesterase was greater than the summed activities of the individual

preparations. The crude enzyme contained an activator, molecular weight 40,000 Daltons (Da), which was removed from the purified enzyme during purification. This activator effectively reconstituted the activity of the purified enzyme. Teo and Wang (1973), using bovine heart cAMP, identified the cyclic 3', 5'-nucleotide phosphodiesterase activator as a calcium-binding protein. Activation of the phosphodiesterase enzyme required the simultaneous presence of both Ca^{2+} ions and the protein activator. Cheung *et al.* (1978) named this activator protein ***calmodulin***.

The discovery that the plasma membrane calcium pump is regulated by calmodulin was a result of two seemingly unrelated observations in 1972. Scharff (1972) reported that the specific activity of the red cell calcium pump isolated in media with calcium was higher than that of membranes prepared in media with EGTA. This was later confirmed by Schatzmann (1973). The effects of the calcium chelators was reversible. This prompted Scharff and Foder (1977) to propose two conformational states of the calcium pump. State A is a low calcium affinity, low maximum velocity enzyme obtained when the membranes are prepared in the presence of chelators and state B a high calcium affinity, high maximum velocity enzyme obtained when membranes are prepared in the presence of calcium. The second observation, by Bond and Clough (1972), was that the red blood cell cytosol contained a soluble protein that activated the calcium pump. Three years later, Quist and Roufogalis (1975) reported that removal of a water soluble factor from red cell membranes reversibly decreased the apparent calcium affinity of the calcium pump. It was soon established that calcium chelators drove the pump into a low affinity and low activity state because, in the absence of calcium, Bond and Clough's activator was removed from binding sites in the membrane.

Jarret and Penniston (1977) showed that the purified phosphodiesterase activator had the same effect on calcium pump activity and apparent affinity for calcium as the partially purified activator protein obtained from the red cell cytosol. They also showed that calmodulin co-electrophoresed with the red cell activator

protein. A year later they purified the activator protein to homogeneity enabling its identity as calmodulin to be confirmed (Jarret and Penniston, 1978).

Calmodulin is a 148 aminoacid polypeptide, molecular weight 16,700 Da, with an isoelectric pH of 3.5-4.5. It is resistant to heating to 95°C for a few minutes (Schatzmann 1982). It is a calcium-modulated protein whose tertiary structure has four similar domains, each with a calcium binding site. The aminoacid sequence around the calcium binding sites is highly preserved, even in different species including protozoa and plants. Estimates on the intracellular calmodulin concentration range from 1 μM (Foder and Scharff 1981) to 10-50 μM (Barritt 1992).

1.4. ISOLATION AND PURIFICATION OF THE RED CELL CALCIUM PUMP

Early attempts to solubilize and reconstitute the Ca^{2+}-Mg^{2+}-ATPase enzyme were unsuccessful for three main reasons (Carafoli and Zuruni 1982): *Firstly*, it is present in the membrane in low concentrations. There are approximately 2,000 copies of the calcium pump per red cell membrane constituting 0.02 - 0.1% of the total membrane protein. *Secondly*, it has solubilization properties similar to those of band III, one of the most abundant components of the red cell membrane. *Thirdly*, the solubilized enzyme is very labile.

Wolf *et al.* (1977) performed the first successful solubilization and partial purification of the human red cell membrane calcium pump using Sepharose CL-6B gel chromatography. Tween 20 and Triton X-100 were used as detergents and phosphatidylcholine as stabilizer. All isolation and purification procedures were carried out in the presence of protease inhibitors. The purification protocol increased the specific activity of the enzyme from 0.02 U/mg protein of membrane-bound enzyme to 3.1 U/mg protein of purified enzyme. Three subunits were identified on gel electrophoresis:

α subunit, molecular weight 145,000 ± 5,000 Da

β subunit, molecular weight 115,000 ± 5,000 Da

γ subunit, molecular weight 105,000 ± 5,000 Da.

The α subunit could be labelled by ^{32}P-ATP indicating that the active site of the pump was located on this subunit.

Peterson *et al.* (1978) partially purified and reconstituted the human red cell Ca^{2+}-Mg^{2+}-ATPase enzyme by two methods. After solubilization by Triton X-100 and removal of detergent, the protein was *either* selectively incorporated into liposomes with a defined lipid content *or* isoelectrically focused prior to incorporation. Isoelectric focusing purified the enzyme 60-fold.

By 1979 two important facts were known about the calcium pump and its modulator, calmodulin. *Firstly*, in the presence of calcium, calmodulin directly and reversibly interacted with high affinity with the ATPase. *Secondly*, in red cells, Ca^{2+}-Mg^{2+}-ATPase was the only component binding calmodulin with high affinity that remained after washing with low ionic strength solutions. These observations provided the necessary rationale for the use of ***calmodulin affinity chromatography*** first employed by Niggli *et al.* (1979) for purifying the calcium pump protein. The calmodulin column was loaded with a Triton X-100 extract of red cell ghosts from which endogenous calmodulin had been removed by washing in EDTA. The detergent, column and elution buffers contained the acidic phospholipid phosphatidylserine to maintain the solubilized enzyme in an active state. The enzyme was purified 147-570 times compared to that in the original ghosts but was not activated by calmodulin. On sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) a single major band appeared at molecular weight 125,000 Da and a minor band (11% of the total protein) at 205,000 Da. Both bands were phosphorylated by ATP suggesting that they were active forms of the purified ATPase. The 205,000 Da protein may well have represented an aggregated form of the monomeric enzyme in SDS.

Gietzen *et al.* (1980) employed deoxycholate as detergent and phosphatidylcholine as stabilizer in their calmodulin affinity chromatography procedure. The enzyme was purified 207-fold and unlike that of Niggli *et al.* (1979) was activated by calmodulin. On SDS-PAGE its molecular weight was 135,000-150,000 Da.

Following on from their earlier work, Niggli *et al.* (1981), using phosphatidylcholine as stabilizer, were able to purify the Ca^{2+}-Mg^{2+}-ATPase enzyme, molecular weight 140,000 Da, which was stimulated by calmodulin and had a higher specific activity. They achieved a 245-fold purification using phosphatidylcholine and a 365-fold purification using phosphatidylserine. They suggested that the lower molecular weight of their earlier preparation was due to a high percentage of acrylamide in the gels resulting in slow migration of the enzyme, hence a less accurate molecular weight estimate. It is also possible that the earlier enzyme was partially proteolysed (Gietzen *et al.* 1980).

Calmodulin affinity chromatography is now widely used for isolating the Ca^{2+}-Mg^{2+}-ATPase enzyme. The specific and calcium-dependent association of the detergent solubilized ATPase with the column-bound calmodulin eliminates contaminating proteins in a single step, while the presence of acidic phospholipids throughout the entire process limits the inactivation of the enzyme.

1.5. SUMMARY

In human cells there exists an inwardly directed calcium gradient of over 10^4. Schatzmann (1966) proposed and later (Schatzmann and Vincenzi 1969) confirmed the existence of an active calcium extrusion mechanism (the calcium pump or Ca^{2+}-Mg^{2+}-ATPase enzyme, molecular weight, M_r, 138,000-140,000 Da) that maintains this gradient in the human red cell. The calcium pump has subsequently been found in all the cell types studied to date. The discovery of the modulator protein calmodulin which, in the presence of calcium, reversibly binds with high affinity to

the calcium pump has enabled the isolation and purification of the calcium pump using calmodulin-affinity chromatography.

Chapter 2

CALCIUM TRANSPORT AND PROPERTIES OF THE CALCIUM PUMP

2.1. CALCIUM DISTRIBUTION

Intracellular calcium is maintained at 0.01-0.1 μM, with an extracellular concentration of approximately 1.5-2.5 mM. Calcium channels and transporters in the plasma membrane maintain the calcium gradient between the extracellular fluid and the cytoplasm and also provide the long-term control of the amount of calcium in the cell. There are two reasons for maintaining such a low intracellular calcium concentration. *Firstly*, this prevents the precipitation of calcium phosphate in the cytoplasmic space. *Secondly*, considerably less energy is required to reversibly increase intracellular calcium concentration 5- to 10-fold than would be expended if the resting free intracellular calcium concentration was higher than 0.1 μM (Barritt 1992). Intracellular calcium is distributed between the cytoplasm and intracellular organelles, predominantly the sarcoplasmic or endoplasmic reticula and the mitochondria. Intracellular calcium concentration is controlled by the rates of movement of calcium between the cytoplasmic space and both the extracellular space and the intracellular stores. Most of the cytoplasmic and intraorganelle calcium is bound to proteins, metabolites and membrane phospholipids leaving only 0.1-1% of the total cytoplasmic calcium in the free state.

The main function of the endoplasmic or sarcoplasmic reticulum calcium stores is to release calcium into the cytoplasm in response to stimuli. The mitochondrial calcium stores act as a sink for calcium during prolonged increases in intracellular calcium concentration to levels above 1 μM (Exton 1986).

2.2. MOVEMENTS OF CALCIUM ACROSS PLASMA MEMBRANES

Calcium moves in both directions across the plasma membrane and across the membranes of the intracellular calcium stores by two processes. One is in the direction of the calcium gradient and the other against it (Figure 2.1).

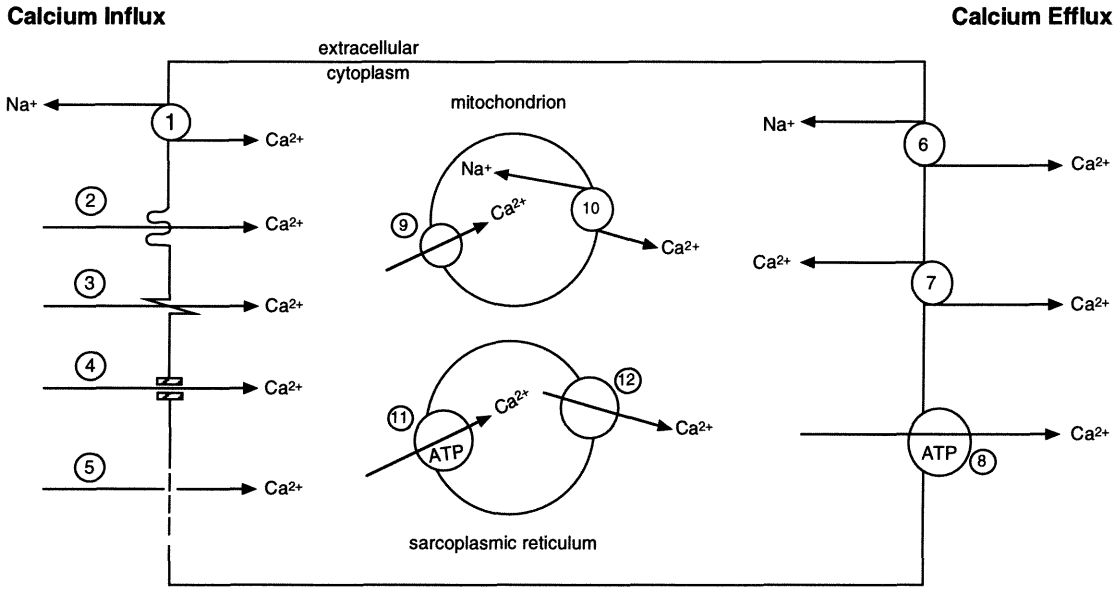

Figure 2.1. Intracellular calcium regulation

(1, 6, 10) Na^+ - Ca^{2+} exchange;
(2) Receptor - activated channels;
(3) Voltage-operated channels;
(4) Facilitated diffusion;
(5) Non-specific leak;
(7) Ca^{2+} - Ca^{2+} exchange;
(8, 11) Calcium pump;
(9) Calcium uptake process;
(12) Calcium release channels.

(1, 4, 5) constitute basal calcium influx and
(2, 3) agonist-stimulated calcium influx. Intracellular [Ca^{2+}]: 0.01 - 0.1μM, extracellular [Ca^{2+}]: 1.5 - 2.5 mM.

Adapted from Triggle (1992)

2.2.1. Calcium influx

In the majority of animal cells, calcium influx can be divided into *basal influx* which is independent of external stimuli, and *agonist-stimulated influx* which is dependent on an external stimulus (Figure 2.1). The relative contributions of these systems to total calcium influx depend on the cell type. In nerve and muscle cells, for example, calcium influx is predominantly agonist-stimulated, in red blood cells it is by basal mechanisms, and in liver cells both mechanisms are important (Barritt 1992). Some investigators have proposed the existence of calcium channels in red cells following the observations that calcium influx was saturable and inhibited by calcium channel blockers at concentrations similar to those used in heart and smooth muscle cells (Neyses *et al.* 1985).

2.2.1.1. Basal calcium influx

There are two main mechanisms, non-specific leakage and facilitated diffusion. A third possible mechanism is sodium-calcium exchange.

2.2.1.1.1. Non-specific leakage

This includes the diffusion of calcium through the phospholipid membrane bilayer itself and through gap junctions, as well as calcium influx mediated in a non-specific manner by proteins involved in the movement of other ions. Calcium penetrates into cells continuously by passive diffusion down its chemical gradient. This influx is favoured by an external versus internal calcium concentration ratio, $[Ca_o^{2+}]/[Ca_i^{2+}]$, of approximately 10^4 and a negative membrane potential. However, passive permeability of the cell membrane to calcium is very low. Rega (1986) detected no net calcium uptake in human red cells suspended in a calcium-containing buffer at 37°C. In another study, Schatzmann and Vincenzi (1969) showed that after one week in calcium-containing buffer in the cold, i.e. in conditions that reduce active transport, intracellular calcium content was only 1.8% of the starting extracellular value.

2.2.1.1.2. Facilitated diffusion

This process is thought to occur through specific protein channels. The amount of calcium transported by this mechanism per unit area of plasma membrane is very low in some cells, such as nerve, muscle and red blood cells and high in others, such as liver cells (Barritt 1992).

2.2.1.1.3. Sodium-calcium (Na^{+}- Ca^{2+}) exchange

In excitable cells (Baker 1978) a component of calcium influx may be dependent on intracellular sodium concentration and is a Ca_o^{2+}- Na_i^{+} exchange mechanism. Three sodium ions are exchanged for one calcium ion.

There is no Na^{+}- Ca^{2+} exchange in the red cell.

2.2.1.2. Agonist-stimulated calcium influx

Two mechanisms are involved in this system, namely voltage-activated and receptor-activated calcium channels. The sources of the initial rise in intracellular calcium following the binding of agonists to receptors or depolarization of the plasma membrane are the extracellular fluid and the intracellular calcium stores, predominantly the sarcoplasmic or endoplasmic reticulum. The relative contributions of these two sources vary according to the cell type. In skeletal muscle, for example, most of the increase in intracellular calcium concentration is from the sarcoplasmic reticulum whereas in platelets the extracellular fluid is the major source of calcium.

2.2.1.2.1. Voltage-activated calcium channels

The voltage-activated calcium channel is a water-filled pore with a gating mechanism to determine whether the channel is open or closed, and a selectivity filter to determine the nature of the ions to be admitted (Stanfield 1986). Compared to calcium, magnesium is poorly transported across these channels. Voltage-operated

calcium channels exist in three states - open, closed and inactivated. An inactivated channel is one that has passed into a refractory state.

2.2.1.2.2. Receptor-activated calcium influx

The binding of an agonist to a membrane receptor can activate calcium influx but, unlike voltage-operated channels, channel opening is not dependent on depolarization of the plasma membrane. There are at least two types of receptor-activated calcium inflow systems, channels which are opened by an intracellular messenger such as inositol polyphosphate or calcium itself and those which are opened by interaction of the channel protein with a GTP-binding regulatory protein (Barritt 1992).

2.2.2. Calcium efflux

Once inside the cell calcium can be rapidly and reversibly complexed to non-membranous anionic ligands such as HPO_4^{3-}, calcium-binding proteins and membrane phospholipids or temporarily sequestered by the two intracellular calcium buffering organelles - the mitochondrion and the sarcoplasmic/endoplasmic reticulum. The long-term maintenance of the 20,000-fold calcium gradient in the presence of continuous influx requires that calcium be pumped out of the cell across the plasma membrane. Two main mechanisms, the sodium-calcium exchange and the calcium pump, work in parallel with a minor third component, the internal and external calcium (Ca^{2+}_i- Ca^{2+}_o) exchange system.

2.2.2.1. Sodium-calcium (Na^+- Ca^{2+}) exchange

This mechanism, which is dependent on external sodium concentration, is a carrier-mediated Na_o^+-Ca_i^{2+} exchange system accounting for 10-50% of the total calcium efflux (Baker 1978). One Ca^{2+} ion moves out in exchange for three Na^+ ions. Because of the imbalance in charge transfer this system is electrogenic. The energy for the outward movement of Ca^{2+} ions is derived from both the Na^+ concentration

gradient across the plasma membrane created by the sodium pump and the membrane potential. In addition, ATP stimulates the movement of calcium by increasing the affinity of the transport system for both calcium and sodium without altering the maximum rate of transport. The Na_o^+-Ca_i^{2+} exchanger is a high capacity and low affinity calcium transport system which is present only in excitable cells and ejects large amounts of calcium whenever the intracellular calcium concentration rises to levels high enough (in the micromolar range) to overcome its low affinity for calcium.

It follows from this and section 2.2.1.1.3 that the Na^+-Ca^{2+} exchange mechanism can operate in either direction to extrude or take up calcium. The resting sodium gradient and membrane potential would favour calcium efflux via Na^+-Ca^{2+} exchange, and Ca^{2+} influx would be mediated by this exchange diffusion carrier during depolarization of the cell only (Exton 1986).

Recently, it has been proposed that the Na^+-Ca^{2+} exchanger can exist in either a Na^+-conformation or a Ca^{2+}-conformation (Missiaen *et al.* 1991). In the Na^+-conformation, the exchanger can operate either in the forward mode as a Ca^{2+} extrusion system (Na_o^+-Ca_i^{2+}) or in an alternative (Na_o^+-Na_i^+)-exchange mode. In the Ca^{2+}-conformation, the exchanger operates either in the reverse mode as a Ca^{2+} influx pathway (Na_i^+-Ca_o^{2+}) or in the alternative (Ca_o^{2+}-Ca_i^{2+})-exchange mode.

2.2.2.2. Ca_i^{2+}- Ca_o^{2+} exchange

This mechanism accounts for less than 10% of calcium movements across the plasma membrane. Because equal amounts of calcium are exchanged there is no net movement of calcium using this system.

2.2.2.3. Ca^{2+}-Mg^{2+}-ATPase enzyme (the calcium pump)

The calcium pump, which is independent of the external concentrations of both sodium and calcium, is the so-called residual calcium efflux. It is an active transport system which accounts for 50-70% of the total calcium efflux in some cell types. The

enzyme catalyses the electro-neutral exchange of one Ca^{2+} ion for two hydrogen (H^{+}) ions (Barritt 1992, Carafoli and Zuruni 1982). Calcium transport by this system is independent of membrane potential, deriving its energy from the hydrolysis of ATP with magnesium as a cofactor.

The calcium pump is present in all the cell types studied to date. In non-excitable cells its role in the maintenance of the calcium gradient across the plasma membrane is predominent but in excitable cells its role in calcium extrusion is minor compared to that of the larger capacity Na^{+}-Ca^{2+} exchanger (Carafoli *et al.* 1992).

2.2.2.3.1. Structure of the calcium pump

The calcium pump is an integral membrane protein of molecular weight 138,000-140,000 Da (138-140 kDa). It is a single polypeptide whose 1,249 aminoacid sequence has been fully characterized (Carafoli and Zuruni 1982, Strehler *et al.* 1990). Four isoforms of the plasma membrane calcium pump (PMCA1-4), coded for by different genes, have been described. PMCA1 has a broad distribution, PMCA2 is found in brain, heart and liver, PMCA3 in brain and skeletal muscle, and PMCA4 in red cells and intestinal mucosa (Carafoli *et al.* 1992, Missiaen *et al.* 1991 for a review). Western blotting analysis using monoclonal and polyclonal antibodies has demonstrated up to four bands in the 130-140 kDa range, the relative proportions of these bands being tissue-specific.

The purified Ca^{2+}-Mg^{2+}-ATPase enzyme has no carbohydrate moieties, is almost devoid of calmodulin and has a tendency to aggregate. Controlled proteolysis using trypsin has revealed that the 138 kDa enzyme is degraded to a 90 kDa molecule which still behaves as a calmodulin-sensitive ATPase. Further degradation produces an 85 kDa molecule that still binds to but is not stimulated by calmodulin, then to an 81 kDa protein that has fully expressed ATPase activity but is insensitive to calmodulin (Carafoli 1988, Zuruni *et al.* 1984). These findings have led to the proposal that the calmodulin interacting domain of the ATPase consists of a 4 kDa sequence that contains the calmodulin binding site proper and a 5 kDa sequence that

is essential for the expression of calmodulin stimulation (Benaim *et al.* 1984, Carafoli 1988, Figure 2.2). The calmodulin binding domain is located near the COOH-terminus of the calcium pump and is a positively charged segment (Carafoli *et al.* 1992) of some 30 aminoacids.

The 3-dimensional structure of the calcium pump is unknown but calculations based on its composition suggest that the calcium pump is moderately hydrophobic (Carafoli and Zuruni 1982) with 80% of its structure protruding into the cytoplasm. There are 10 transmembrane helical domains connected on the external side by very short loops and 3 main protuding domains on the internal side (Carafoli *et al.* 1992). The first of the internal units is a β-strand which couples ATP hydrolysis to the translocation of calcium and the second, the largest, has the ATP binding site. The third, which follows the tenth transmembrane helix, is 150 aminoacids long and contains important regulatory sites including the calmodulin-binding domain, substrate sites for protein kinases A and C, and possible calcium-binding sites. The sites involved in channeling calcium across the membrane - 'the catalytic calcium-binding sites' - are in the transmembrane domain (Carafoli *et al.* 1992).

2.2.2.3.2. Kinetic properties of the calcium pump

Whereas the sodium-calcium exchanger is a low affinity, high capacity transport system whose optimal activity is reached when intracellular free calcium concentration is in the micromolar range, the Ca^{2+}-Mg^{2+}-ATPase enzyme pumps calcium with higher affinity but lower capacity and is the system that maintains the low levels of cytosolic calcium at rest. It is stimulated only by intracellular calcium (Schatzmann and Vincenzi 1969) and magnesium is essential for its activity (Sarkadi *et al.* 1978). It displays calcium-dependent phosphorylation by ATP and ATP- and magnesium-dependent dephosphorylation (Schatzmann 1982, Foldes-Papp 1992). The plasma membrane calcium pump is a 'P-type' cation transport ATPase because it forms a covalently phosphorylated intermediate during its reaction cycle.

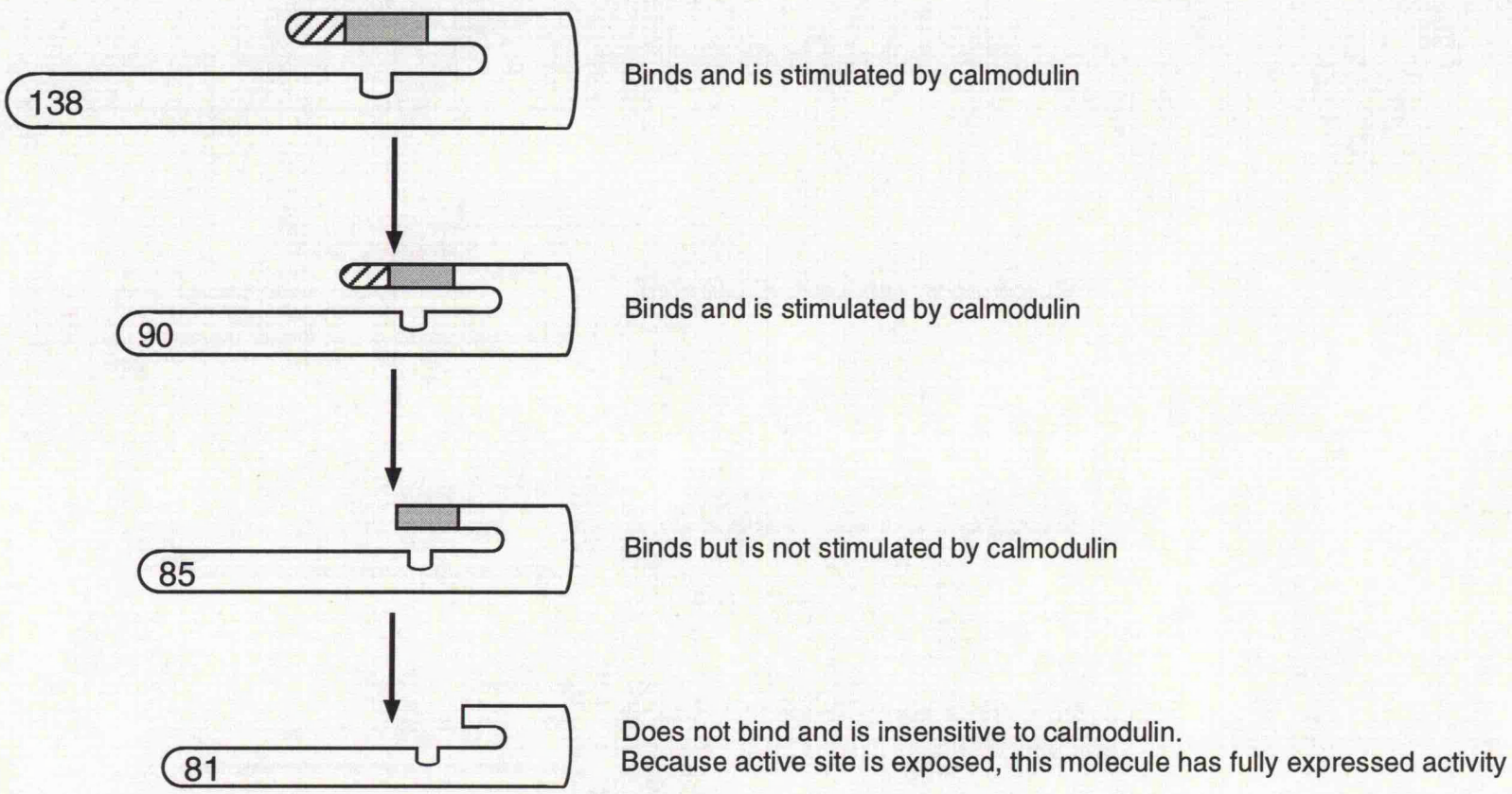

Figure 2.2. Proposed structure of the calcium pump based on trypsin digestion of the 138 kDa molecule (schematic)

The active site is shielded by a 9 kDa sequence consisting of a 4 kDa molecule that binds calmodulin (▭) and a 5 kDa sequence that is required for the expression of calmodulin stimulation (▨).

Adapted from Carafoli 1988

There are two basic conformations of the red cell calcium pump. A low activity (low V_{max}), low affinity A-state is converted to a high activity, high affinity B-state (Scharff and Foder 1977, Scharff 1978) by calcium-induced calmodulin binding and by calcium-independent processes including mild proteolysis which removes the regulatory domain (Muallem and Karlish 1980), phosphorylation by protein kinases - cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C (Missiaen *et al.* 1991) - and by lipid modification in the inner membrane leaflet. Acidic phospholipids, long chain polyunsaturated fatty acids (Sarkadi *et al.* 1982), heparin, salicylic acid, alkali metals (Na^+, K^+) and NH_4^+ all increase the affinity of the calcium pump for calcium and hence stimulate the enzyme. The negatively charged, acidic phospholipids are powerful activators of red cell and synaptosomal calcium pump (Carafoli and Zuruni 1982, Missiaen *et al.* 1991), in the order phosphatidylinositol-4,5-bisphosphate > phosphatidylinositol-4-phosphate > phosphatidylinositol = phosphatidylserine = phosphatidic acid. They increase both V_{max} and calcium affinity due to direct binding of the phospholipid to the calcium pump but higher concentrations inhibit the enzyme by reducing V_{max} (Missiaen *et al.* 1991). The proposition that phosphatidylinositol is involved in calcium-mediated cell responses makes this an interesting observation.

A possible mechanism of action of acidic phospholipids, fatty acids and the calmodulin-calcium complex is the introduction of a relatively hydrophobic environment into the neighbourhood of the active site. This alters the conformation of the protein in a way that makes the active site more accessible. Since the calcium-calmodulin complex is anionic and partially hydrophobic, for reasons of complementarity, the regulatory domain has to be hydrophilic and cationic (Carafoli *et al.* 1992). A domain with these properties would bind, in addition to the calcium-calmodulin complex, anionic amphiphiles such as acidic phospholipids and unsaturated fatty acids. Trypsin digestion of the regulatory domain has the same effect of exposing the active site (Carafoli and Zuruni 1982).

On conversion from the A-state to the B-state V_{max} and calcium affinity increase 3- to 5-fold. The preparation of ghost membranes in the absence of calcium removes calmodulin, giving an A-state Ca^{2+}-Mg^{2+}-ATPase enzyme. The presence of calcium, even in the form of impurities, during the preparation process gives the B-state enzyme (Scharff and Foder 1977).

2.2.2.3.3. Calmodulin and calcium pump activity

Calmodulin is present in all cells and interacts with a wide variety of proteins, including the calcium pump, called calmodulin-binding proteins. It acts by either binding directly to the protein or activating calmodulin-dependent protein kinases. With regard to the calcium pump, ^{125}I-calmodulin binding studies favour the former mechanism with a calmodulin:calcium pump stoichiometry of 1:1 (Barritt 1992). The fact that the solubilized enzyme can be activated by calmodulin lends further support to the direct binding mechanism. This binding is absolutely dependent on calcium. Only the calcium-calmodulin complex has the right conformation to interact with the Ca^{2+}-Mg^{2+}-ATPase enzyme.

The calmodulin concentration in red cells is 50-100 times higher than the Ca^{2+}-Mg^{2+}-ATPase enzyme concentration but its affinity for calcium is relatively low and calcium-induced calmodulin binding to the pump protein is also relatively low. Foder and Scharff (1981) concluded that *in vivo* most of the red cell calmodulin is dissociated from the calcium pump and that optimal calcium binding to calmodulin and the subsequent activation of the pump require a cytoplasmic calcium concentration of 1-10 μM. Therefore, the resting cytosolic calcium concentration of 0.1 μM is less than that needed to saturate the calcium binding sites of calmodulin. In stimulated cells, the large pool of cytosolic calmodulin ensures a rapid but not instantaneous response of the calcium pump to transient increases in cytosolic calcium concentrations. Intracellular calmodulin concentrations are well above those necessary to establish maximally effective concentrations of calcium-calmodulin

complexes. This suggests that calmodulin is not the limiting factor in intracellular reactions in which it participates.

At first it was thought that three calcium ions and one magnesium ion bind to calmodulin which, in turn, forms a 1:1 complex with the pump protein. If four rather than three calcium ions bind there is self-inhibition on calmodulin (Schatzmann 1982). This explains the inhibitory effects on the calcium pump of high calcium concentrations. Other reports, however, suggest that all four calcium binding sites on the calmodulin molecule are occupied by calcium (Klee *et al.* 1983). If so, the mechanism by which calcium inhibits the Ca^{2+}-Mg^{2+}-ATPase enzyme needs to be re-examined. Calcium concentrations above 0.1 mM inhibit the enzyme but this inhibition is overcome by increasing the magnesium concentration (Dunham and Glynn 1961) because magnesium competes with calcium at all the calcium binding sites on the calmodulin molecule. Above a magnesium concentration of 1 mM, magnesium shifts the calcium concentration for half maximal ATPase activation, K_m, to higher values i.e. high magnesium concentrations drive the ATPase into the A-state. In the presence of physiological intracellular concentrations of magnesium (1 μM), an increase in intracellular calcium concentration leads to increased calcium-calmodulin binding. The resulting conformational change allows this complex to bind to a diversity of enzymes and other proteins involved in such activities as calcium transport, glycogen and cyclic nucleotide metabolism, and neurotransmitter synthesis and release (Winkler *et al.* 1987). Upon binding to a target enzyme, the calcium-calmodulin complex increases its V_{max}, with little effect on K_m for the substrate.

To assess the quantitative effects of added calmodulin in intact membranes three conditions must be fulfilled. *Firstly*, the membrane must be devoid of endogenous calmodulin. This is achieved by either preparing the membranes in the presence of or treating the prepared membranes with calcium chelators. *Secondly*, the pump must be protected against endogenous proteolysis since partial proteolysis mimicks the effects of calmodulin. This is achieved by preparing the membranes in

the presence of proteolysis inhibitors. However, calcium pump activity may be impaired in the presence of proteolysis inhibitors (Madden *et al.* 1981). *Thirdly*, the membrane lipid fraction must be poor in acidic lipids which mimick the effects of calmodulin.

The solubilized Ca^{2+}-Mg^{2+}-ATPase enzyme is very labile. At 0°C its half-life is 1-2 days, increasing to 1 week if calcium and calmodulin are added to final concentrations of 50 μM free calcium and 5 μg/ml calmodulin (Steiger and Luterbacher 1981). In the presence of calcium and calmodulin it can be maintained in liquid nitrogen for several weeks. The purified enzyme shows affinities for calcium, magnesium and ATP similar to those in intact membranes. In intact membranes, calmodulin increases the maximum rate of ATPase activity (ATP hydrolysis) and/or active calcium transport 2- to 9-fold. In the purified enzyme calmodulin induces an 8- to 10-fold increase in activity and a 20-fold increase in apparent affinity for calcium (Gietzen *et al.* 1980, Steiger and Luterbacher 1981). The purified enzyme is inhibited at a higher calcium concentration than the membrane-bound enzyme (Niggli *et al.* 1981). If the enzyme is purified in the presence of phosphatidylcholine it can be activated by calmodulin whereas that purified in the presence of phosphatidylserine is fully activated without calmodulin (Niggli *et al.* 1979, 1981; Steiger and Luterbacher 1981). However, Adeoya *et al.*, in hypertension studies in the rat model (1989) and in humans (1992), were able to stimulate their phosphatidylserine-stabilised solubilized enzyme at least 3-fold, albeit with a calmodulin concentration 10 times higher than that used in their intact membranes.

Both membrane-bound and solubilized enzymes are inhibited by low concentrations of vanadate, a phosphate analogue. There is an inverse relationship between the concentration of vanadate causing inhibition of the pump and that of magnesium. Other inhibitors include: lanthanides by binding to calcium sites, phenothiazines by eliminating hydrophobic interactions between the calcium-calmodulin complex and the enzyme molecule, high calcium concentrations by

binding to the magnesium site of calmodulin, α-adrenergic antagonists and a number of antidepressant drugs. Drugs which inhibit calmodulin do so by interacting only with the calcium-calmodulin complex and not with free calmodulin.

Evidence that the Ca^{2+}-Mg^{2+}-ATPase enzyme in intact red blood cell membranes is an active calcium pump was presented in chapter 1. That calcium transport is coupled to ATP hydrolysis was also demonstrated very early during the reconstitution attempts on the purified ATPase (Niggli *et al.* 1981). The calcium:ATP stoichiometry is probably 1:1 in the red cell and 2:1 in the sarcoplasmic reticulum (Schatzmann 1982). During calcium transport the calcium pump undergoes calcium-dependent phosphorylation and ATP- and magnesium-dependent dephosphorylation reactions via an enzyme-phosphate intermediate. Phosphorylation requires ATP at the high affinity, catalytic site and calcium at the transport site of the enzyme (Katz and Blostein 1975). Magnesium affects the rate but not the steady-state level of phosphorylation and is effective only from the cytoplasmic surface of the membrane. The link between ATP hydrolysis and calcium transport is presented in a simplified and schematic form in Figure 2.3. This system is reversible. Hence, if calcium is forced across the pump inwards ATP is synthesized from ADP and inorganic phosphate, P_i (Schatzmann 1982). For this to occur intracellular ATP concentration should be low, with high intracellular concentrations of ADP and P_i, and high extracellular calcium relative to intracellular calcium concentrations. The synthesis of ATP is catalysed by the calcium pump using the energy of the calcium concentration gradient.

2.3. IMPORTANCE OF THE CALCIUM PUMP

The importance of the Ca^{2+}-Mg^{2+}-ATPase enzyme can be appreciated from a review of the diverse intracellular functions of the Ca^{2+} ion.

Adrenaline and noradrenaline (acting via α1 adrenergic receptors), acetylcholine (ACh, via cholinergic receptors), cholecystokinin and angiotensin II

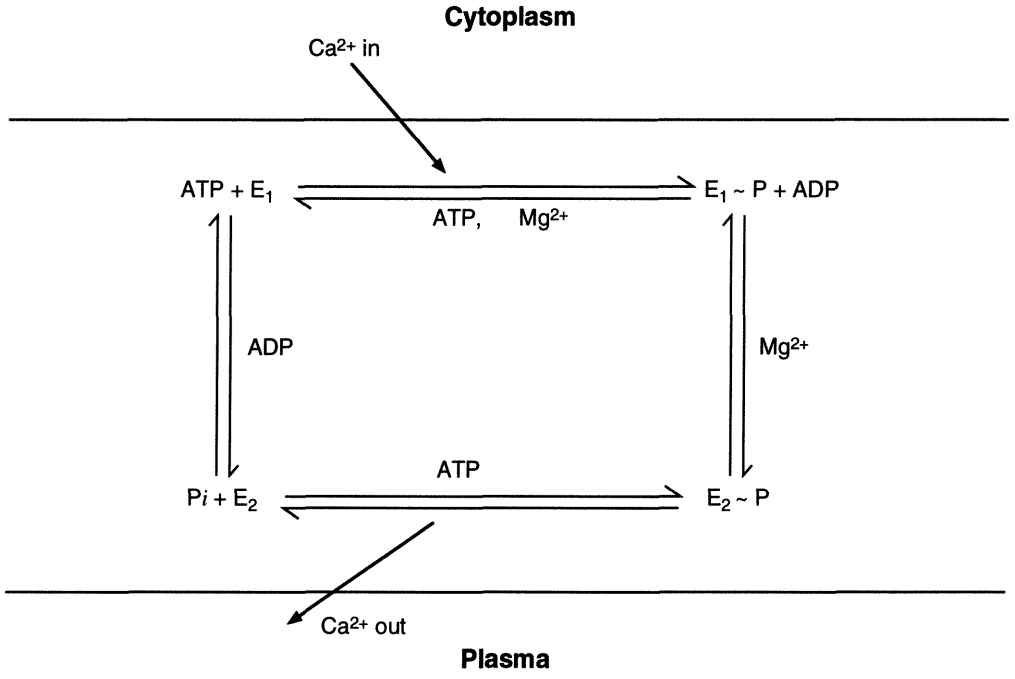

Figure 2.3. Red cell membrane calcium pump in situ (schematic)

E_1 and E_2 are different conformations of the same enzyme. Calcium binding sites face the cytoplasm in E_1 and the cell exterior in E_2. E_1 possesses high affinity and E_2 low affinity sites for ATP. The two ATP sites do not coexist in the same molecule, but are different states of the same site.

are major agonists that exert their biological effects by increasing the levels of intracellular calcium in their target cells (Exton 1986). The mechanism by which agonists acting at the cell surface are able to mobilize calcium from internal stores has been elucidated. Upon binding to a specific plasma membrane receptor the agonist induces rapid hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) by the enzyme phospholipase C, generating two compounds - *myo*-inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) - which act as second messengers. IP_3 is the headgroup and DAG the lipid portion of the PIP_2 molecule. Diacylglycerol remains in the membrane where it stimulates the Ca^{2+} and phospholipid (especially phosphatidylserine)-dependent protein kinase C to phosphorylate specific proteins. The water-soluble IP_3 diffuses into the cytosol where it binds to specific intracellular receptors that promote calcium efflux from selected storage sites (Berridge 1992). Calcium therefore acts as a third messenger in the stimulus-effector cascade.

Because intracellular calcium stores are limited and the calcium pump is very active in extruding calcium, in order to raise intracellular calcium concentrations for an appreciable period of time there needs to be either an increased influx of calcium or an inhibition of the calcium pump or both. Evidence for the inhibition of the calcium pump exists in liver cells (Lin *et al.* 1983, Prpic *et al.* 1984) and uterine smooth muscle (Soloff *et al.* 1982). Polyphosphoinositides, on the other hand, stimulate the calcium pump (Penniston 1983). Since agonists deplete the membrane of polyphosphoinositides, this could be a possible means of regulating calcium pump activity. The reasons for maintaining low resting intracellular calcium concentrations were discussed in section 2.1. Were calcium pump activity to be permanently depressed the resulting sustained increase in intracellular calcium concentration would impair cellular function.

The major intracellular target for calcium is the Ca^{2+}-dependent regulatory protein calmodulin which binds calcium with high affinity. The resulting calcium-calmodulin complex interacts with a variety of enzymes (multifunctional calmodulin-dependent protein kinases and other more specific protein kinases)

altering their function. A small number of enzymes are also calcium-dependent but calmodulin-independent. Examples of calcium dependent processes include muscle contraction, secretory processes, neurotransmission, glycogenolysis, platelet aggregation, phagocytosis and cell division and differentiation.

2.4. SUMMARY

Cells maintain low resting intracellular concentrations of ionised calcium in the face of large and inwardly directed concentration and electrochemical gradients. The several processes which regulate intracellular calcium concentration are not of equal importance in every cell type. There are two main calcium extrusion mechanisms. The calcium pump (Ca^{2+}-Mg^{2+}-ATPase enzyme), which is magnesium-dependent and calcium-stimulated in the presence of the activator protein calmodulin, is a high affinity and low capacity system, whereas the Na^{+}-Ca^{2+} exchanger extrudes calcium with low affinity and high capacity. There is no Na^{+}-Ca^{2+} exchange in red cells.

Chapter 3

MEMBRANE FLUIDITY AND THE CALCIUM PUMP

3.1. RED CELL MEMBRANE STRUCTURE

According to the Singer and Nicolson (1972) model, a biological membrane consists of a phospholipid bilayer in which various proteins are located.

3.1.1. Membrane lipids

In the red cell membrane the lipid content is approximately 40% of the membrane dry weight and consists of 40% unesterified cholesterol, 50% phospholipids and 10% glycolipids. Sphingomyelin, phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine make up 95% of the total phospholipid content (Roelofsen *et al.* 1981). The phospholipids are asymmetrically distributed in the bilayer, with the choline-containing sphingomyelin and phosphatidylcholine in the outer leaflet and phosphatidylserine and phosphatidylethanolamine in the inner leaflet (Bretscher and Raff 1975). All the glycolipids are located on the outer leaflet with their sugar residues exposed to the cell exterior. Together with glycoproteins, glycolipids determine the antigenic properties of the cell (Furthmayr 1977). The fatty acyl chains of the phospholipids have differing lengths and degrees of unsaturation (Rice-Evans and Chapman 1981, Shinitzky 1984).

Cholesterol is located within the bilayer matrix, with more of it in the outer than in the inner membrane leaflet (Roelofsen *et al.* 1981). Interestingly, when red cell membranes are depleted of cholesterol a disproportionate amount of that cholesterol is derived from the inner leaflet, whereas when they are enriched with cholesterol a disproportionate amount is incorporated into the outer leaflet (Flamm and Schachter 1982).

3.1.2. Membrane proteins

Human red cell membrane proteins are divided into two groups, integral proteins and peripheral proteins (Bennett 1985, Furthmayr 1977, Guidotti 1972).

3.1.2.1. Integral proteins

Integral proteins are transmembrane proteins that span the whole width of the phospholipid membrane bilayer. There are two major integral proteins, band 3 and glycophorin.

3.1.2.1.1. Band 3 (M_r 89 kDa).

Band 3 constitutes 25% of the total membrane protein content (Furthmayr 1977) and contains a large water-soluble cytoplasmic domain (M_r 43 kDa) associated with glycolytic enzymes, aldolase, phosphofructokinase and glyceraldehyde-3-phosphodehydrogenase (Bennett 1985). The cytoplasmic domain is also associated with spectrin, band 4.2, and ankyrin (Bennett 1980, Hargreaves *et al.* 1980, Weaver *et al.* 1984). Band 3 is involved in facilitated diffusion of anions across membranes (Furthmayr 1977, Luna and Hitt 1992), and in the transport of glucose (Lin and Spudick 1974) and water (Brown *et al.* 1975).

3.1.2.1.2. Glycophorins

Glycophorin A (M_r 31 kDa) is the major sialic acid-containing glycoprotein (or sialoglycoprotein) and has blood group antigens and binding sites for lectins and viruses (Marchesi *et al.* 1976). Glycophorin B (M_r 23 kDa) and glycophorin C (M_r 29 kDa) are minor sialoglycoproteins.

3.1.2.2. Peripheral proteins

Approximately 50% of the red cell membrane proteins are water-soluble and located on the cytoplasmic surface of the membrane (Bennett 1985) and are termed peripheral proteins. These include spectrin, ankyrin, actin, the band 4 complex (4.1, 4.2 and 4.9), band 7, band 8, tropomyosin, myosin and glyceraldehyde-3-phosphodehydrogenase. A number of these proteins fulfil a structural role.

3.1.2.2.1. Spectrin

Spectrin consists of two polypeptide chains, an α-chain (M_r 260 kDa) and a β-chain (M_r 225 kDa) arranged into a $(\alpha\beta)_2$ tetramer. It has specific binding sites for a number of proteins, including actin, ankyrin, band 4.1 (Fowler *et al.* 1981) and calmodulin (Berglund *et al.* 1984).

3.1.2.2.2. Ankyrin (M_r 215 kDa)

Ankyrin is a monomeric phosphoprotein that links spectrin to the cytoplasmic domain of band 3 (Bennett 1985).

3.1.2.2.3. Actin (M_r 43 kDa)

Actin associates with band 4.9 and spectrin.

3.1.2.2.4. Band 4.1 (M_r 78 kDa)

Band 4.1 has a membrane-binding site (Bennett 1985) and also binds to spectrin and to actin (Pinder *et al.* 1984).

3.1.3. Organization of the red cell cytoskeleton

Spectrin forms the backbone of the membrane cytoskeleton and forms a meshwork with actin and associated proteins (Bennett 1985). Ankyrin and band 4.1 link this meshwork to the plasma membrane. In addition, ankyrin links band 3 (the anion exchanger) to the β-subunits of the spectrin molecule. Spectrin and band 4.1 bind with low affinity to negatively charged plasma membrane phospholipids, interactions that may help to stabilize the lipid bilayer and maintain its phospholipid asymmetry (Shukla *et al.* 1978).

3.2. DEFINITIONS

3.2.1. Membrane fluidity and microviscosity

The fluid nature of membranes is now well documented and widely accepted (Singer and Nicolson 1972, Cherry 1975). In an isotropic fluid, viscosity, η, is a measure of its resistance to an applied shearing force. Fluidity (ϕ), the reciprocal of viscosity, is a measure of the tendency of the fluid to flow. Under a given set of environmental conditions, a single coefficient of viscosity is sufficient to describe a given isotropic system fully.

Biological membranes are highly anisotropic, two-dimensional molecular or submolecular microdomains. In these systems fluidity and microviscosity are used to describe the tightness of organization or order of the membrane components (structural information) and/or how easily the membrane components move (dynamic information). These concepts are inherently ambiguous when applied to biological membranes. For example, the frictional force opposing the movement of a small molecule across the plane of the membrane is different from that opposing the movement of the same molecule within the membrane plane. At a molecular level, the lipid bilayer itself is not a homogeneous system. The orientational order of its phospholipid molecule decreases and the rate of motion increases in the direction from the glycerol backbone towards either the polar headgroup or the hydrocarbon chains - the so called 'fluidity gradient' (Levine *et al.* 1972, Stubbs 1983). Each microdomain exhibits a different microviscosity which contributes to the average microviscosity of the system (Shinitzky 1984). Membrane microviscosity, $\bar{\eta}$, is a weighted average of the microviscosities of these domains and is used to relate these intrinsic viscosities to that of an isotropic macroscopic system. Although it predominantly refers to the phospholipid bilayer, microviscosity covers other membrane components, in particular thermal mobility of membrane proteins (Cherry 1975, Inbar and Sachs 1973, Inbar *et al.* 1973). Therefore, unlike an isotropic fluid, a

single fluidity or viscosity coefficient cannot adequately characterize a biological membrane.

Structural and dynamic information on the membrane can be expressed in terms of fluidity and microviscosity or, alternatively, fluorescence polarisation and anisotropy.

3.2.2. Fluorescence polarisation and fluorescence anisotropy

When a fluorophore is excited by polarized light some of the light energy is absorbed and the molecule attains a transient excited state. Fluorophore molecules have definite axes and only those whose axes are in the direction of the polarised light will attain an excited state, a process called photoselection (Udenfriend 1969). On returning to the ground state the fluorophore emits light of lower energy and longer wavelength than that of the excitation light. This **lifetime of the excited state, *t***, is in the order of 10^{-9} seconds but varies for any given probe under different conditions. The greatest fluorescence intensity is detected if the excitation and emission dipoles are parallel to each other. The resulting emission which should also be polarised can be depolarised for several reasons resulting in reduced fluorescence intensity. Rotational diffusion of the fluorophore is one such cause. A group of fluorophores will have their axes completely randomised due to Brownian motion. If we observe only those with the same orientation over a period of time they too become disorganised. The time taken to change from an organised to a disorganised state is called the **relaxation time** of the fluorophore (Udenfriend 1969). In media of high viscosity and at low temperature Brownian motion is lowest and fluorescence intensity is highest. The observed fluorescence intensity is also influenced by the lifetime of the excited state of the fluorophore. If the relaxation time is shorter than the lifetime of the excited state, by the time emission occurs the excited molecules are already disorganized and the resulting fluorescence is depolarised. If, on the other hand, relaxation time is longer than the lifetime of the excited state disorientation is incomplete and the fluorescence will be partially polarised.

If we consider a fluorophore molecule orientated in the direction of the polarised excitation light and whose absorption and emission dipoles are parallel, two kinds of angular displacement of the dipoles can occur: displacement of the emission dipole by an angle θ from the excitation light and displacement of the absorption and emission dipoles by an angle α. ***Fluorescence polarisation***, P, and ***fluorescence anisotropy***, r, refer to these angular displacements of the fluorophore dipoles which occur between the absorption and the emission of a photon (Lakowicz 1983). Partially polarised light travelling along a given axis can be resolved into a vertical (y) and two horizontal (x, z) components. Fluorescence polarisation is the ratio of the polarized light in the x and y planes, whereas fluorescence anisotropy is the ratio of the polarised component to the total light intensity (x, y, and z planes). The observed anisotropy is affected by both intrinsic depolarisation (photoselection and angular displacement of the dipoles) and extrinsic depolarisation which includes rotational depolarisation of the fluorophore and energy transfer among the fluorophore molecules. Rotational depolarisation is minimal if the relaxation time is much longer than the lifetime of the excited state. Inefficient energy transfer is minimal if the fluorophore concentration in solution is less than 13 mM (Lakowicz 1983). Light scattering, light reabsorption and misalignment of the polarisers also affect fluorescence anisotropy.

Although fluorescence polarisation and fluorescence anisotropy are properties of the fluorophore they are influenced by the environment of the membrane in which they are embedded and are related directly to membrane microviscosity and inversely to membrane fluidity (Shinitzky and Inbar 1974). When a fluorophore molecule labels a membrane domain, how easily that molecule rotates depends on how tightly packed the membrane components are. The more rigid the membrane the less the fluorophore rotates and the greater the observed fluorescence intensity. The less rigid (i.e. the more fluid) the membrane the more the fluorophore molecule rotates and the less intense the resulting fluorescence. This principle is employed in ***fluorescence spectroscopy*** and is discussed in greater detail later in this chapter.

Several factors influence membrane microviscosity. These can be divided into those intrinsic to the probe, those affecting the fluorophore in solution (both discussed above) and those intrinsic to the membrane (membrane environment). If the same probe is used under identical experimental conditions, useful information on the immediate environment of various membrane preparations can be obtained.

3.3. FACTORS AFFECTING MEMBRANE FLUIDITY

There are three main determinants of membrane microviscosity: cholesterol/phospholipid mole ratio, length and degree of unsaturation of acyl chains and relative amounts of sphingomyelin in the system (Shinitzky 1984, Shinitzky and Barenholz 1978, Shinitzky and Inbar 1976).

3.3.1. Cholesterol/phospholipid mole ratio

This is the most important determinant of membrane microviscosity. Phospholipids and cholesterol are the two major constituents of biological membranes, cholesterol being the prominent sterol in all animal cell membranes. Essentially a solute in a phospholipid solvent, where it is only loosely bound, cholesterol freely distributes among its potential sites of solubilization within lipoproteins and cell membranes. Total cell membrane cholesterol is influenced by the membrane phospholipid content, the fraction of this phospholipid available to solubilize cholesterol, and the cholesterol/phospholipid ratio of plasma lipoproteins in equilibrium with cell membranes (Shinitzky 1984). For example, cholesterol enrichment of plasma lipoproteins and cell membranes occurs in some patients with cirrhosis in whom liver injury is such that the cholesterol/phospholipid ratio within the exchangeable pool is maintained at 50-75% above normal (Cooper *et al.* 1974). Equilibrium is established in terms of: *simple equilibrium exchange* - a molecule of membrane cholesterol enters the plasma and a molecule of plasma cholesterol enters the membrane, a process with a half life of two and a half hours, and the

cholesterol/phospholipid mole ratio (normally less than 1.0) in these various compartments, an equilibrium partition with a half life of 8-10 hours.

The 3β-hydroxyl group, δ 5 double bond, central planar ring and branched aliphatic chain at C-17 of the cholesterol molecule provide proper alignment with the phospholipid acyl chain and modulate the microviscosity of the lipid bilayer. Cholesterol increases both the order and the rigidity of the lipid bilayer through the interaction of its rigid backbone with the phospholipid acyl chains. This interaction between cholesterol and phospholipids imposes a degree of immobility on the portions of the lipid molecules that are nearest the membrane surface and increases the freedom of motion deeper in the hydrophobic core - creating an 'intermediate state of fluidity'. Being shorter than the fatty acyl chains, cholesterol restricts angular motion of the first 10 carbons by reducing the range but not the rate of motion of fatty acyl chains (Stubbs 1983). It also acts as a spacer, increasing the separation between phospholipid groups, thus disrupting intermolecular attractions between the phospholipid heads and freeing them to engage in more rapid rotational motion (Cullis *et al.* 1976). In the fluid phase of lipids, increasing the cholesterol/phospholipid ratio increases membrane microviscosity (Shinitzky and Inbar 1976, Straume and Litman 1987). Below the phase transition, T_c, which in most cases is physiologically irrelevant, cholesterol increases membrane fluidity by perturbing its regular structure. That the amount of membrane cholesterol correlates with the fluidity of the membrane has been demonstrated in liposomes (Shinitzky and Inbar 1976, Straume and Litman 1987), red cells (Shinitzky and Inbar 1976) and platelets (Cooper 1977).

It follows therefore that translocation of cholesterol may be used to change and control membrane microviscosity under various physiological conditions. An increase in the cholesterol/phospholipid ratio increases microviscosity in both liposomes and cell membranes. Increasing the ratio from 0 to 1 causes a 4-fold increase in microviscosity (Shinitzky and Inbar 1976), but increasing the ratio above 2 causes no further change in microviscosity (Shinitzky 1984).

3.3.2. Length and degree of unsaturation of the phospholipid acyl chains

Increased acyl chain unsaturation promotes headgroup and acyl chain mobility (Levine *et al.* 1972). Acyl chains with cis-double bonds are more fluid than those with trans-double bonds because of a greater reduction in the compactness of side chain packing with cis-double bonds. The increase in fluidity is greater when saturated acyl chains are desaturated than when unsaturated chains are further desaturated (Shinitzky 1984).

Fatty acyl chain length determines the thickness of the membrane bilayer.

3.3.3. Relative amount of sphingomyelin in the system

Phospholipids are asymmetrically distributed in the plasma membrane (section 3.1.1). In addition, phosphatidylethanolamine can be sequentially methylated in the lipid bilayer to lecithin with a subsequent increase in membrane fluidity. Alterations in the lipid composition of cell membranes can therefore influence membrane fluidity (Stubbs and Smith 1984). An increase in the sphingomyelin/phosphatidylcholine ratio increases microviscosity. Glycolipids increase membrane fluidity, sphingolipids decrease it (Shinitzky 1984). However, some investigators have found no change in membrane fluidity when fatty acid composition and the degree of fatty acyl chain unsaturation are altered (Rand *et al.* 1986).

Other factors affecting membrane microviscosity include:

3.3.4. Presence of neutral lipids

At relatively high levels of neutral lipids, such as triglycerides, microviscosity correlates better with the cholesterol/total lipid ratio than with the cholesterol/phospholipid ratio (Shinitzky and Inbar 1976).

3.3.5. Membrane proteins

Membrane proteins have qualitatively the same effect on fluidity as cholesterol, especially at cholesterol/phospholipid ratios less than 0.5 (Shinitzky and Barenholz 1978). Fluidity and functional properties of biological membranes are therefore achieved by full collaboration between proteins and lipids.

3.3.6. Presence of amphipathic molecules

Amphipathic molecules such as lyophosphatides increase membrane fluidity (Cooper 1977).

Physical factors also modulate membrane fluidity (Shinitzky 1984). These include:

3.3.7. Temperature

Microviscosity is exponentially dependent on the reciprocal of absolute temperature.

3.3.8. Membrane potential

The formation of an electrical potential across a lipid bilayer causes a small but significant increase in microviscosity.

3.3.9. pH

Changes in pH can affect the charges on phospholipid headgroups and, consequently, lipid-lipid interactions.

3.3.10. Calcium

At low concentrations calcium has no effect on microviscosity. Above a concentration of 0.1 mM calcium reduces membrane fluidity (Viret and Leterrier 1976) by cross-linking with negatively charged phospholipids.

3.4. MEMBRANE FLUIDITY IN CLINICAL PRACTICE

In systems where lipid metabolism is minimal, such as red cells and platelets, serum and membrane lipids are in dynamic equilibrium (Shohet 1972, Shinitzky and Barenholz 1978). Membrane fluidity should therefore be affected by clinical conditions in which lipid metabolism is abnormal.

Increased red cell membrane cholesterol reduces membrane fluidity, red cell deformability and survival. In spurr-cell anaemia this results from an increased cholesterol/phospholipid ratio and in abetalipoproteinaemia from an increased sphingomyelin/lecithin ratio (Cooper 1977). A reduction in platelet membrane fluidity increases platelet sensitivity to aggregatory substances such as adenosine 5'-diphosphate (ADP).

In the respiratory distress syndrome (RDS), the main cause of death in premature neonates, there is an improper lipid composition of the pulmonary surfactant. Most of the lipids in amniotic fluid originate from foetal lung surfactant. Microviscosity of amniotic fluid decreases steadily as pregnancy progresses. At birth fluorescence polarization (P) values are in the range 0.20-0.30. If P is greater than 0.32 the risk of respiratory distress syndrome increases substantially (Shinitzky *et al.* 1976).

Membrane fluidity could have a central role in the pathogenesis of atherosclerosis. An increase in serum total cholesterol leads to increased membrane cholesterol content and reduced membrane fluidity. In order to regulate membrane fluidity cholesterol is esterified into an end product of no functional significance which is then stored in blood vessel walls and other structures - cholesterol esters do not readily enter the membrane (Cooper 1977, Ray *et al.* 1969).

Membrane fluidity increases in malignancy, as demonstrated by studies on normal lymphocytes and lymphoma cells in rats and mice (Shinitzky and Inbar 1974) and in human leukaemia cells (Ben-Bassat *et al.* 1977, Shinitzky and Inbar 1976). Malignancy is associated with a reduced serum cholesterol content but which of

these is the primary event remains uncertain. Interestingly, Shinitzky and Inbar (1976) found an increase in membrane microviscosity in malignant hamster fibroblasts. The plasma is thought to be the main source of membrane cholesterol in lymphoma cells, whereas intracellular synthesis is the source in solid tumours. Indeed, solid tumours like hepatoma (Siperstein and Fagan 1964) and colonic malignancy (Burkitt 1975, Wynder and Reddy 1975) have an elevated cholesterol content.

3.5. MEMBRANE FLUIDITY AND CELL FUNCTION

Changes in membrane fluidity may alter various cellular functions which depend on the dynamics of the cell membrane such as transport processes, signal transmission across the membrane and cell division and differentiation. (During normal cell division microviscosity is maximal in mitosis, falls at the G_1 and S phases and increases at the G_2 phase (Shinitzky 1984)). Increased fluidity increases permeability to water and hydrophilic molecules and also increases lateral mobility of integral proteins. If the active site of an integral protein is in the hydrophobic region, increased fluidity may alter its function.

3.5.1. Membrane receptors

How much of a membrane receptor is exposed to the aqueous environment depends on membrane fluidity. Increased membrane fluidity causes receptors to sink into the fluid environment and decreased fluidity exposes more of the receptor (Borochov and Shinitzky 1976, Gleason *et al.* 1991). An increase in lipid microviscosity reflects an overall increase in lipid-lipid interactions and packing density. Such an increase will diminish the protein-lipid interactions and hence the solubilization capacity which, in turn, will displace the proteins vertically. If one considers the membrane proteins as being 'dissolved' in membrane lipids, increasing the membrane lipid content literally forces the proteins out of 'solution'. This could explain the increased

platelet sensitivity when their membrane cholesterol is increased. For example, in familial hypercholesterolaemia increased platelet membrane cholesterol content decreases membrane fluidity and increases platelet sensitivity to aggregation by ADP and adrenaline (Shattil and Cooper 1976). Increased membrane cholesterol content in macrophages reduces membrane fluidity and phagocytosis. On the other hand an abnormally low membrane cholesterol content depresses endocytosis in L cells (Cooper 1977). Thus, membrane cholesterol must be maintained within a narrow range to permit normal membrane-dependent cellular functions.

3.5.2. Membrane transport

A direct relationship between membrane transport and membrane fluidity has been demonstrated in red cells. Increased cholesterol/phospholipid ratio decreases membrane permeability, carrier-mediated transport and facilitated diffusion e.g. frusemide sensitive Na^+/K^+-cotransport (Wiley and Cooper 1975) and anion transport. On the other hand the carrier systems for monocarboxylates e.g. lactate and for monosaccharides e.g. arabinose are activated by cholesterol and inhibited by its removal (Grunze *et al.* 1980). Decreased membrane cholesterol increases diffusion of electrolytes e.g. Na^+ and K^+, and non-electrolytes e.g. glycerol, and possibly increases active sodium flux (Shinitzky 1984) but decreases calcium influx (Locher *et al.* 1984, Roelofsen and Schatzmann 1977, Rosier *et al.* 1986). Gleason *et al.* (1991) reported an increase in both calcium influx and calcium efflux when smooth muscle cell membrane cholesterol content was increased.

3.5.3. Enzyme activity

Alterations in membrane bilayer fluidity might regulate enzyme activity by varying either the lateral diffusion of the protein or the vertical position of the enzyme in the bilayer or the ability of the enzyme to change its conformation.

Increased membrane cholesterol (decreased membrane fluidity) increases basal adenylyl-cyclase activity in platelets (Sinha *et al.* 1977) but inhibits kidney

Na^{+}-K^{+}-ATPase and sarcoplasmic reticulum Ca^{2+}-Mg^{2+}-ATPase activities (Cooper *et al.* 1977). Cholesterol depletion in red cell membranes increases ATPase associated sodium transport (Pagnan *et al.* 1989) although excess cholesterol has no effect. Squier *et al.* (1988), using sarcoplasmic reticulum from rabbit white muscle, showed that a decrease in membrane fluidity consistently produced a decrease in calcium pump activity. This was in contrast to the earlier work of Warren *et al.* (1975) in which increased membrane cholesterol content, although reducing membrane fluidity, had no effect on enzyme activity.

There is little information in the literature on the role of membrane environment on the activity of the red cell membrane Ca^{2+}-Mg^{2+}-ATPase enzyme. Three human studies have been reported to date. In red cell ghosts, cholesterol depletion by itself did not lead to an inactivation of the enzyme (Roelofson and Schatzmann 1977). However, when the membrane was treated with phospholipase C the calcium pump was inactivated - the degree of inactivation being proportional to the percentage by which the phospholipid fraction in the inner membrane layer was degraded. After complete inactivation the enzyme was reactivated by the addition of any of the phospholipids - phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and lyso-phosphatidylcholine - but not by the addition of sphingomyelin, free fatty acids or the detergent Triton X-100. At a ratio of 2 μmol phosphatidylserine to 1 mg of membrane protein the activity of the reactivated enzyme was over 90% of the control value and was similar to that in the presence of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine in the molar ratio 2:5:3 (the same ratio as in the inner membrane leaflet). They concluded that any of the phospholipids in the human red cell membrane are involved in the maintenance of the Ca^{2+}-Mg^{2+}-ATPase activity, and in particular that fraction of these phospholipids located in the inner half of the membrane. Rosier *et al.* (1986) showed that cholesterol depletion reduced calcium influx leading to a fall in intracellular calcium concentration. This, in turn, caused a fall in calcium efflux through the calcium pump. The pump characteristics were not modified by

cholesterol depletion, the relative inactivation of the pump being a direct result of the lower intracellular calcium concentration. Adeoya *et al.* (1992) found that Ca^{2+}-Mg^{2+}-ATPase activity in intact red cell membranes from hypertensive patients was lower than that from normotensive controls. When the enzyme was extracted from the membrane its activity in the two groups was similar, suggesting that membrane environment is important in determining the activity of the calcium pump in hypertension.

Most of the apparent discrepancies between membrane cholesterol, fluidity and calcium pump activity (Cooper *et al.* 1977, Roelofsen and Schatzmann 1977, Rosier *et al.* 1986, Warren *et al.* 1975) can be explained by the membrane *phospholipid annulus*. In the sarcoplasmic reticulum, Ca^{2+}-Mg^{2+}-ATPase is the major intrinsic membrane protein and is known to depend for its activity on the surrounding phospholipids although these can be replaced by suitable detergents without loss of activity (Dean and Tanford 1978). The protein interacts directly with a slowly exchanging annulus of about 30 phospholipid molecules, also called the boundary lipids. Cholesterol is excluded from this annulus which protects the enzyme from any change in bulk membrane fluidity caused by the incorporation of cholesterol into the membrane (Hesketh *et al.* 1976). Cholesterol does not affect the activity of the sarcoplasmic reticulum Ca^{2+}-Mg^{2+}-ATPase with a complete phospholipid annulus but as cholesterol replaces phospholipids in the annulus enzyme activity is progresssively inhibited. When the phospholipid content in the annulus falls below 30 molecules, enzyme activity declines progressively until it becomes negligible below 15 phospholipid molecules (Warren *et al.* 1975). Without the annulus the calcium pump is maintained in a stable but inactive conformation. An increase in boundary lipid fluidity increases calcium pump activity but there is an optimum fluidity above which a further increase in fluidity causes a fall in enzyme activity (Bigelow and Thomas 1987). Thus, changes in membrane fluidity consistently modulate Ca^{2+}-Mg^{2+}-ATPase activity as long as they include the boundary lipids.

A further explanation for the apparent disparity in the effect of cholesterol on calcium pump activity is the observation that Ca^{2+}-Mg^{2+}-ATPase activity of native membranes prepared in the absence of protease inhibitors and reducing agents, such as dithiothreitol, is considerably higher than in membranes prepared in the presence of these agents (Madden *et al.* 1981). Inhibitory effects of cholesterol on the calcium pump are masked if the membrane is prepared in the presence of protease inhibitors and reducing agents.

The thickness of the membrane bilayer may be a major factor in determining enzyme activity (Johannsson *et al.* 1981). The protein conformation for optimal enzyme activity is maintained by lipids whose chain lengths match the dimensions of the hydrophobic part of the trans-membrane protein with the polar headgroups apposed to the more polar parts of the protein structure at the surface of the membrane bilayer. Disturbing the optimal bilayer thickness displaces this optimal interaction and reduces enzyme activity (Johannsson *et al.* 1981).

3.6. MEASUREMENT OF MEMBRANE FLUIDITY

Current technology provides average values of membrane fluidity. Electron spin resonance (e.s.r.), magnetic resonance imaging (MRI), fluorescence polarization and infra-red and laser-raman spectroscopy provide information on membrane microviscosity. X-Ray diffraction provides structural information on the membrane bilayer and differential scanning calorimetry (DSC) gives information on phase transitions (Stubbs 1983). The large number of available probes, the high sensitivity and the relatively inexpensive instrumentation favour the use of fluorescence-based techniques.

3.6.1. Fluorescence spectroscopy

When inserted into a membrane bilayer fluorescent probes can be used to provide information on their immediate environment. Two methods are used in fluorescence

polarization studies: steady state fluorescence anisotropy and decay fluorescence polarization. ***Steady state fluorescence anisotropy*** (section 3.2.2.) is the most widely used method in biological systems because of its sensitivity and flexibility. When fluorophores in the excited state return to the ground state they produce fluorescence. The emitted light intensity, I, is detected through an analyser orientated parallel or perpendicular to the direction of polarization of the excitation light. Emission is generally measured at right angles to the excitation beam. With vertically polarized excitation light the vertically (I_y) and horizontally (I_x) polarized components of the emitted light are measured. Fluorescence polarization, P, refers to the fluorescence intensity of the polarized light in the vertical and horizontal planes and is given by the equation

$$P = \frac{I_y - I_x}{I_y + I_x} \quad \text{.....(3.1)}$$

Fluorescence anisotropy, r, is the ratio of the polarized component in the x and y planes to the total polarized light intensity in the x, y, and z planes,

$$r = \frac{I_y - I_x}{I_y + I_x + I_z} \quad \text{.....(3.2)}$$

but the polarized light intensities in the two horizontal planes are equal,

$$I_z = I_x \quad \text{.....(3.3)}$$

hence,

$$r = \frac{Iy - Ix}{I_y + 2I_x} \quad \text{.....(3.4)}$$

Expressed in their more familiar forms,

$$P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \quad \text{.....(3.5)}$$

$$r = \frac{I_{VV} - I_{VH}}{I_{VV} + 2 \times I_{VH}} \quad \text{.....(3.6)}$$

where I_{VV} & I_{VH} are measured fluorescence intensities with vertically polarized excitation light and the analyser vertically and horizontally orientated respectively.

When the emitted light passes through the analyser its grating imposes a degree of polarization on the light. A grating correction factor, G, must therefore be introduced. The G factor is the ratio of intensities of the vertically and horizontally polarized fluorescence emissions with horizontally polarized excitation light,

$$G = \frac{I_{HV}}{I_{HH}} \quad \text{.....(3.7)}$$

This ratio must be determined for each probe used. In theory G equals unity but in practise the ratio is always less than one. Using the G correction factor, fluorescence polarization becomes

$$P = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + G \times I_{VH}} \quad \text{.....(3.8) (Chen and Bowman 1965)}$$

Fluorescence polarization and fluorescence anisotropy are related by the equations:

$$P = \frac{3 \times r}{2 + r} \quad \text{.....(3.9)}$$

$$r = \frac{2 \times P}{3 - P} \quad \text{.....(3.10)}$$

Steady state fluorescence polarization has been employed in the present study. An extrinsic probe is embedded in the phospholipid bilayer and fluorescence polarization is observed under continuous illumination by vertically polarized light. Microviscosity is the average of the vectorial viscosities which oppose the various rotational modes of the probe. For probes whose limiting relaxation time and lifetime of the excited state are known microviscosity is given by the formula

$$\bar{\eta} = \frac{2 \times P}{0.46 - P} \quad \text{.....(3.11)}$$

These techniques measure primarily either average orientational order or average rate of motion or in some cases both parameters. These two parameters are normally but not always inversely related.

Fluorescence polarization can be readily applied to complex systems like isolated biological membranes, the polarized signals are highly sensitive and reproducible and the data are easy to interpret. However, for the reasons given at the beginning of this chapter, the resolution of the highly heterogeneous fluid regions in biological membranes is at best only partial.

The employed probe should possess a series of well-defined spectral and physical properties (Shinitzky *et al.* 1971), including:

(1) rigid structure to avoid depolarization due to rotations of side groups,

(2) lifetime of the excited state in the range 1-8 nanoseconds (ns),

(3) high values for the extinction coefficient and quantum yield since these will increase the fluorescence signal,

(4) minimum overlap between absorption and emission spectra, to eliminate depolarization due to energy transfer in case of high local concentration of the probe.

Fluorescent probes fall into two main classes. The first class can incorporate spontaneously into a well defined lipid region of the membrane. The fluorescence polarization characteristics of such probes directly relate to the microviscosity of the labelled region. 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), which were employed in the present study, are two such probes. The second class are covalently attached to fatty acids or phospholipids at different loci. Some rotational modes of the fluorescent moiety in such compounds are markedly hindered, hence the correlation between microviscosity and fluorescence polarization is even more approximate than in the first class.

3.6.1.1. 1,6-diphenyl-1,3,5-hexatriene (DPH)

DPH is a polyene hydrocarbon with a stable all-trans configuration of an elongated rectangular shape. All-trans is the exclusive configuration which contributes to the fluorescence. It has outstanding properties for a fluidity probe (Shinitzky and Barenholz 1974), including an absorption maximum around 355 nm with an extinction coefficient of about 80,000 M^{-1} cm^{-1} and a fluorescence decay time of 11.4 ns. The absorption and emission transition moments are essentially but not completely collinear, one with the other and with the symmetry axis. This makes DPH an excellent probe for studies of order in lipid bilayers since even a small displacement of the symmetry axis results in depolarization of fluorescence emission which is easily detected and measured (Prendergast *et al.* 1981). Incorporation of DPH into the lipid bilayer is followed by a steep increase in fluorescence intensity. The degree of fluorescence polarization is an intrinsic parameter and is therefore independent of fluorescence intensity over a wide range of concentrations.

DPH is localized almost exclusively to the hydrocarbon core of the lipid structure and there is debate as to whether it does or does not perturb membrane structure. Although it is sufficiently soluble in the hydrocarbon domain of the bilayer that we cannot be sure that it does not change location or undergo transitional motion during the lifetime of its excited state the general consensus is that DPH is evenly distributed in the lipid bilayer and that the derived microviscosity is therefore a weighted average of all the lipid domains (Shinitzky and Barenholz 1978). However, DPH cannot detect the asymmetry of the lipid bilayer.

3.6.1.2. 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH)

TMA-DPH is a cationic analogue of DPH used for studying membrane fluidity in the outer regions of the membrane bilayer (near the lipid-water interface). The positive charge is responsible for its interactions with the polar heads of the membrane phospholipids with its DPH moiety intercalated between the upper portions of the fatty acyl chain (Prendergast *et al.* 1981). The length of the hydrophobic part of the molecule is about that of a 10 carbon aliphatic chain (Le Quan Sang *et al.* 1991). In solution, the lifetime of the excited state for TMA-DPH is less than 1.5 ns which increases to 7 ns when the probe is embedded in lipid bilayers. The lifetime of the excited state for TMA-DPH is very sensitive to polar solvents (Prendergast *et al.* 1981). TMA-DPH has an extinction coefficient of 30,200 M^{-1} cm^{-1}.

In dimethylformamide, TMA-DPH is stable for over a month, provided it is not directly exposed to light (Kuhry *et al.* 1985).

Fluorescence studies using DPH and TMA-DPH have shown that cholesterol restricts probe reorientation in both the hydrophobic core and the outer regions of the membrane (Shinitzky and Barenholz 1978, Straume and Litman 1987). There are suggestions that increased membrane cholesterol content increases the order of the core substantially more than that of the interfacial region (Straume and Litman 1987).

3.7. SUMMARY

All cells are surrounded by a phospholipid bilayer membrane in which cholesterol and various proteins reside. The interactions between these various components and other physical factors determine membrane fluidity, with the membrane cholesterol/phospholipid ratio being the most important determinant. Changes in membrane dynamics can alter cell function. Thus, for example, changes in membrane fluidity consistently affect calcium pump activity as long as they involve the phospholipid annulus surrounding the enzyme.

The most widely employed technique for measuring membrane fluidity, which was also used in the present studies, is DPH and TMA-DPH steady state fluorescence anisotropy. DPH anisotropy is inversely related to the fluidity of the hydrophobic domain and TMA-DPH anisotropy is inversely related to the fluidity of the outer regions of the membrane bilayer.

Chapter 4

PLASMA LIPIDS, DIABETES MELLITUS AND THE CALCIUM PUMP

4.1. DEFINITIONS

Lipids are hydrophobic, organic molecules which have a hydrocarbon as a major part of their structure. There are two kinds of lipid, simple and complex lipids.

4.1.1. Simple lipids

There are two classes of simple lipids, cholesterol and fatty acids. Cholesterol, a steroid alcohol, is a major structural component of biological membranes and is a precursor of many other steroids such as steroid hormones and bile acids. Fatty acids are either saturated (no double bonds in their hydrocarbon structure), monounsaturated (one double bond), or polyunsaturated (two or more double bonds).

4.1.2. Complex lipids

Complex lipids are formed when a fatty acid combines with an alcohol to form an ester. There are two groups of esters depending on whether the fatty acid is complexed to cholesterol (cholesterol esters) or to glycerol (glycerol esters). If all the three hydroxyl groups on a glycerol molecule are esterified with fatty acids the resulting non-polar molecule is a triacylglycerol or triglyceride. If the terminal hydroxyl group on the glycerol molecule is esterified with a phosphate-containing molecule the resulting polar molecule is a phospholipid.

4.1.3. Lipoproteins

Because lipids are insoluble in aqueous solution, they are transported in plasma complexed with specialized amphipathic proteins called apolipoproteins or apoproteins in a spherical structure called a lipoprotein. The polar phospholipid headgroups and the alcohol groups of unesterified cholesterol project from the lipoprotein surface into the aqueous environment, whereas the non-polar parts of the phospholipids and cholesterol, together with cholesterol esters and triglycerides, are contained within the lipoprotein core (Feher and Richmond 1991).

Lipoproteins can be separated by ultracentrifugation into five major density classes depending on their protein content (Feher and Richmond 1991). *Chylomicrons,* (CYM, density <1 g/ml) transport diet-derived triglycerides. *Very low density lipoproteins* (VLDL, density 1-1.006 g/ml) transport endogenous triglycerides which are produced in the liver. There are two types of VLDL: $VLDL_1$ have a higher triglyceride content and are larger than $VLDL_2$, the form present in normal individuals. *Intermediate density lipoproteins* (IDL, density 1.006-1.019 g/ml) are derived from VLDL and are metabolised to *low density lipoproteins* (LDL, density 1.019-1.063 g/ml) which transport cholesterol from the liver to the periphery. *High density lipoproteins* (HDL, density 1.063-1.25 g/ml) transport cholesterol from the periphery to the liver, a process called reverse cholesterol transport. There are three types of HDL: Nascent HDL are the newly synthesized disc-shaped particles which are devoid of cholesterol esters and are converted to HDL_3 and the latter to HDL_2 by accepting cholesterol from triglyceride-rich particles. HDL_3 are small, lipid-poor and dense (density 1.125-1.250 g/ml) whereas HDL_2 are larger, more lipid-rich and less dense (density 1.063-1.125 g/ml). Serum HDL_3 levels are fairly constant, while those of HDL_2 vary widely, accounting for the variability of an individual's total HDL-cholesterol levels (Gotto *et al.* 1991). High levels of HDL_2 favour reverse cholesterol transport.

LDL carries 70% of the serum total cholesterol and HDL 20%, making LDL and HDL the main cholesterol-carrying lipoproteins. Because they carry most of the plasma cholesterol LDL particles are the most atherogenic of the lipoproteins, the smaller the particles the more atherogenic they are.

The main triglyceride carrying lipoproteins are chylomicrons and VLDL. After a 12 hour fast chylomicrons are absent from the blood and VLDL then carries 60% of the total serum triglycerides (Feher and Richmond 1991).

4.1.4. Apoproteins

At least fourteen apoproteins have been identified in human plasma (Brewer *et al.* 1988). Apoproteins serve one or more of three functions (Feher and Richmond 1991): *structural* e.g. apoA-I in HDL, apoB-100 in VLDL, IDL and LDL, apoB-38 in chylomicrons; *binding ligands* e.g. apoB-100 binds to the LDL receptor and apoE to the chylomicron remnant receptor in the liver; *enzyme activation* e.g. apoA-I activates lecithin cholesterol acyl transferase (LCAT), apoC-II activates lipoprotein lipase.

4.1.5. Lipoprotein metabolism

Most of the cholesterol in tissues is synthesized *de novo*, the liver being its main site of synthesis. Several enzymes are involved in lipoprotein metabolism (Feher and Richmond 1991):

(a) *Lipoprotein lipase* removes triglycerides from VLDL and chylomicrons.

(b) *LCAT*, which is synthesized in the liver, esterifies the free cholesterol acquired by nascent HDL and HDL_3 by the transfer of a fatty acid from lecithin.

(c) *Cholesterol ester transfer protein (CETP)* is involved in the transfer of cholesterol esters from mature HDL_2 to triglyceride-rich particles. Triglycerides are simultaneously transferred in the reverse direction.

(d) *Hepatic triglyceride lipase (HTGL)* or *hepatic lipase*, which is similar to lipoprotein lipase but situated in endothelial cells in hepatic capillaries, is involved in the conversion of triglyceride-rich HDL_2 to HDL_3.

Lipoprotein metabolism and reverse cholesterol transport are shown in Figure 4.1.

4.2. COMMON HYPERLIPIDAEMIAS

An elevation of VLDL and/or chylomicron lipoproteins manifests as hypertriglyceridaemia and an elevated LDL as hypercholesterolaemia. Due to the

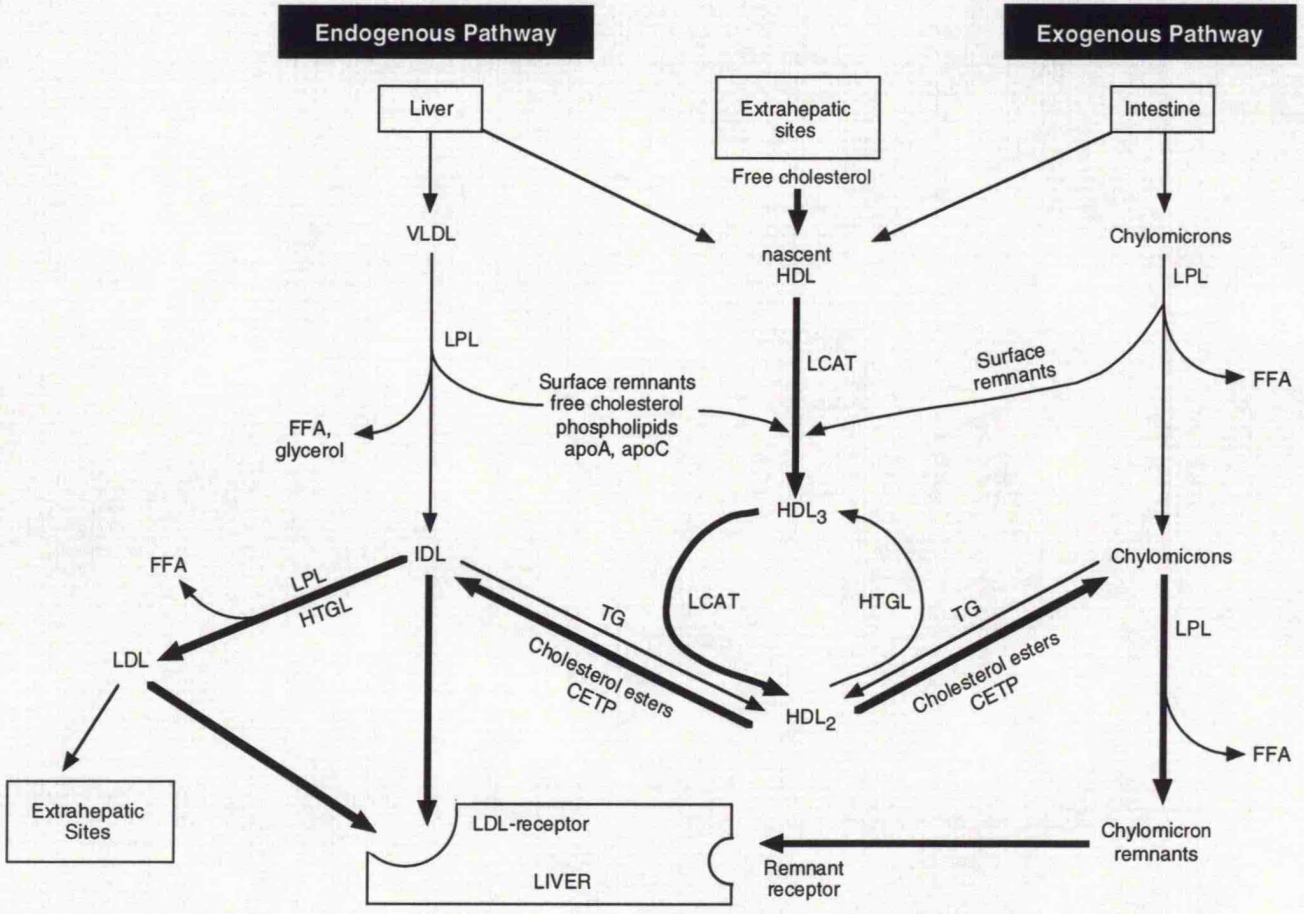

Figure 4.1. **Lipoprotein metabolism**

➡ Reverse cholesterol transport
apo- apoprotein,
CETP- cholesterol ester transfer protein
FFA- free fatty acids
HDL- high density lipoprotein
HTGL- hepatic triglyceride lipase (hepatic lipase)
IDL- intermediate density lipoprotein
LCAT- lecithin cholesterol acyl transferase
LDL- low density lipoprotein
LPL- lipoprotein lipase
TG- triglycerides.

presence of small amounts of cholesterol in VLDL and chylomicrons, severe hypertriglyceridaemia results in varying degrees of hypercholesterolaemia giving rise to a combined hyperlipidaemia.

Hyperlipidaemia can either be primary or secondary to a co-existing disease. Secondary causes of hyperlipidaemia include: *hypercholesterolaemia* (total serum cholesterol > 6.5 mmol/l) - hypothyroidism and biliary obstruction; *hypertriglyceridaemia* (triglycerides > 2.3 mmol/l) - diabetes mellitus, obesity and alcohol; *combined hyperlipidaemia* (total serum cholesterol > 6.5 mmol/l, triglycerides > 2.3 mmol/l) - diabetes mellitus, chronic renal failure, nephrotic syndrome, myeloma and thiazide diuretics.

4.2.1. Hyperlipidaemia in diabetes mellitus

Serum lipid profiles in people with well controlled insulin-dependent diabetes mellitus (IDDM) are either similar to those in the general population or even anti-atherogenic (Taskinen 1990). In poorly controlled IDDM, VLDL-triglycerides and LDL are increased and HDL is reduced. In addition there is increased glycosylation of apoB in LDL (Gibbs 1993, Sosenko *et al.* 1993).

Several quantitative and qualitative changes occur in lipoproteins in non-insulin dependent diabetes mellitus (NIDDM) resulting in abnormal lipoprotein function. The insulin resistance that is associated with this type of diabetes promotes lipolysis in adipose tissue and increases the delivery of free fatty acids to the liver. In addition, the associated reduction in lipoprotein lipase activity reduces VLDL clearance (Dunn *et al.* 1984). This results in an overall increase in total serum and $VLDL_1$-triglycerides. The levels of serum and $VLDL_1$-triglycerides in non-insulin treated diabetes are proportional to the degree of diabetic control (Taskinen *et al.* 1988).

HDL levels, in particular HDL_2, are reduced by 10-20% due to a combination of impaired VLDL metabolism secondary to reduced lipoprotein lipase

activity and to increased apoA-I catabolism secondary to increased hepatic lipase activity (Taskinen *et al.* 1988).

Although apoB levels are increased in mild untreated NIDDM, LDL levels are normal since both LDL production and clearance are increased (Taskinen 1990). In uncontrolled NIDDM, there is increased production of $VLDL_1$ and LDL, and reduced clearance of LDL (Howard 1987). The triglyceride content of LDL is also increased. Furthermore, poor diabetic control increases both the oxidation and glycosylation of LDL. The altered LDL is then cleared by the scavanger pathway (Witztum *et al.* 1982). In addition to the above changes the dynamic exchange of core lipids between HDL, LDL and VLDL is disturbed in NIDDM.

Studies on the prevalence of hyperlipidaemia in diabetes mellitus have included both insulin- and non-insulin-dependent diabetes (West *et al*, 1983; Wilson *et al.* 1985), although the prevalence in insulin-dependent diabetes is probably the same as in the general population (Taskinen 1990). In a recent survey in a United Kingdom diabetic clinic 28% of 299 diabetic patients had hyperlipidaemia (Paterson *et al.* 1991) - 59% hypercholesterolaemia, 15% hypertriglyceridaemia and 24% combined hyperlipidaemia. Hypercholesterolaemia was present in 17% of men and 5% of women with type I diabetes compared to 29% and 55%, respectively, with type II diabetes. However, Finnish studies have suggested that hypercholesterolaemia in NIDDM is no more common than in the general population (Taskinen 1990). In the study of Winocour *et al.* (1989) in 205 insulin-dependent diabetic patients 40% had hyperlipidaemia. Whilst the prevalence of hypertriglyceridaemia and combined hyperlipidaemia was higher in patients than in non-diabetics, this was not the case for hypercholesterolaemia. Hypertriglyceridaemia is about 2-3 times more common in the diabetic population (Barrett-Connor *et al.* 1982). In the PROCAM study (Assman and Schulte 1988) the prevalence of combined hyperlipidaemia was higher (28.1%) than in the general population (12.6%).

4.2.2. Hyperlipidaemia in the general population

In a recent American report, 20% of adults in the general population had serum cholesterol levels greater than or equal to 6.21 mmol/l, and 29% of adults should be on dietary therapy (Sempos *et al.* 1993). In the United Kingdom 55% of 20-59 year-olds have serum total cholesterol levels above 5.5 mmol/l, 25% have levels above 6.5 mmol/l and 4% have levels above 8.0 mmol/l (Miller 1989).

4.3. THE CALCIUM PUMP IN HYPERLIPIDAEMIA

There have been no reports in the literature on Ca^{2+}-Mg^{2+}-ATPase activity in hyperlipidaemia. Extrapolation from studies on liposomes and malignant cells suggests that, because serum and membrane lipids are in dynamic equilibrium, membrane microviscosity is increased and calcium pump activity is reduced in hypercholesterolaemia. No *in vivo* or *in vitro* studies have been carried out on membrane environment and calcium pump activity in hypertriglyceridaemia.

4.4. THE CALCIUM PUMP IN DIABETES MELLITUS

Only four studies have been published on the calcium pump in diabetes (Gonzalez Flecha *et al.* 1990, Gronda *et al.* 1986, Schaefer *et al.* 1987, Zemel *et al.* 1987). In the study of Zemel *et al.* (1987) in Black hypertensive type II diabetic patients, red cell calcium pump activity was reduced by 70% in hypertensive diabetic patients and by 20% in non-diabetic hypertensive patients compared to normal control subjects. In another study on poorly controlled type I diabetic patients basal and calmodulin-stimulated calcium pump activities were reduced by 62% and 28% respectively compared to healthy volunteers with normal glucose tolerance (Schaefer *et al.* 1987). This reduction was partly explained by non-enzymatic glycosylation of calmodulin. In the above studies serum lipids were not measured, hence altered membrane

dynamics secondary to hyperlipidaemia could not be excluded as a cause of the reduction in enzyme activity.

4.5. SUMMARY

Lipids circulate in the plasma in lipoproteins. Chylomicrons and VLDL transport serum triglycerides. LDL carries cholesterol from the liver to the peripheral tissues and HDL carries cholesterol in the opposite direction. Serum lipid profiles in well-controlled type I diabetes are probably similar to those in the general population but those in type II diabetes are atherogenic, with raised serum triglycerides and low HDL.

Intracellular calcium, whose concentration is determined in part by the calcium pump, is important to normal cell function. Although calcium pump activity has been investigated in liposomes, malignant cells and the sarcoplasmic reticulum, few studies have been carried out on the red cell in hyperlipidaemia and in diabetes, conditions that could affect membrane environment and the physical properties of the enzyme respectively.

4.6. AIMS OF THE PRESENT PROJECT

Previous studies in the department have shown the importance of membrane environment in determining red cell calcium pump activity in hypertension (Adeoya *et al.* 1989, 1992). Calcium pump activity was lower in intact membranes from both hypertensive rats (1989) and patients (1992) but when the protein was isolated from the membrane enzyme activity was similar to that in the respective normotensive controls.

The present project, an extension of the previous work, investigates membrane environment and calcium pump activity in subjects with primary

hyperlipidaemia and those with hyperlipidaemia secondary to NIDDM. Several hypotheses were proposed:

(1) *According to the membrane hypothesis hyperlipidaemia should alter the membrane environment by increasing its lipid content.*

(2) *The altered membrane environment should impair calcium pump activity.*

(3) *In diabetic subjects with hyperlipidaemia there is a further reduction in calcium pump activity due to glycosylation of the Ca^{2+}-Mg^{2+}-ATPase enzyme itself.*

The red cell was chosen for this study for several reasons: it is the most extensively studied system and was used in the human studies of the calcium pump quoted in the literature; red cells are easily obtainable in large enough numbers with good techniques for their isolation and handling of their plasma membranes; red cells have no intracellular organelles hence one can be certain that only the plasma membranes are being studied; lipid metabolism in red cells is minimal, hence membrane lipids should mirror those in the plasma.

Diabetes mellitus was selected as an example of a condition that, in addition to affecting the membrane environment, should also affect the Ca^{2+}-Mg^{2+}-ATPase enzyme itself. Non-insulin treated diabetes was ideal because of the higher prevalence of lipid abnormalities than in insulin-treated diabetes and to avoid any possible confounding effects of exogenous insulin on calcium pump activity.

Chapter 5

MATERIALS AND METHODS

This project was approved by the Leicestershire Health Authority Ethics Committee. All participants gave informed consent after the study protocol had been fully explained to them.

5.1. MATERIALS

All the reagents used were of analytical grade unless stated otherwise. Materials (name, formula, formula weight in grams) and sources are given below:

Aldrich Chemical Company, Gillingham, Dorset, England
1,6-diphenyl-1,3,5-hexatriene (DPH, $C_{18}H_{16}$, 232.33)

Amersham International, Amersham, England
gamma-^{32}P-adenosine triphosphate, [γ-^{32}P]ATP

BDH Chemicals Ltd, Poole, England
acrylamide ($CH_2CHCONH_2$, 71.08), ammonium persulphate (ammonium peroxodisulphate, $(NH_4)_2S_2O_8$, 228.19), bromophenol blue dye, Coomassie brilliant blue G250, Coomassie brilliant blue R250, copper sulphate ($CuSO_4.5H_2O$, 249.68), Nonidet p40, sodium dodecyl sulphate (SDS, $C_{12}H_{25}O_4SNa$, 288.4), sucrose ($C_{12}H_{22}O_{11}$, 342.30), Temed:- N,N,N'N'-tetramethylethylene diamine [$(CH_3)_2NCH_2CH_2N(CH_3)_2$, 116.2]

Fisons plc, Loughborough, England
acetic acid, "glacial" (CH_3COOH, 60.05), ammonium molybdate {$(NH4)_6Mo_7O_{24}.4H2O$, 1235.86}, butan-2-ol (sec-butyl alcohol, $CH_3CH_2CH(OH)CH_3$, 74.12), butylated hydroxytoluene {BHT, 2,6-di-tert-butyl-cresol, $[(CH_3)_3C]_2C_6H_2(CH_3).OH$, 220.36}, calcium chloride ($CaCl_2.2H_2O$, 147.02), chloroform (HPLC grade, $CHCl_3$, 119.38), Folin Ciocalteu's phenol

reagent, hydrochloric acid (HCl, 34.46), magnesium chloride ($MgCl_2.6H_2O$, 203.31), methanol (CH_3OH, 32.04), potassium chloride (KCl, 74.56), potassium dihydrogen orthophosphate (KH_2PO_4, 136.09), potassium hydroxide (KOH, 56.11), propan-2-ol (isopropyl alcohol, $(CH_3)_2CHOH$, 60.1), sodium carbonate (Na_2CO_3, 105.99), sodium chloride (NaCl, 58.44), sodium hydroxide (NaOH, 40.0), sulphuric acid (H_2SO_4, 98.07), tetrahydrofuran [$CH_2(CH_2)_2CHO$, 72.11], trichloroacetic acid (CCl_3COOH, 163.39)

Gibco BRL, Paisley, Scotland (UK)

N,N'-methylenebisacrylamide ($C_7H_{10}N_2O_2$, 154.2)

Molecular Probes Inc., Eugene, OR, USA

1-[(4-trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene, (TMA-DPH, $C_{21}H_{24}N$, 461.0)

Pharmacia LKB, St Albans, Herts, England

optiphase 'safe', optiscint 'safe'

Sigma Chemical Company, St Louis, MO, USA

adenosine 5'-triphosphate (disodium salt, grade 1, $C_{10}H_{14}N_5O_{13}P_3Na_2$, 507.2), albumin bovine (BSA, RIA grade), amido black, N,N-dimethylformamide (C_3H_7NO, 73.09), ethylenediaminetetraacetic acid (EDTA, $C_{10}H_{16}N_2O_8$, 292.2), ethyleneglycol-bis (β-aminoethylether)-N,N,N'N'-tetraacetic acid (EGTA, $C_{14}H_{24}N_2O_{10}$, 380.4), glycine ($C_2H_5NO_2$, 75.07), N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid) (HEPES, $C_8H_{18}N_2O_4S$, 238.3), phosphodiesterase 3',5'-cyclic nucleotide activator (calmodulin), polyoxyethylenesorbitan monolaurate (Tween 20), potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$, 282.23), N-lauroyl-sarcocine (sodium salt, $C_{15}H_{28}NO_3Na$, 293.4), α-tocopherol (vitamin E, $C_{29}H_{50}O_2$, 430.7), Trizma base [Tris (hydroxymethyl)-amino methane, 121.1]

5.2. PATIENTS

Patients with hyperlipidaemia were recruited from the Lipid Clinic, Glenfield Hospital. Diabetic patients were recruited from both the Lipid Clinic and the Leicester Royal Infirmary Diabetic Clinics. Most of the normolipidaemic controls were recruited from elective orthopaedic and general surgical admissions, with a small number of volunteers from the staff of the Glenfield Hospital. All subjects, adults aged 30-65 years inclusive, had normal renal, liver and thyroid fuctions and were not on lipid lowering medication or any treatment known to affect lipid metabolism or calcium pump activity.

5.3. METHODS

5.3.1. Collection of blood samples

For accurate assessment of serum lipid profiles venous blood samples were collected with minimal venous stasis, using the vacutainer™ system (Becton Dickinson, Meylan, France) after a 12 hour fast. From each subject, 10 ml of blood were collected into a plain tube for serum lipid (serum total cholesterol, HDL-cholesterol and serum triglycerides) measurement using the Kodak Ektachem Clinical Chemistry Slides (Kodak Clinical Products, Rochester, NY, USA), 4 ml into a tube with EDTA as anticoagulant for glycosylated haemoglobin (HbA_1) estimation by electrophoresis (except for the normal control group), and 10 ml into a tube with lithium-heparin as anticoagulant for the preparation of red cell membranes and for membrane cholesterol extraction. The lithium-heparin sample was put on ice immediately after collection and processed within 20 mins of collection. All subsequent manipulations were at $4^{o}C$ unless stated otherwise.

5.3.2 Preparation of red cells

The lithium-heparin sample was transferred into a polycarbonate tube and centrifuged for 10 mins at 1,900 x g, 4°C. The supernatant and buffy coat were removed and the cells washed twice with 5 volumes of buffer containing 130 mM KCl and 20 mM Tris-HCl, pH 7.4, with centrifugation for 10 mins at 5,900 x g, 4°C, between washes. One millilitre (1 ml) of the washed cells was used for the extraction of membrane cholesterol and the remainder for red cell membrane preparation based on the method of Niggli *et al.* (1981).

5.3.3. Preparation of red cell membranes

The washed red cells were haemolysed in 5 volumes of buffer containing 2 mM EDTA and 1 mM Tris-HCl, pH 8.0 and stirred gently on a spiramix for 5 mins to increase the membrane protein yield. The haemolysate was then centrifuged for 10 mins at 25,000 x g, 4°C. A fluffy pink pellet contained the ghost membrane fragments. To collect this pellet the supernatant was carefully aspirated and a small white pellet, which contained membrane-bound proteases, near the bottom of the tube carefully removed with a cotton wool bud while gently tilting the membrane pellet away from it. The membrane pellet was washed two more times with 5 volumes of the haemolysis buffer, with centrifugation at 25,000 x g between washes as before. The membrane pellet was washed a further 2-3 times in 5 volumes of 10 mM Tris-HCl buffer, pH 7.4, and then suspended in 2-3 drops of the 10 mM Tris-HCl buffer, pH 7.4.

5.3.4. Determination of membrane protein content

5.3.4.1. Reagents

(1) Bovine serum albumin (BSA) standard, 5 mg to 10 ml with deionised water

(2) Folin Ciolcalteu's phenol reagent :- 10 ml of reagent diluted with 9 ml of deionised water

(3) 500 ml Solution A :- 100 mM NaOH and 189 mM Na_2CO_3

(4) Solution B :- 100 ml of 35 mM potassium sodium tartrate and a separate 100 ml solution of 20 mM $CuSO_4$. One millilitre (1 ml) of each of these solutions were mixed together just before use to form solution B.

(5) Solution C:- the freshly prepared solution B was mixed with 98 ml of solution A.

5.3.4.2. Methods

Membrane protein concentration was determined in triplicate by the method of Lowry *et al.* (1951) with BSA standards (appendix, section 11.1, validated in section 6.1).

25 μl of freshly prepared red cell membranes were diluted with 475 μl of deionised water. Three millilitres (3 ml) of solution C were added to the tubes at 15 second intervals, mixing well on a vortex between additions. The tubes were left at room temperature for 10 mins. Three hundred microlitres (300 µl) of diluted Folin Ciocalteu's phenol reagent were then added to each tube at 15 second intervals in the same order as for solution C, mixing well between additions. The tubes were allowed to stand in the dark (cupboard) at room temperature for an hour. Absorbance was read in a Gallenkamp Visi-Spec spectrophotometer (Fisons Gallenkamp, Crawley, England) at 550 nm against the reagent blank. Membrane protein concentration was calculated from the standard curve and expressed in mg of protein/ml of membrane extract.

5.3.5. Assay for ATP hydrolysis activity in ghost membranes

5.3.5.1. Principles

The commonest method of measuring ATPase activity under constant conditions is to measure inorganic phosphate, P_i, production using deproteinized extracts of the

incubation mixture. The released P_i reacts with acid ammonium molybdate to form a phosphomolybdate complex which is then quantified by spectrophotometric or radioisotopic methods (Brown 1982). Radioisotopic methods are more sensitive and rapid, require small incubation volumes and allow many samples to be processed simultaneously. Because the assay conditions also favour the hydrolysis of acid-labile organic phosphates, the organic and inorganic phosphates in the reaction mixture are separated before P_i determination. The released P_i is then measured as $^{32}P_i$ activity in the organic phase after extraction of the phosphomolybdate complex into that phase.

5.3.5.2. Reagents

(1) Acid ammonium molybdate was prepared by dissolving 25 g of ammonium molybdate in 200 ml of 10 M sulphuric acid in a fume cupboard. The solution was left stirring overnight. Deionised water was then added to a final volume of 500 ml (i.e. 5% ammonium molybdate), and the solution stored at 4°C.

(2) 55% TCA was prepared by carefully dissolving 55 g of trichloroacetic acid in 100 ml of deionised water. TCA improves deproteination which occurs at the same time as phosphomolybdate formation.

(3) Ca^{2+}-Mg^{2+}-ATPase buffer consisted of 125 mM KCl, 1.5 mM $MgCl_2$, 45 mM Hepes, pH 7.4.

(4) labelled adenosine 5'-triphosphate was prepared by dissolving 3.04 mg of ATP in 199 μl of Ca^{2+}-Mg^{2+}-ATPase buffer and adding 1 μl of tracer [γ-^{32}P]ATP (1 μCi/ml) to form a 30 mM ATP solution.

(5) 0.4 mM calcium chloride.

(6) 2 μM calmodulin (in 20 mM Tris/HCl buffer, pH 7.4).

(7) 1 M KH_2PO_4 solution. The phosphate acts as a carrier for $^{32}P_i$ during the extraction, preventing loss of radioactivity through non-specific binding e.g. to the membrane fraction, plasticware, etc.

5.3.5.3. Methods

Calcium pump (Ca^{2+}-Mg^{2+}-ATPase) activity was estimated using a modification of the method of Brown (1982, validated in sections 6.3.3. and 6.4). All experiments were carried out in triplicate.

Four 1.5 ml eppendorf tubes were set up on ice as in Table 5.1, but the 5 µl of ATP were added just before incubation.

tube	buffer (µl)	membrane (µl)	calcium (µl)	calmodulin (µl)	HCl (µl)	ATP (µl)
1. Stimulated	75	10	5	5	-	5
2. Basal	80	10	5	-	-	5
3. Blank	90	-	5	-	-	5
4. Hydrolysis	-	-	5	-	90	5

Table 5.1. Assay for ATP hydrolysis. Tube 1 - calmodulin stimulated activity, tube 2 - basal activity, tube 3 - blank, tube 4 - hydrolysis, buffer - Ca^{2+}-Mg^{2+}-ATPase buffer, membrane - red cell membrane suspension, calcium - calcium chloride solution. Total reaction volume was 100 µl and final concentrations of ATP, calcium and calmodulin in the reaction mixture were 1.5 mM, 20 μM and 0.1 μM respectively.

To initiate the reaction 5 μl of [γ-^{32}P]-labelled ATP stock solution were added to each tube sequentially at 30 second intervals, and the reaction mixture vortexed for 15 seconds before incubating in a shaking waterbath at 37°C. Because of the small incubation volume, it is best to mix the incubation components by vortexing because if they are shaken a large proportion of the mixture sticks to the cap of the tube leading to poor temperature regulation during the incubation. At 30 mins (tube 1) and 60 mins (tubes 2-4) the tubes were placed on ice for 15 mins to stop any further reaction. While the tubes were on ice, a molybdate mixture of 2 ml acid ammonium molybdate, 0.4 ml TCA and 12 μl of 1 M KH_2PO_4 was prepared. The molybdate mixture was prepared within an hour of use since a yellow precipitate forms if it is

stored even for a few hours. After the 15 mins on ice, the tubes were transferred to a racked plastic box at 4°C. One hundred microlitres (100 μl) of cold deionised water, 100 μl of the molybdate mixture and 800 μl of ice cold butan-2-ol were added to each eppendorf in that order. To extract the released inorganic phosphate, $^{32}P_i$, the tubes were carefully but vigorously shaken for 15 seconds (*the mixture turns yellow*) and then allowed to stand for 5 mins at 4°C before repeating the agitation process. More than one agitation of the extraction mixture was necessary because at 0-4°C this process is slow, hence the aqueous and organic phases must be mixed well. Furthermore, extraction of the phosphomolybdate complex is more complete with shaking than with vortexing. After the third agitation the tubes were centrifuged at 3,000 x g in a microcentaur (Fisons Gallenkamp, Crawley, England) at room temperature for 30 seconds. Three hundred microlitres (300 μl), in duplicate, from the organic phase (the top layer) were dissolved in 4 ml of a toluene based scintillant, Optiscint 'Safe' and counted in a Packard LS 1500 beta counter (Pangbourne, Berks, England) in which a quench curve had been previously installed. Five microlitres (5 μl) of the [γ-^{32}P]-labelled ATP stock solution were dissolved in 4 ml of Optiphase 'Safe' and counted together with the above samples. From the released radioactivity (dpm), corrected for spontaneous ATP hydrolysis (blank, tube 3) the amount of ATP hydrolysed per milligram of membrane protein per hour was calculated from the formula

$$\frac{15 \times Y}{H \times p \times t} \quad \mu\text{mol ATP hydrolysed/mg/h} \qquad (5.1)$$

where Y = dpm in 300 µl of reaction mixture (tube 1 or 2)

H = dpm in 300 µl of hydrolysis sample (tube 4)

p = membrane protein concentration in mg/ml

t = time of reaction in hours.

The full derivation of this formula is given in the appendix, section 11.2.

Calcium pump activity can also be assessed by the rate of uptake of $^{45}Ca^{2+}$ into inside-out-vesicles (IOVs). Adeoya *et al.* (1989, 1992) used both methods to assess calcium pump activity in hypertension with similar results. The less cumbersome [γ-^{32}P]ATP hydrolysis assay was employed in the present study.

5.3.6. Determination of red cell membrane fluidity (validated in sections 6.3.2. and 6.3.3.)

5.3.6.1. Reagents

(1) 1 mg DPH in 2.152 ml tetrahydrofuran (2 mM)

(2) 1.984 mg TMA-DPH in 2.152 ml dimethylformamide (2 mM)

5.3.6.2. Methods

A Perkin-Elmer LS-3 fluorescence spectrometer (Beaconsfield, Bucks, England) fitted with a Heidolph unit and temperature-controlled (37^{o}C) cell holder was switched on at least an hour before the experiment to ensure a constant intensity of the excitation light.

Red cell membranes (section 5.3.3.) were diluted to a protein concentration of 100 μg/ml in 10 mM Tris-HCl buffer, pH 7.4. A freshly prepared stock solution of 2 mM DPH in tetrahydrofuran was diluted to a working concentration of 1 μM in 10 mM Tris-HCl buffer, pH 7.4. Two millilitres (2 ml) of diluted membranes were mixed with an equal volume of the 1 μM DPH solution and incubated in a shaking water bath at 37^{o}C. A total of 4 tubes were incubated, tube 1 at time 0, tube 2 fifteen seconds later, tube 3 at exactly 10 mins and tube 4 fifteen seconds after tube 3. This staggering of the tubes allowed for continuous reading of fluorescence intensities. At 30 mins (tubes 1 and 3) and 35 mins (tubes 2 and 4) fluorescence intensities were measured at excitation and emission wavelengths of 365 nm and 456 nm respectively, with the polarizers in the four positions:

	excitation	*emission*
(i)	90°	0°
(ii)	90°	90°
(iii)	0°	0°
(iv)	0°	90°

$$G \text{ (equation 3.7)} = \frac{\text{(i)}}{\text{(ii)}}$$

$$P \text{ (equation 3.8)} = \frac{\text{(iii)} - G \times \text{(iv)}}{\text{(iii)} + G \times \text{(iv)}}$$

Using the average of all four P values measured at steady state, fluorescence anisotropy, r, was calculated as in equation 3.10.

TMA-DPH fluorescence polarisation was measured and fluorescence anisotropy calculated using an identical procedure to that for DPH. Aliquots of the TMA-DPH stock solution were stored in the dark at 0°C and used within a month (Kuhry *et al.* 1985).

5.3.7. Membrane cholesterol extraction

5.3.7.1. Reagents

(1) Propan-2-ol with 9 mg/ml butylated hydroxytoluene (BHT)

(2) Chloroform

(3) 1 μg/ml α-tocopherol in chloroform

5.3.7.2. Methods

This was based on the method of Rose and Oklander (1965). One millilitre (1 ml) of washed red blood cells from section 5.3.2. was haemolysed in an equal volume of deionised water in a 30 ml glass container on ice for 15 mins. Eleven millilitres (11 ml) of propan-2-ol containing 9 mg/ml butylated hydroxytoluene (BHT) as an antioxidant were added. The mixture was left on ice for one hour, then 7 ml of chloroform were added. After a further hour on ice, with intermittent shaking, the mixture was centrifuged for 10 mins at 1,000 x g, 4°C. Forty microlitres (40 µl) of the antioxidant α-tocopherol in chloroform (1 μg/ml) were added to the supernatant and the sample stored at -20°C until the estimation of membrane cholesterol content.

5.3.8. Estimation of membrane cholesterol content (validated in section 6.3.1.)

Membrane cholesterol content was estimated using a cholesterol test combination kit (Boehringer Mannheim, Lewes, England) with some modifications.

5.3.8.1. Principles

Cholesterol is oxidized by cholesterol oxidase to cholest-4-en-3-one (cholestenone). In the presence of catalase, the hydrogen peroxide produced in this reaction oxidizes methanol to formaldehyde. The latter reacts with acetylacetone forming a yellow lutidine-dye in the presence of NH_4^+-ions. The concentration of the lutidine-dye (3,5-diacetyl-1,4-dihydrolutidine) formed is stoichiometric with the amount of cholesterol and is measured by the increase of absorbance in the visible range at 405 nm.

5.3.8.2. Reagents

(1) Solution 1:- catalase/phosphate buffer

(2) Solution 2:- acetylacetone/methanol solution

(3) Solution 3:- cholesterin-oxidase

(4) Cholesterol standard, 1 mg/ml (2.59 µM).

5.3.8.3. Methods

Solutions 1 and 2 were thawed at room temperature. Three parts of solution 1 were then mixed with 2 parts of solution 2 by gentle inversion, covered with aluminium foil and allowed to stand at room temperature for 1-2 hours (solution 4).

Five millilitres (5 ml) of the membrane extracts (section 5.3.7.) were dried at 50°C under oxygen-free nitrogen and reconstituted in 1 ml of propan-2-ol. Two hundred microlitres (200 µl) of the reconstituted extracts were mixed with 1.8 ml of solution 4 in a glass tube and 1 ml of this mixture transferred to a second glass tube. To one tube 5 μl of solution 3 were added (sample), the other tube acting as the blank. Both tubes were incubated in a shaking waterbath at 37°C for one hour and then allowed to stand at room temperature for 10 mins before reading absorbances at a wavelength of 405 nm. The difference in absorbance between the sample and the blank was used to calculate membrane cholesterol content from a standard curve (validated in section 6.2) prepared together with the samples as in Table 5.2.

cholesterol content (µg)	cholesterol standard (µl)	isopropanol (µl)	solution 4 (ml)
100	200	0	1.8
90	180	20	1.8
80	160	40	1.8
60	120	80	1.8
40	80	120	1.8
20	40	160	1.8
10	20	180	1.8

Table 5.2. Standard curve for the estimation of membrane cholesterol content.

5.3.9. Western blotting techniques (appendix 11.3)

5.3.9.1. Principles of enhanced chemiluminescence (ECL) detection

There are several types of luminescence, each characterized according to the source of energy for light emission. Chemiluminescence occurs when the energy from a chemical reaction is emitted in the form of light. Enhanced chemiluminescence (ECL) Western blotting is a light emitting non-radioactive method for detecting immobilized specific antigens, conjugated directly or indirectly with horseradish peroxidase (HRP)-labelled antibodies. Horseradish peroxidase catalyzes the oxidation of luminol (a cyclic diacylhydrazide) in the presence of hydrogen peroxide. The oxidised luminol is in an excited state and as it returns to the ground state emits light which is detected on an autoradiograph. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by HRP in the presence of chemical enhancers such as phenols. This increases the light output about 1,000-fold and extends the time of light emission. The system has been optimized for use with nitrocellulose membranes, such as Hybond™-ECL (ECL™ Western blotting protocols, Amersham, England).

The proteins of interest are separated by electrophoresis and transferred to a nitrocellulose membrane. Following the blockage of non-specific sites the proteins are incubated, sequentially, with a primary antibody, biotinylated secondary antibody, HRP-streptavidin complex, ECL reagents and then exposed to light-sensitive film.

5.3.9.2. Reagents

Western blotting reagents are listed in appendix 11.3.1.

5.3.9.3. Methods

5.3.9.3.1. Gel electrophoresis

A 1.5 mm linear gradient 4-12% polyacrylamide gel (appendix 11.3.2) was prepared using the 4% and 12% gel ingredients (appendix 11.3.1). When the gel had set 10-20 wells were cast in a 4% stacking gel on top. B x 1 solution was poured onto the stacking gel to wash the wells and remove unpolymerised acrylamide.

The red cell membranes (section 5.3.3) were diluted with H x 3 solution containing dithiothreitol (DTT, 18 mg/ml) at a ratio of 30 µl H x 3 to 100 µl membranes. The membranes were then denatured by placing in boiling water for 5 mins.

The B x 1 solution was carefully aspirated from the wells in the stacking gel using a needle and syringe. Sigma Dalton low and high molecular weight standard proteins (29-205 kDa, appendix, section 11.3.3) were loaded into the first track. Fifty micrograms (50 µg) of membrane protein (determined in section 6.5) were loaded into each of the other wells and B x 1 solution used to fill up the wells completely. The proteins were electrophoresed for approximately 16 hours at a constant voltage of 40 volts.

5.3.9.3.2. Immunoblotting

While the gel (section 5.3.9.3.1.) was running, blot buffer was prepared and degased on ice. Two 15 cm x 15 cm pieces of 3 mm Whatman filter paper, and one piece of 15 cm x 15 cm Hybond™-Super C (Amersham, England) nitrocellulose paper were prepared. From the latter piece, a 1.5 cm x 15 cm strip was cut off for the standard track. (Gloves must be worn when handling the nitrocellulose paper to avoid the transfer of proteins from fingerprints).

Electrophoresis was terminated when the dye had migrated to within 0.5-1 cm of the bottom of the gel. The stacking gel was removed and the proteins transferred from the main gel onto nitrocellulose paper in a Western blot sandwich

(Figure 11.1), in a blotting tank cooled externally with ice and internally by a cycled water cooling system, at 80 volts for 2.5 hours (details in appendix 11.3.4).

The side of the nitrocellulose paper bearing the blotted proteins was placed face upwards during all subsequent manipulations. The 1.5 cm x 15 cm standard track was stained with amido black for 2 mins and destained for 5 mins. The rest of the nitrocellulose paper was incubated in a rocking sandwich box for an hour at room temperature in 200 ml of the 5% milk solution to block remaining protein binding sites. The paper was then washed twice in 100 ml of sarcosyl buffer, 5 mins each wash.

Twenty millilitres (20 ml) of the primary antibody, mouse anti-Ca^{2+}-Mg^{2+}-ATPase antibody, diluted 1:500 in overlay buffer was applied and left rocking at 4°C (to minimise evaporation of antibody) overnight. The primary antibody was retrieved and stored at 4°C. The paper was washed in the rocking tray with 2 changes of 100 ml sarcosyl buffer, 5 mins each wash. Twenty millilitres (20 ml) of the linking antiboby, biotinylated rabbit anti-mouse antibody, diluted 1:500 in overlay buffer was applied for 1 hour at room temperature. The antibody was then discarded and the paper washed twice, 5 mins each wash, with 100 ml of sarcosyl buffer before incubating for 1 hour in 20 ml streptavidin-biotinylated horseradish peroxidase, diluted 1:400 with overlay buffer. The nitrocellulose paper was washed twice, 5 mins each wash, in 100 ml sarcosyl buffer before incubating with 10 ml of ECL reagents 1 and 2 (5 ml each) for 1 minute exactly. The detection reagents were drained off and the paper carefully wrapped in Saran™ wrap and exposed to Hybond™-Super C film (Amersham, England) in the dark for 30 sec, 60 sec and 3 mins, respectively, to obtain a range of exposures. A standard curve was plotted, on log paper, from the mobility of the standard peptides from which the molecular weights of the blotted proteins were determined.

Chapter 6

OPTIMIZATION EXPERIMENTS

6.1. SERUM LIPID ANALYSIS

Serum lipids (total- and HDL-cholesterol and triglycerides) were measured using the quality-controlled Kodak Ektachem Clinical Chemistry Slides (Kodak Clinical Products, Rochester, NY, USA) with coefficients of variation of 3.2% and 4% for cholesterol and triglyceride measurements respectively.

6.2. RED CELL MEMBRANE PROTEIN CONCENTRATION

Membrane protein concentration was determined using the Folin-phenol reagent as described by Lowry *et al.* (1951). At an absorbance of 550 nm the standard curve was linear at all the protein concentrations used (Figure 6.1).

6.3. INTRA-ASSAY AND INTER-ASSAY VARIABILITY STUDIES

The variability of the assays was assessed in one experiment on 2 successive days using one or two membrane samples. The coefficient of variability is given by the equation

$$\frac{\text{standard deviation}}{\text{mean}} \times 100\ \% \quad \text{.....(6.1)}$$

6.3.1. Red cell membrane cholesterol content

Membrane cholesterol concentration was determined by a colorimetric method using a Boehringer Mannheim (Lewes, England) biochemical analysis test-combination with modifications described in the methods section 5.3.8. At an absorbance of 405 nm the standard curve was linear to a cholesterol concentration of 80 μg/ml

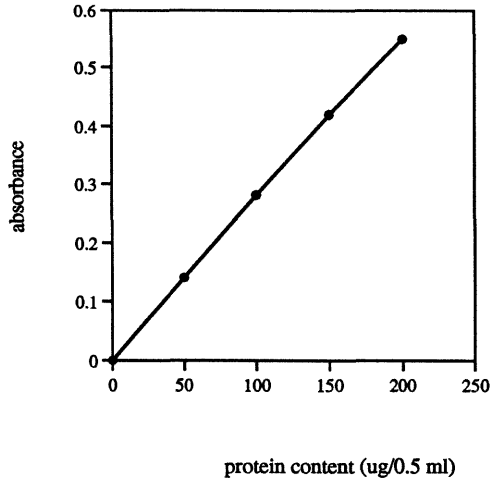

Figure 6.1. Standard curve for membrane protein estimation. Absorbance was read at a wavelength of 550 nm. Average of 3 experiments.

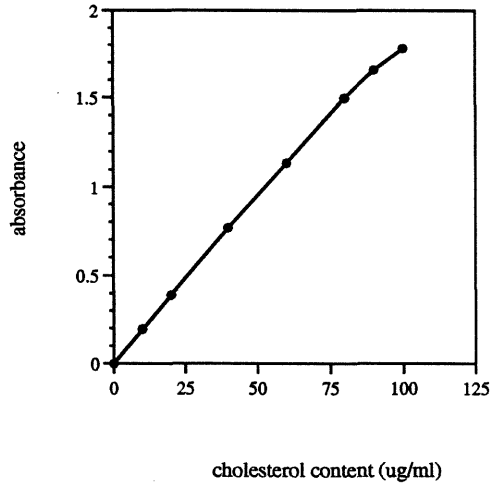

Figure 6.2. Standard curve for membrane cholesterol estimation. Absorbance was read at 405 nm. Average of 2 experiments.

(1.036 μmol/l, Figure 6.2). At the dilutions used the absorbance of the cholesterol extracts was on the linear part of the standard curve. The coefficients of variation for the entire protocol of membrane cholesterol extraction and estimation were: intra-assay 2.6%, and inter-assay 2.7% (Table 6.1).

	day 1	day 2
membrane 1	1.636	1.616
	1.616	1.600
	1.712	1.552
	1.681	1.681
	1.620	1.681
	1.636	1.650

Table 6.1. Intra-assay and inter-assay variability in membrane cholesterol concentration (mmol/l of cells).

6.3.2. DPH fluorescence anisotropy

Intra-assay and inter-assay variabilities were 1.3% and 3.3% respectively (Table 6.2).

6.3.3. TMA-DPH fluorescence anisotropy

Intra-assay and inter-assay variabilities were 1.4% and 1.8% respectively (Table 6.3).

6.3.4. Ca^{2+}-Mg^{2+}-ATPase activity

Intra-assay and inter-assay variabilities were assessed for both basal and calmodulin-stimulated Ca^{2+}-Mg^{2+}-ATPase activities. Enzyme activity was expressed in micromoles of ATP hydrolysed per milligram of membrane protein per hour (μmol ATP/mg/h).

	day 1	day 2
membrane 1	0.210	0.213
	0.210	0.213
	0.213	0.214
	0.213	0.209
membrane 2	0.226	0.223
	0.226	0.225
	0.228	0.226
	0.223	0.216

Table 6.2. Intra-assay and inter-assay variability in DPH fluorescence anisotropy.

	day 1	day 2
membrane 1	0.258	0.256
	0.264	0.261
	0.262	0.256
	0.263	0.267
membrane 2	0.270	0.269
	0.265	0.269
	0.262	0.268
	0.262	0.268

Table 6.3. Intra-assay and inter-assay variability in TMA-DPH fluorescence anisotropy.

6.3.4.1 Basal Ca^{2+}-Mg^{2+}-ATPase activity

Intra-assay and inter-assay variabilities were 4.4% and 4.6% respectively (Table 6.4).

6.3.4.2. Calmodulin-stimulated Ca^{2+}-Mg^{2+}-ATPase activity

Intra-assay and inter-assay variabilities were 7.0% and 7.5% respectively (Table 6.5).

6.4. KINETIC STUDIES ON THE Ca^{2+}-Mg^{2+}-ATPASE ENZYME

Normal control membranes were used for the following series of enzyme kinetic experiments. Normolipidaemic diabetic membranes were also used for comparison.

6.4.1. Time-course studies

Ca^{2+}-Mg^{2+}-ATPase activity was measured by an [γ-^{32}P]ATP hydrolysis assay as described previously (Adeoya *et al.* 1989, 1992). In 100 µl of reaction mixture were 20-50 µg of membrane protein, 1.5 mM final ATP concentration, 20 µM final calcium concentration and 0.1 µM final calmodulin concentration. It was therefore important to exclude the effects of substrate depletion and/or product inhibition on enzyme activity over 30 minutes for calmodulin-stimulated activity or 60 minutes for basal activity by confirming linearity in enzyme activity with time. Figures 6.3 and 6.4 confirm this relationship in one normal control and one normolipidaemic diabetic control membrane respectively over 35-40 minutes (calmodulin-stimulated) and 80 minutes (basal).

	day 1	day 2
membrane 3	1.83	1.73
	1.96	1.79
	1.81	1.81
	1.90	1.80
	1.91	1.72
membrane 4	1.78	1.92
	2.00	2.00
	1.78	1.98
	1.91	1.83
	1.85	1.83

Table 6.4. Intra-assay and inter-assay variability in basal Ca^{2+}-Mg^{2+}-ATPase activity (μmol/mg/h).

	day 1	day 2
membrane 3	4.31	4.89
	4.21	4.98
	4.52	4.82
	4.61	4.43
	3.90	4.95
membrane 4	4.85	5.29
	4.71	5.58
	4.76	5.40
	4.76	4.82
	4.75	5.51

Table 6.5. Intra-assay and inter-assay variability in calmodulin-stimulated Ca^{2+}-Mg^{2+}-ATPase activity (μmol/mg/h).

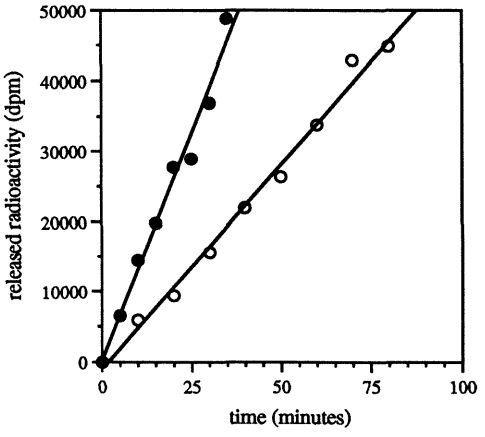

Figure 6.3. Rate of release of radioactivity (dpm) by basal (○) and calmodulin-stimulated (●) calcium pump. One experiment using a normal control membrane.

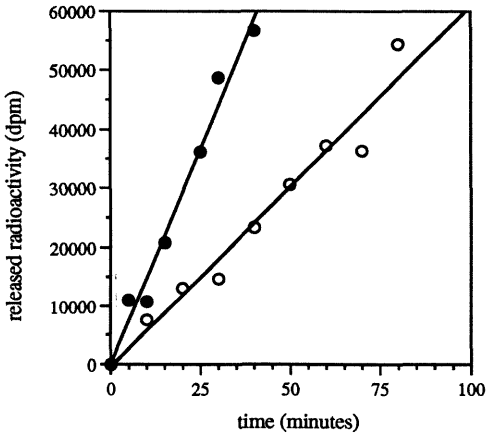

Figure 6.4. Rate of release of radioactivity (dpm) by basal (○) and calmodulin-stimulated (●) calcium pump. One experiment using a normolipidaemic diabetic control membrane.

6.4.2. Calcium concentration studies

High concentrations of calcium are known to inhibit calcium pump activity (Dunham and Glynn 1961, Schatzmann and Rossi 1971). The effect of added calcium concentrations below and above 20 μM was therefore assessed to ensure that, in the normal controls, enzyme activity was at maximum velocity, V_{max}.

In a preliminary experiment with final added calcium concentrations up to 40 μM, it was noted that the enzyme was fully active even in the absence of added calcium (Figure 6.5). Because small amounts of calcium, even in the form of impurities, can fully stimulate the calcium pump (Scharff and Foder 1977), these results suggested the presence of enough calcium in the system to stimulate the enzyme fully. Since the membranes were all prepared in the presence of EDTA the most likely source of calcium contamination was the Ca^{2+}-Mg^{2+}-ATPase buffer. Therefore, new buffer was prepared using filtered deionised water [Milli-Q™ water purification system (Millipore, Watford, England)]. It was also found in a previous study that EGTA to a final concentration of 27 μM was sufficient to sequester calcium (Booth *et al.* 1988). Using the new buffer and a wider range of calcium concentrations calcium pump activity was measured in 3 normal control and 2 diabetic control membranes. To one sample EGTA to a final concentration of 80 μM was added. The results are shown in Figures 6.6 and 6.7 respectively. As in the preliminary study, there was substantial enzyme activity in the absence of added calcium. However, with added EGTA enzyme activity was absent (basal) or markedly reduced (calmodulin-stimulated) confirming the presence of calcium as an impurity in assay buffers. Figure 6.6 shows that 20 μM final added calcium concentration gave maximum enzyme activity in normal controls. These results also confirm that ATP-hydrolysis was Ca^{2+}-dependent and not attributable to other ion-transporting ATPases present in the membrane e.g. Na^{+}-K^{+}-ATPase.

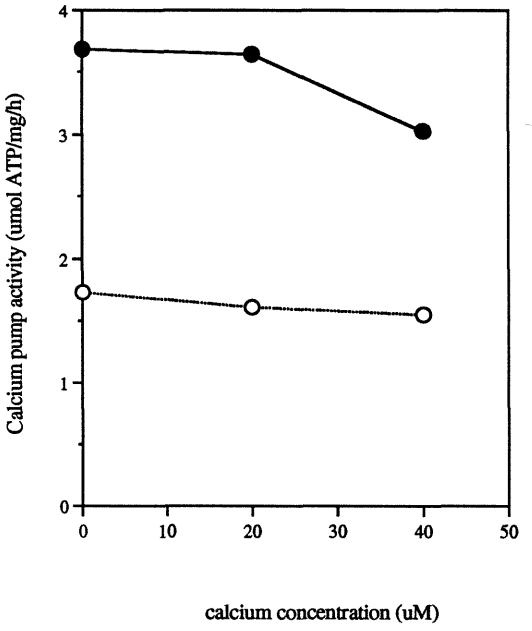

Figure 6.5. Effect of calcium on basal (○) and calmodulin-stimulated (●) calcium pump activity. One experiment using a normal control membrane.

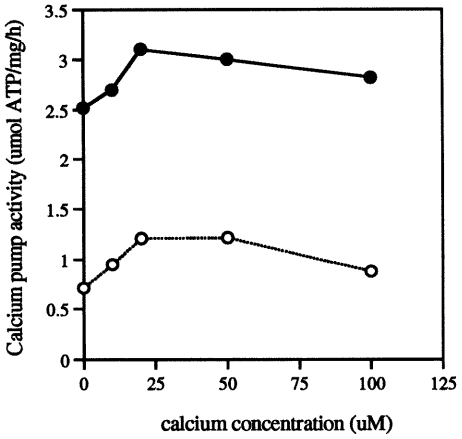

Figure 6.6. Effect of added calcium on basal (○) and calmodulin-stimulated (●) calcium pump activity in normal control membranes. With 80 uM EGTA and no added calcium, basal activity was absent and calmodulin-stimulated activity was 0.15 umol ATP/mg/h. Average of 3 experiments.

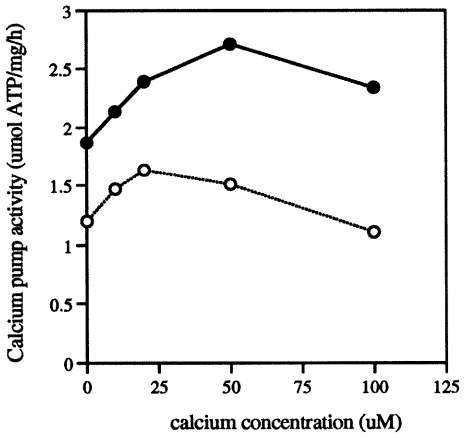

Figure 6.7. Effect of added calcium on basal (○) and calmodulin-stimulated (●) calcium pum activity in normolipidaemic diabetic control membranes. With 80 uM EGTA and no added calcium, both activities were absent. Average of 2 experiments.

6.5. WESTERN BLOTTING TECHNIQUES

To determine the optimal protein concentration for gel electrophoresis and immunoblotting as described in section 5.3.9 a preliminary experiment using a wide range of membrane protein concentrations (0.2 μg-180 μg per well) was performed. Figure 6.8 shows the ECL detection at an exposure time of 30 seconds. Fifty micrograms (50 μg) of membrane protein gave the clearest band separation in the region of the calcium pump (molecular weight 138-140 kDa) and were used in all the western blotting experiments.

The possible identities of the various bands and the limitations of this technique are discussed in section 8.5.5.

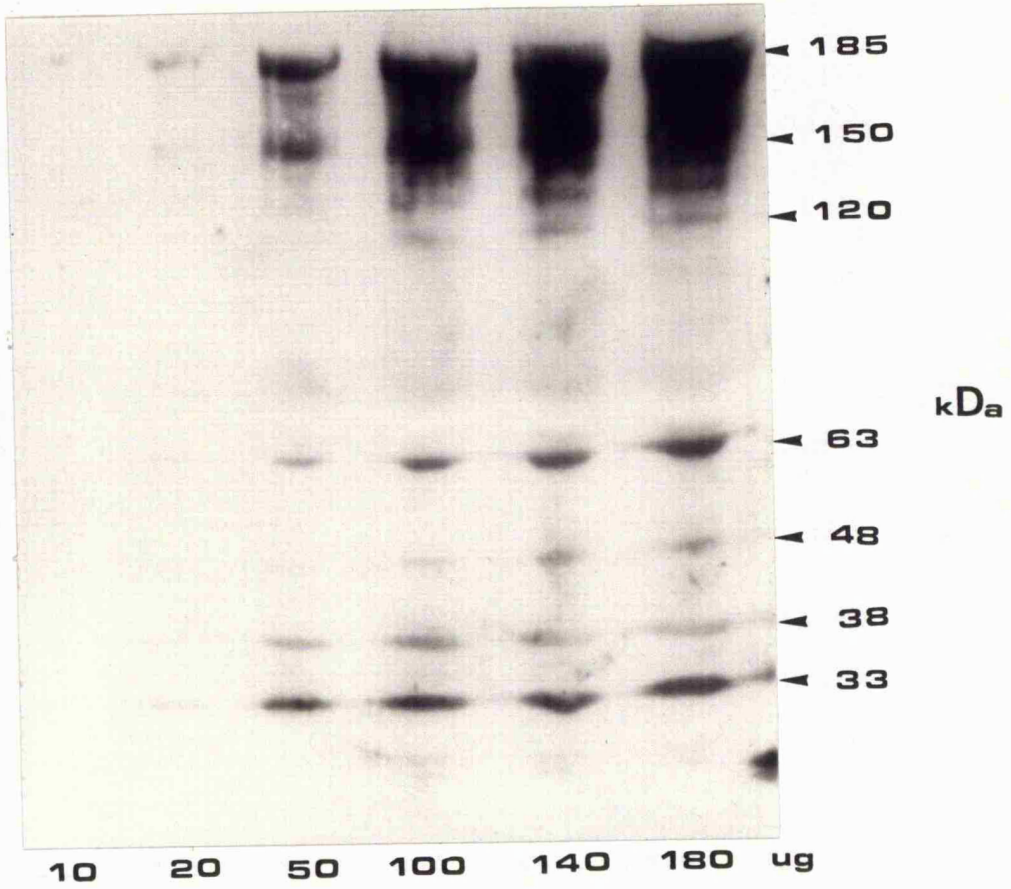

Figure 6.8. Membrane protein mobility on Western blotting. Fifty micrograms (50 μg) of membrane protein gave the clearest band separation. The 150 kDa band is the intact calcium pump (Ca^{2+}-Mg^{2+}-ATPase enzyme). The 185 kDa and the minor 170 kDa bands are most likely partially proteolysed Ca^{2+}-Mg^{2+}-ATPase dimers. At higher protein concentrations minor bands appear at 120, 132 and 143 kDa which may represent partially proteolysed Ca^{2+}-Mg^{2+}-ATPase enzyme. The 38-63 kDa bands are a result of non-specific staining (see figure 8.2).

Chapter 7

EFFECT OF LOWERING SERUM CHOLESTEROL ON HUMAN RED CELL MEMBRANE CHOLESTEROL AND FLUORESCENCE ANISOTROPY

7.1. INTRODUCTION

The dynamic properties of biological membranes have been extensively studied using liposomes as plasma membrane models (Shinitzky and Inbar 1974, 1976; Straume and Litman 1987). Other systems that have been employed in membrane dynamics studies include human red cells (Cooper *et al.* 1974, Shinitzky and Inbar 1976), human platelets (Shattil *et al.* 1975, Stuart *et al.* 1980), human lymphocytes, lymphoma and leukaemia cells (Shinitzky and Inbar 1976), lymphocytes and lymphoma cells in rats and mice (Shinitzky and Inbar 1974), normal and malignant fibroblasts in hamsters (Shinitzky and Inbar 1976), and rat hepatocytes (Shinitzky and Inbar 1976). In these studies increasing the membrane cholesterol/phospholipid mole ratio increased membrane microviscosity and vice versa. In addition, liposomes and red cell membranes with similar phospholipid composition had similar membrane microviscosities (Shinitzky and Inbar 1976). In rats and mice, lymphoma cells had more fluid membranes than normal lymphocytes, but increasing their membrane cholesterol content by incubating with lecithin/cholesterol liposomes increased membrane microviscosity (i.e. reduced membrane fluidity) to the same level as in the normal lymphocytes. The converse was also true: incubating normal lymphocytes with lecithin liposomes reduced their microviscosity to a value near that of lymphoma cells (Shinitzky and Inbar 1974). These changes occurred after incubating for only 3 hours. These results were taken to indicate that the introduction of cholesterol into plasma membranes of lymphoma cells *in vivo* can be conducted, in principle, by lipoproteins provided that the cholesterol levels in the plasma were high enough.

In red cells and platelets, where lipid metabolism is minimal, serum and membrane cholesterol are in dynamic equilibrium (Shohet 1972, Shinitzky and Barenholz 1978). Hence, an increase in serum cholesterol should be mirrored by an increase in membrane cholesterol content and, consequently, an increased membrane microviscosity. The converse should also hold.

7.2. AIMS

The present study assesses the effect of lowering serum cholesterol on membrane cholesterol and fluorescence anisotropy in the human red cell.

7.3. PATIENTS AND METHODS

7.3.1. Patients

Patients with primary hypercholesterolaemia were recruited from the Lipid Clinic. Primary hypercholesterolaemia was defined as serum total cholesterol > 7.0 mmol/l and triglycerides < 3.0 mmol/l on a fasting venous blood sample, in the absence of secondary causes of hyperlipidaemia such as renal, liver and thyroid dysfunction and diabetes mellitus. Patients were also excluded from the study if they had symptoms or a history of gallstones and/or biliary obstruction. Normolipidaemic volunteers (fasting serum total cholesterol < 6.5 mmol/l and triglycerides < 2.3 mmol/l) in the University Departments of Medicine and Surgery, Glenfield General Hospital, were recruited to control for operator and instrument variability. Because this was a paired study, where each subject acted as his or her own control, no attempt was made to match the two groups.

7.3.2. Methods

Patients whose total serum cholesterol was higher than 7.0 mmol/l after 3 months of a low fat, weight-reducing diet were recruited into the study. The following parameters were measured at baseline, using the methods described in chapter 5; height, in metres, without shoes; weight, in kilograms, with light indoor clothes and no shoes (body mass index was calculated by weight/height^2 (kg/m^2)); sitting blood pressure after 5 minutes rest (diastolic blood pressure at phase V); serum lipids (total cholesterol, triglycerides, HDL-cholesterol) after a 12 hour fast, using the Kodak

Ekctachem Clinical Chemistry Slides (Kodak Clinical Products, Rochester, NY, USA); red cell membrane cholesterol and fluorescence anisotropy. Anisotropy was measured using both DPH and TMA-DPH fluorescent probes.

Patients were then commenced on the cholesterol-lowering resin, colestipol, at a dose of 10 g once daily taken with breakfast, in addition to the low fat, weight reducing diet. The dose of colestipol was doubled to 10 g twice a day if the fasting serum total cholesterol had not fallen by more than 1 mmol/l one month after starting treatment. The normolipidaemic group received no active intervention.

At three months on lipid-lowering therapy the parameters measured at baseline were re-measured in both groups.

7.3.3. Statistical analyses

Statistical analysis within each group was by the Wilcoxon signed ranks test as described by Siegel and Castellan Jr. (1988) and between group comparisons were by the Mann Whitney U-test. Correlations were assessed by Spearman's rank correlation. The Minitab Release 8 statistical software (Clecom Ltd, Birmingham, England) was used for the Mann-Whitney U-test and Spearman's rank correlations. Results are expressed as median (inter-quartile range). Statistical significance was taken at $p < 0.05$, with p values > 0.1 expressed as NS, non-significant.

7.4. RESULTS

Sixteen patients and 8 normolipidaemic subjects were recruited. The characteristics of the participating subjects are shown in Table 7.1. Although they were not matched, the two groups were compared for interest. Patients were significantly older than the normolipidaemic subjects but blood pressure profiles were similar. Table 7.2 shows all the parameters measured at baseline. Although the median triglyceride levels were within the normal range (0-2.3 mmol/l) in both groups,

	hyperlipidaemic patients	normolipidaemic subjects	differences between medians (95% CI)	p value
number (M : F)	16 (5 : 11)	8 (3 : 5)		
age (years)	60 (45.8 - 65.8)	44.5 (35.3 - 48.8)	-14.5 (-22 to 1)	0.04
systolic blood pressure (mmHg)	160 (133 - 170)	130 (122 - 148)	-19 (-40 to 2)	0.08, NS
diastolic blood pressure (mmHg)	90 (80 - 96)	88 (79 - 94)	-2 (-10 to 6)	NS

Table 7.1. Demography and clinical characteristics of participating subjects. Results are expressed as median (inter-quartile range) and difference between medians (95% confidence intervals). Statistical analysis was by the Mann-Whitney U-test. *Abbreviations*: CI, confidence intervals; NS, non-significant.

	hyperlipidaemic patients	normolipidaemic subjects	difference between medians (95% CI)	p value
body mass index (BMI, kg/m^2)	25.2 (23.2 - 27.8)	24.0 (21.8 - 26.6)	-1.1 (-4.3 to 2.5)	NS
serum total cholesterol (mmol/l)	8.7 (8.1 - 9.3)	5.6 (5.1 - 5.8)	-3.2 (-4.0 to -2.7)	< 0.001
serum HDL-cholesterol (mmol/l)	1.51 (1.08 - 1.82)	1.45 (1.02 - 1.71)	-0.08 (-0.50 to 0.33)	NS
serum triglycerides (mmol/l)	1.92 (1.17 - 2.30)	0.78 (0.69 - 1.34)	-0.8 (-1.5 to -0.34)	0.002
membrane cholesterol (mmol/l of cells)	1.500 (1.352 - 1.753)	1.629 (1.461 - 1.675)	0.102 (-0.156 to 0.285)	NS
membrane DPH anisotropy	0.221 (0.219 - 0.224)	0.223 (0.220 - 0.224)	0.001 (-0.002 to 0.003)	NS
membrane TMA-DPH anisotropy	0.266 (0.264 - 0.267)	0.267 (0.264 - 0.269)	0.001 (-0.002 to 0.004)	NS

Table 7.2. A comparison of baseline parameters between the treated and the normolipidaemic groups. Results are expressed as median (inter-quartile range) and difference between medians (95% confidence intervals). Statistical analysis was by the Mann-Whitney U-test. *Abbreviations*: NS, non-significant.

the patient group had significantly higher levels than the normolipidaemic group. Despite a significant difference in serum total cholesterol levels, there were no differences in membrane cholesterol levels, or both DPH and TMA-DPH fluorescence anisotropies between the normolipidaemic and hypercholesterolaemic groups. BMI and serum HDL-cholesterol levels were also similar between the two groups.

After three months of lipid-lowering therapy there was a significant fall (of 13%) in serum total cholesterol with the expected rise (of 9%) in serum triglycerides and no significant change in serum HDL-cholesterol levels in the hyperlipidaemic group. There were significant falls in both membrane cholesterol and DPH and TMA-DPH anisotropies (Table 7.3). BMI did not change significantly. No significant changes in the baseline parameters occurred in the normolipidaemic group (Table 7.4)

In both groups there were no correlations between serum and membrane cholesterol levels or between membrane cholesterol and fluorescence anisotropy (Table 7.5). There was a consistent negative correlation between serum triglycerides and DPH anisotropy which reached statistical significance in the pre-treatment hyperlipidaemic group, r_S = -0.5, p = 0.03, but just failed to reach statistical significance in the treated group at 3 months, r_S = -0.5, p = 0.055. (Figure 7.1). There was a positive correlation between TMA-DPH anisotropy and serum triglycerides in the normolipidaemic subjects at baseline only.

7.5. DISCUSSION

For most of the parameters the dotplots were not normally distributed and the n-scores were not linear even after log transformation {Minitab release 8, Clecom Ltd, (Birmingham, England)}. It was felt inappropriate to assume that the lipid distribution in the general population would apply to the samples. Furthermore, because the populations from which the samples were selected were either

	baseline	3 months	difference between medians (95% CI)	p value
body mass index ($BMI, kg/m^2$)	25.2 (23.2 - 27.8)	25.1 (23.2 - 27.4)	-0.1 (-2.8 to 2.5)	NS
serum total cholesterol (mmol/l)	8.7 (8.1 - 9.3)	7.6 (6.8 - 8.2)	-1.1 (-1.8 to -0.5)	< 0.001
serum HDL-cholesterol (mmol/l)	1.51 (1.08 - 1.82)	1.35 (1.14 - 1.58)	-0.11 (-0.40 to 0.26)	NS
serum triglycerides (mmol/l)	1.92 (1.17 - 2.30)	2.09 (1.59 - 2.95)	0.45 (0.13 to 0.67)	<0.01
membrane cholesterol (mmol/l of cells)	1.500 (1.352 - 1.753)	1.319 (1.054 - 1.429)	-0.233 (-0.465 to -0.31)	<0.01
membrane DPH anisotropy	0.221 (0.219 - 0.224)	0.219 (0.216 - 0.221)	-0.003 (-0.005 to -0.0001)	< 0.01
membrane TMA-DPH anisotropy	0.266 (0.264 - 0.267)	0.263 (0.262 - 0.265)	- 0.003 (-0.005 to -0.001)	< 0.05

Table 7.3. Parameters at baseline and at 3 months in the treated group. Results are expressed as medians (inter-quartile range), and difference between medians (95% confidence intervals). Statistical analysis was by the Wilcoxon signed ranks test, except differences between medians which was by the Mann-Whitney U-test. *Abbreviations*: NS, non-significant.

	baseline	3 months	difference between medians (95% CI)	p value
body mass index (BMI, kg/m^2)	24.0 (21.8 - 26.6)	24.3 (21.8 - 26.7)	0 (-3.4 to 3.5)	NS
serum total cholesterol (mmol/l)	5.6 (5.1 - 5.8)	5.6 (5.0 - 6.4)	0.1 (-0.7 to 1.0)	NS
serum HDL-cholesterol (mmol/l)	1.45 (1.02 - 1.71)	1.24 (1.06 - 1.43)	-0.18 (-0.54 to 0.25)	NS
serum triglycerides (mmol/l)	0.78 (0.69 - 1.34)	0.86 (0.80 - 1.13)	0.07 (-0.30 to 0.30)	NS
membrane cholesterol (mmol/l of cells)	1.629 (1.461 - 1.675)	1.461 (1.309 - 1.692)	-0.086 (-0.311 to 0.196)	NS
membrane DPH anisotropy	0.223 (0.220 - 0.224)	0.223 (0.219 - 0.226)	0.001 (-0.003 to 0.004)	NS
membrane TMA-DPH anisotropy	0.267 (0.264 - 0.269)	0.264 (0.263 - 0.268)	-0.002 (-0.005 to 0.003)	NS

Table 7.4. Parameters at baseline and at 3 months in the normolipidaemic group. Results are expressed as median (inter-quartile range), and difference between medians (95% confidence intervals). Statistical analysis was by the Wilcoxon signed ranks test, except differences between medians which was by the Mann-Whitney U-test. *Abbreviations*: NS, non-significant.

	hyperlipidaemic patients	normolipidaemic subjects
serum cholesterol v membrane cholesterol	(a) -0.05 (-0.5 to 0.5) (b) 0.1 (-0.4 to 0.6)	0.06 (-0.4 to 0.5) -0.05 (-0.7 to 0.7)
membrane cholesterol v DPH anisotropy	(a) 0.1 (-0.4 to 0.6) (b) 0 (-0.5 to 0.5)	0.2 (-0.6 to 0.8) 0.1 (-0.6 to 0.8)
membrane cholesterol v TMA-DPH anisotropy	(a) 0.3 (-0.2 to 0.7) (b) -0.2 (-0.6 to 0.3)	0 (-0.6 to 0.8) 0.5 (-0.3 to 0.9)
serum triglycerides v DPH anisotropy	(a) -0.5 (-0.8 to -0.05)* (b) -0.5 (-0.8 to 0.03)	-0.6 (-0.9 to 0.1) -0.3 (-0.8 to 0.5)
serum triglycerides v TMA-DPH anisotropy	(a) -0.3 (-0.7 to 0.3) (b) 0.2 (-0.4 to 0.6)	0.8 (0.2 to 0.96)* 0.2 (-0.7 to 0.7)

Table 7.5. Correlations between selected parameters. Results are expressed as Spearman's rank correlation coefficient, r_S, (95 % confidence interval). * $p < 0.05$. (a) baseline, (b) at 3 months.

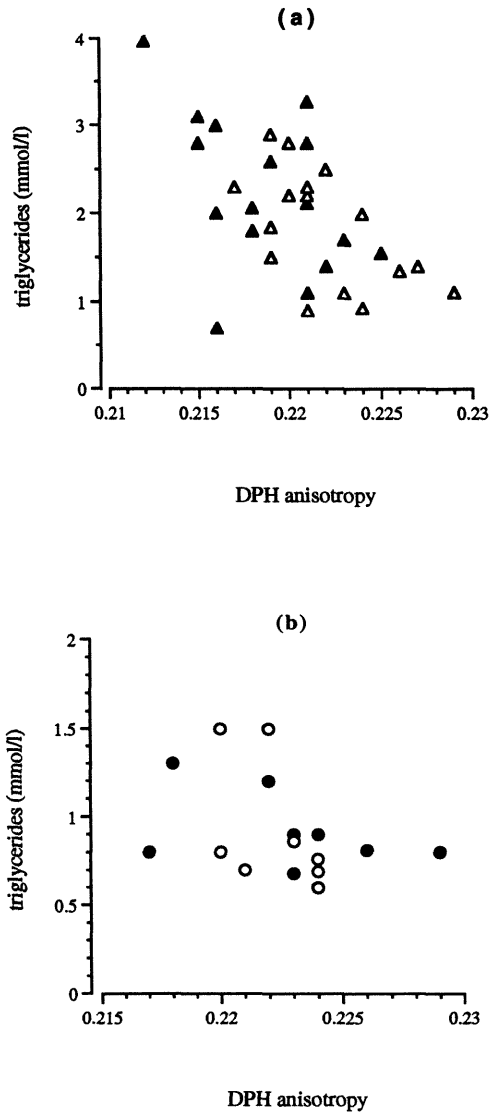

Figure 7.1. Relationship between serum triglycerides and DPH anisotropy in the (a) hypercholesterolaemic and (b) normolipidaemic groups. Open symbols are at baseline and closed symbols at 3 months.

normolipidaemic (controls) or hyperlipidaemic (patient group) the lipid distribution in the population would be skewed. Non-parametric tests were, therefore, more appropriate (J. Imeson, C. Jagger, personal communications) and were employed in all analyses in the present study. Spearman's rank correlations were determined for serum and membrane cholesterol, and for membrane cholesterol and fluorescence anisotropy. Because membrane triglycerides could not be measured, correlations involving triglycerides employed serum values. The correlations in the present study must be intepreted with caution due to a number, albeit small, of multiple comparisons.

There are a number of interesting observations in the present study. Despite the differences in serum total cholesterol levels at baseline membrane cholesterol levels were similar in the patient and normolipidaemic groups. There are two possible explanations for this finding. *Firstly*, membranes can alter their cholesterol content to maintain an optimal fluidity and cholesterol/phospholipid ratio (Inbar *et al.* 1973), a form of homeoviscous adaptation. In this context Yawata *et al.* (1984) described the self-adaptive modification of red cell membrane lipids to maintain normal membrane fluidity in a case of LCAT deficiency. The metabolic defect leads to high levels of free cholesterol in both the plasma and the red cell membrane and to reduced membrane fluidity. Yawata *et al.* (1984) found that to counteract this the membrane phospholipid and fatty acid composition were altered; phosphatidylcholine (PC), shorter chain fatty acids (16:0) and double bonds (18:2) were increased, whereas sphingomyelin (SM), phosphatidylethanolamine (PE), and longer chain fatty acids (18:0, 24:0) were reduced. In the studies of Popp-Snijders *et al.* (1986) and Hagve *et al.* (1991, 1993) dietary supplementation with (n-3) fatty acids rich in eicosapentaenoic acid, EPA C20:5 (n-3), and docosahexaenoic acid, DHA C22:6 (n-3), in healthy volunteers for 4-8 weeks resulted in an increase in membrane C20:5 (n-3) and C22:6 (n-3) fatty acids and a reduction in membrane C18: 2 (n-6) fatty acid, with no changes in cholesterol/phospholipid ratio and in membrane fluidity. Membrane fluidity was maintained by modifications in the

membrane phospholipid subclasses. In the studies of Hagve *et al.* (1991, 1993), there was a transient decrease in red cell osmotic fragility due to an increase in membrane (n-3) fatty acids. Membrane osmotic fragility returned to normal by day 28 of supplementation due to a redistribution of the membrane phospholipid subclasses. However, a study by Hornstra and Rand (1986) in human platelets showed that (n-3) fatty acid supplementation for 6 weeks increased both membrane (n-3) fatty acids and membrane fluidity.

Secondly, *in vitro* models, in which such correlations have been described, may not reflect what happens *in vivo*. Thus, for example, Giannettini *et al.* (1991) found that the order parameters of red cell membranes from healthy human volunteers were higher than those of liposomes prepared from the lipid extracts of ghost membranes prepared from these subjects. In another study, *in vivo* exposure of rat brain synaptosomes to toluene had no effect on their membrane fluidity whereas *in vitro* exposure increased membrane fluidity (Edelfors and Ravn-Jonsen 1989). Against the homeoviscous adaptation theory are the observations that red cell membrane cholesterol/phospholipid ratio is increased in patients with cirrhosis (Cooper *et al.* 1974) and in animals fed diets rich in cholesterol (Sardet *et al.* 1972).

Baseline membrane anisotropies were similar in the hyperlipidaemic and normolipidaemic groups despite their age differences. This could be partly explained by the similarities in membrane cholesterol in the respective groups, although there was no correlation between membrane cholesterol and membrane anisotropy measured by either fluorescent probe. The lack of correlation could be due to the small number of subjects in the present study giving a narrow range of lipid values. Although patients had significantly higher serum triglyceride levels than normolipidaemic volunteers, they were much lower than those in the study of Dowd *et al.* (1993b), 1.92 mmol/l (0.9-2.9) vs 4.87 mmol/l (1.8-8.6), [median (range)], respectively. This could also explain why, unlike the above study, membrane fluidity was similar in the present hyperlipidaemic and normolipidaemic groups. Muller *et al.* (1990) also found no difference in both DPH and TMA-DPH anisotropy between 12

patients with type IIA hyperlipidaemia (familial hypercholesterolaemia) and 15 normal controls using sealed and unsealed red cell membranes and intact platelets. In that study, patients with type IIA hyperlipidaemia also had significantly higher serum triglyceride levels than the normal controls. Similar DPH and TMA-DPH anisotropy results have been reported by Bharaj *et al.* in human red cell (1993, 1994) and platelet (1994) membranes in familial hypercholesterolaemia. In the study of Dowd *et al.* (1993b) when their 2 hyperlipidaemic groups were combined and membrane anisotropy analysed by the type of hyperlipidaemia, both DPH and TMA-DPH anisotropy were similar between 19 normal controls, 18 type IIA and 9 polygenic hypercholesterolaemic subjects. Le Quan Sang *et al.* (1991), on the other hand, using platelets from mild to moderately hypertensive patients, found reduced TMA-DPH anisotropy in 2 groups with hypercholesterolaemia and normal fasting serum triglycerides. Those with serum total cholesterol between 6.2 - 6.8 mmol/l (N = 10) had mean $\pm$ s.e.m. anisotropy of 0.270 $\pm$ 0.003, and those with serum total cholesterol > 6.8 mmol/l (N = 7) of 0.271 $\pm$ 0.004, compared to 0.278 $\pm$ 0.003 in those with serum total cholesterol < 6.2 mmol/l (N = 9). When the 2 hypercholesterolaemic groups were combined, the reduction in anisotropy was statistically significant at $p < 0.05$.

In the hyperlipidaemic group in the present study there was a significant negative correlation between serum triglycerides and DPH anisotropy at baseline but after 3 months on treatment just failed to reach statistical significance. The correlation between serum triglycerides and DPH anisotropy in the normolipidaemic subjects was not significant most likely due to the small number of subjects and a narrower range of serum triglyceride values in this group. Muller *et al.* (1990) showed that compared to 15 normal controls DPH anisotropy was reduced in 13 patients with type IIB (combined) hyperlipidaemia in both sealed and unsealed red cell membranes. In platelets DPH anisotropy was reduced in both type IIB and type IV (endogenous hypertriglyceridaemia, N = 7) hyperlipidaemia. When the patient and control groups were combined, Muller *et al.* (1990) found a negative correlation

between serum triglycerides and DPH anisotropy in intact platelets, $r_S = -0.74$, $p < 0.001$, but not in red cell membranes. Malle *et al.* (1991) also found a negative correlation between DPH anisotropy and serum triglycerides, $r_S = -0.45$, $p < 0.05$, in intact platelets from 18 healthy volunteers aged 20 - 26 years. Dowd *et al.* (1993b) had conflicting results on red cell membrane fluidity in two groups of hypercholesterolaemic patients with a wide range of triglyceride values. DPH anisotropy was reduced in the first group of 27 patients, but there was no correlation between DPH anisotropy and serum triglycerides or serum total cholesterol. In a second group of 26 patients, DPH anisotropy was similar to that in 19 normal controls despite the fact that when the two hyperlipidaemic patient groups were compared similar serum lipid values [triglycerides, median (range), 3.1 (0.5 - 11.1) mmol/l vs. 3.8 (0.9 - 8.4) mmol/l; total cholesterol, mean $\pm$ s.e.m., 7.66 $\pm$ 0.28 mmol/l vs. 8.06 $\pm$ 0.38 mmol/l] and similar age and sex distribution were found. The reasons for this discrepancy were not addressed but it seems likely that lipid lowering therapy may have influenced the results: 13/27 in group 1 and 12/26 in group 2 were on therapy including 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors or statins and fibric acid derivatives which can affect membrane dynamics. When the 2 groups were combined and analysed by the type of hyperlipidaemia DPH anisotropy was lower in the group with combined hyperlipidaemia.

Unlike DPH, there was no correlation between TMA-DPH anisotropy and serum triglycerides in the hyperlipidaemic group in the present study. This is an expected finding since TMA-DPH labels the outer regions of the membrane (lipid-water interface). That DPH anisotropy is negatively correlated with and is reduced in hypertriglyceridaemia suggests increased fluidity of the hydrocarbon core of the membrane. The positive correlation between serum triglycerides and TMA-DPH anisotropy in the control group is therefore unexpected and difficult to reconcile with current concepts of membrane fluidity and may be due to the small numbers involved. In the study of Muller *et al.* (1990) TMA-DPH anisotropy was reduced,

indicating an increased fluidity at the membrane lipid-water interface, in type IIB and type IV hyperlipidaemia in sealed and unsealed red cell membranes and in intact platelets. When the patient and control groups were combined there was a negative correlation between TMA-DPH anisotropy and serum triglycerides in unsealed red cell membranes, $r_S = -0.33$, $p < 0.05$, and platelets, $r_S = -0.31$, $p < 0.01$. Malle *et al.* (1991), as in the present study, found no correlation between TMA-DPH anisotropy and serum triglycerides in intact platelets from healthy volunteers. In the study of Dowd *et al.* (1993b) TMA-DPH anisotropy was similar between the hyperlipidaemic groups combined and normal controls. In addition there was no correlation between anisotropy and serum triglycerides in both the hypercholesterolaemic and the combined hyperlipidaemic groups. Carr *et al.* (1993) showed a negative correlation between TMA-DPH anisotropy and serum triglycerides in intact lymphocytes, $r_S = -0.23$, $p = 0.04$. Although lymphocytes metabolise lipids, the anisotropy values, median (range), of 0.267 (0.264 - 0.270) were similar to those in the red cell membranes of the present study.

The picture that emerges from studies on the effect of hyperlipidaemia on DPH fluorescence anisotropy is that, as in the present study, anisotropy is unaltered in isolated hypercholesterolaemia (Bharaj *et al.* 1993, 1994; Dowd *et al.* 1993b; Muller *et al.* 1990). In hypertriglyceridaemia DPH anisotropy is reduced (Dowd *et al.* 1993b; Muller *et al.* 1990). As in the present hyperlipidaemic group, there is a negative correlation between serum triglycerides and DPH anisotropy (Malle *et al.* 1991, Muller *et al.* 1990) but, as in the present normolipidaemic groups, not in all cases (Bharaj *et al.*, personal communication; Dowd *et al.* 1993b).

The effect of hyperlipidaemia on TMA-DPH anisotropy is less clear but, as in the present study, hypercholesterolaemia appears not to alter TMA-DPH anisotropy (Bharaj *et al.* 1993, 1994; Dowd *et al.* 1993b; Muller *et al.* 1990). There is no pattern in hypertriglyceridaemia, with anisotropy either unchanged (Dowd *et al.* 1993b) or reduced (Muller *et al.* 1990). The correlation between TMA-DPH and serum triglycerides is also unclear, ranging from non-existent (Bharaj *et al.*, personal

communication; Dowd *et al.* 1993b; Muller *et al.* 1990), negative (Carr *et al.* 1993, Muller *et al.* 1990), to positive in the present normolipidaemic subjects at baseline.

The inconsistencies in the published results could be partly explained by demography and methodology. For example, Le Quan Sang *et al.* (1991) and Muller *et al.* (1990) examined TMA-DPH anisotropy in platelets from hyperlipidaemic subjects. Whereas Le Quan Sang *et al.* (1991) found reduced anisotropy in hypercholesterolaemia, Muller *et al.* (1990) found no difference in anisotropy between subjects and controls. Le Quan Sang *et al.* (1991) studied 17 hypertensive/hyperlipidaemic subjects and 9 hypertensive/normolipidaemic controls with an unspecified age and sex distribution, whereas Muller *et al.* (1990) studied 12 normotensive/hyperlipidaemic male subjects and 15 normotensive/normolipidaemic male controls with similar mean ages. The platelets were incubated with TMA-DPH at 37°C for 1 hour by Le Quan Sang *et al.* (1991) and at 25°C for an unspecified time period by Muller *et al.* (1990). Carr *et al.* (1993) incubated their lymphocytes at 37°C for 5 minutes only. In the present study on red cell membranes there was a positive correlation between serum triglycerides and TMA-DPH anisotropy in the normolipidaemic subjects at baseline whereas Muller *et al.* (1990) and Carr *et al.* (1993, using intact lymphocytes) described negative correlations. In the latter 2 studies the subjects were heterogeneous, including normolipidaemic subjects, with wide ranges of serum lipids. The sample sizes were also markedly different: 23 in the study of Muller *et al.* (1990), 83 in that of Carr *et al.* (1993) and 8 in the present study.

Although there was no correlation between serum and membrane cholesterol levels in the present study, a 13% reduction in serum total cholesterol and an associated 9% rise in serum triglycerides on treatment resulted in a 12% reduction in membrane cholesterol content and a 1% drop in both DPH and TMA-DPH fluorescence anisotropy. The reduction in membrane fluorescence anisotropy observed after treatment with colestipol is likely to be a result of both the fall in membrane cholesterol and the rise in serum triglycerides. As discussed in section

3.3.4, high levels of neutral lipids affect membrane fluidity. Ray *et al.* (1969) produced some evidence in rat liver plasma membranes that neutral lipids enter the membrane. In that study the membranes were shown to contain, as a percentage of the total plasma membrane lipid content, 18.1% free cholesterol, 7.9% free fatty acids, 3.9% triglycerides and 55.4% phospholipids.

It appears, therefore, that serum triglycerides may be more important than serum cholesterol in determining membrane fluidity. Muller *et al.* (1990), concluded from their study that hyperlipidaemia-associated erythrocyte and platelet fluidity alterations are not related to hypercholesterolaemia but to the triglyceride levels. How triglycerides affect membrane fluidity remains speculative. It is possible that they have an indirect effect via changes in the fatty acid composition of the phospholipid fatty acyl chains or that they reduce the cholesterol/phospholipid ratio of the cell membranes. Studies supporting the latter hypothesis have been published in type IV hyperlipidaemia (Neerhout *et al.* 1968, Vakakis *et al.* 1983). Triglyceride emulsion particles promote the efflux and/or block the influx of cholesterol in cultured macrophages (Aviram *et al.* 1988). In familial hypercholesterolaemia with normal triglycerides the cholesterol/phospholipid ratio in the membranes is unaltered (Cooper 1977), a finding that lends support to both homeoviscous adaptation and the effect of triglycerides on the membrane cholesterol/phospholipid ratio.

Of all the available treatments for hypercholesterolaemia only the bile acid sequestrants or resins are not absorbed systemically. These drugs were chosen for the present study because they should have no direct effect on plasma membrane biophysics. 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors or statins, a common alternative to resins, have been shown to suppress lymphoid cell function *in vitro* (Cutts and Bankhurst 1989). The mechanisms of action of the fibric acid derivatives or fibrates are not fully understood but are thought to include lipoprotein lipase stimulation and HMG CoA reductase inhibition. Using the resin colestipol serum lipid changes were as expected - a 13% fall in serum

total cholesterol, a 9% rise in serum triglycerides and a non-significant change in serum HDL-cholesterol levels.

7.6. SUMMARY

Several observations have been made in the present study:

(1) There is no direct correlation between serum and membrane cholesterol levels. This is most likely due to membrane homeoviscous adaptive mechanisms.

(2) A fall in serum total cholesterol is accompanied by a fall in membrane cholesterol at 3 months.

(3) There is no direct correlation between membrane cholesterol and fluorescence anisotropy, although a fall in membrane cholesterol with a concomitant rise in serum triglycerides is associated with a reduction in fluorescence anisotropy.

(4) As in other studies published previously, there is a negative correlation between serum triglycerides and DPH anisotropy, suggesting an increased fluidity deep in the hydrocarbon core of the membrane bilayer.

(5) Serum triglycerides may be more important than serum cholesterol in the control of plasma membrane fluidity.

Chapter 8

MEMBRANE ENVIRONMENT AND CALCIUM PUMP ACTIVITY IN DIABETES AND THE COMBINED HYPERLIPIDAEMIAS

8.1 INTRODUCTION

Adeoya *et al.* have shown the importance of membrane environment in determining red cell calcium pump activity in hypertension in the rat model (1989) and in human studies (1992).

As discussed in section 4.4 the few studies carried out on the calcium pump in diabetes mellitus have shown a reduction in enzyme activity attributed, in part, to non-enzymatic glycosylation of its modulator protein calmodulin. The roles of serum lipids and membrane environment on calcium pump activity were not investigated in these studies. In the sarcoplasmic reticulum, an increase in membrane cholesterol content causes a reduction in calcium pump activity (Squire *et al.* 1988), although this has not been a consistent finding (Warren *et al.* 1975).

Membrane fluidity also regulates the activity of membrane structures (Stubbs 1983). Thus, for example, above or below an optimal membrane fluidity sarcoplasmic reticulum calcium pump activity is reduced (Bigelow and Thomas 1987). It was shown in chapter 7 that lowering serum cholesterol reduces membrane cholesterol content and, with a concomitant rise in serum triglycerides, increases membrane fluidity. In addition, serum triglycerides appeared to influence membrane fluidity more than serum cholesterol. These results confirm that serum lipids affect membrane environment. Therefore, it is possible that some of the observed reduction in calcium pump activity in the above studies in diabetes was due to altered membrane dynamics associated with abnormal serum and membrane lipid levels.

Raised intracellular calcium concentration increases red cell and platelet rigidity, plasma viscosity (Weed *et al.* 1969, Isogai *et al.* 1981; Schmid-Schonbein and Volger 1976) and smooth muscle proliferation (Gleason *et al.* 1991). Intracellular calcium concentration is controlled by the calcium pump (Schatzmann 1969), hence altered calcium pump activity may play a role in the pathogenesis of the vascular complications of diabetes and hyperlipidaemia.

The present study, an extension of that in chapter 7, assesses the effect of a wider range of serum lipids on membrane environment (membrane cholesterol and membrane fluidity) and the effect of serum lipids and membrane environment on calcium pump activity. Primary combined hyperlipidaemia and combined hyperlipidaemia secondary to type II diabetes were studied. The working hypothesis was that calcium pump activity is reduced in primary combined hyperlipidaemia due to an altered membrane environment and that in diabetic subjects with combined hyperlipidaemia calcium pump activity might be further reduced by glycosylation of the calcium pump protein (Ca^{2+}-Mg^{2+}-ATPase enzyme) itself.

8.2. PATIENTS

Four groups of subject were studied:

(i) normolipidaemic, non-diabetic controls,

(ii) normolipidaemic, non-insulin treated type II diabetic controls,

(iii) patients with primary combined hyperlipidaemia,

(iv) non-insulin treated type II diabetic subjects with combined hyperlipidaemia.

All the participants were not on any medication known to affect lipid metabolism or calcium pump activity. Diabetes mellitus was defined according to the World Health Organization criteria (1985).

8.3. STUDY DESIGN

The study was designed in two stages, with new recruits at each stage.

Stage 1 *to assess calcium pump activity in intact red cell ghosts*

Stage 2 *to assess differences in glycosylation of the calcium pump protein by Western blotting techniques.*

Due to difficulties in recruiting non-insulin treated type II diabetic subjects with combined hyperlipidaemia, stages 1 and 2 were combined. The same difficulties in recruitment meant that exact sex matching of the groups was not always managed.

8.4. CALCIUM PUMP ACTIVITY IN INTACT RED CELL GHOSTS

8.4.1. Methods

Fasting serum lipids (total cholesterol, HDL-cholesterol, triglycerides), glycosylated haemoglobin (HbA_1, except in the normal controls), red cell membrane anisotropy using both DPH and TMA-DPH, membrane cholesterol and basal and calmodulin-stimulated calcium pump activities were measured in the four groups using the methods described in chapter 5. Surplus red cell membranes were stored at -80^{o}C for use later in Western blotting experiments.

8.4.2. Statistical analyses

The Kruskal-Wallis test was used to compare the four groups, and the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr 1988) was used to determine which groups were significantly different. Relationships between selected variables were assessed by Spearman's rank correlation using the Minitab Release 8 statistical software (Clecom Ltd, Birmingham, England). Results are expressed as median (inter-quartile range). Statistical significance was taken at $p < 0.05$, with p values > 0.1 expressed as NS, non-significant.

8.4.3. Results

The characteristics of the participants are shown in Table 8.1. The majority of participants were White Caucasian (n = 65) and the rest were of Asian origin (2 normal controls, 6 diabetic controls, 2 primary combined hyperlipidaemia and 6 hyperlipidaemic diabetic subjects). There were no differences in age and blood

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects	p value
Number (M/F)	20 (9/11)	16 (10/6)	24 (16/8)	21 (15/6)	
Age (yrs)	49 (43.3-58.5)	54 (49.5-60)	50 (39.3-59)	52 (45.5-61)	NS
Systolic BP (mmHg)	140 (120-151)	140 (130-160)	150 (140-160)	160 (135-168)	NS
Diastolic BP (mmHg)	87 (80-90)	80 (80-90)	89 (80-90)	90 (80-98)	NS
BMI (kg/m^2)	23.2 (21.8-28.6)	30 (27.2-34.1)	26.3 (23.6-27.7)	28.3 (25.8-32.4)	0.001

Table 8.1. Demography and clinical characteristics of participating subjects. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test. *Abbreviations*: BP, blood pressure; BMI, body mass index.

pressure profiles between the 4 groups. BMI was different between the groups ($p = 0.001$) because the diabetic groups were heavier than the other 2 groups ($p < 0.05$).

Lipid profiles are shown in Table 8.2a and membrane anisotropy and calcium pump activities in Table 8.2b. As expected, serum total cholesterol and triglycerides were higher in the hyperlipidaemic groups, but despite the differences in serum cholesterol membrane cholesterol levels were similar between all 4 groups. Serum HDL-cholesterol levels were also similar between the groups. In the 3 groups where HbA_1 was measured there were significant differences in glycaemic control ($p < 0.001$) due to the difference between the primary combined hyperlipidaemia and the two diabetic groups ($p < 0.05$).

There were significant differences in DPH anisotropy between the normolipidaemic and the hyperlipidaemic groups, but TMA-DPH anisotropy was reduced only in the diabetic group with combined hyperlipidaemia. Membrane cholesterol levels were similar in all 4 groups, and there was a positive correlation between serum total cholesterol and membrane cholesterol levels in only 2 of the 4 groups - normal controls and primary combined hyperlipidaemia. A strong negative correlation existed between membrane cholesterol and DPH anisotropy in the normal control group only, but there was no correlation between membrane cholesterol and TMA-DPH anisotropy in any of the 4 groups. There was a significant negative correlation between serum triglycerides and DPH anisotropy in the hyperlipidaemic diabetic group only, although there was a non-significant trend towards a negative correlation between serum triglycerides and DPH anisotropy in the other 3 groups (Table 8.3a, Figure 8.1). There was no correlation between serum triglycerides and TMA-DPH anisotropy.

Compared to normal controls basal calcium pump activity was reduced to a similar degree in the other three groups (Table 8.2b), activity being higher in the normal controls than in the primary combined hyperlipidaemic group than in the normolipidaemic diabetic controls than in the group with combined hyperlipidaemia secondary to type II diabetes. This difference was lost on calmodulin stimulation

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects	p value
TC	5.5 (4.8-5.8)	5.00 (3.7-5.8)	8.2 (7.5-9.5)	7.6 (7.0-9.0)	<0.001
HDL	1.12 (1.02-1.46)	0.94 (0.73-1.26)	1.07 (0.96-1.25)	1.02 (0.95-1.26)	NS
TG	1.15 (0.90-1.53)	1.50 (1.40-1.80)	4.25 (3.43-5.46)	4.30 (3.70-5.72	<0.001
m.Chol	1.293 (1.208-1.456)	1.107 (0.985-1.350)	1.327 (1.188-1.390)	1.267 (1.190-1.397)	NS
HbA_1		10.0 (8.2-11.2)	6.8 (6.4-7.5)	8.6 (7.8-9.6	<0.001

Table 8.2a. Serum lipids (mmol/l), membrane cholesterol (mmol/l of cells) and HbA_1 (%) in the four groups. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test. *Abbreviations*: TC, serum total cholesterol; HDL, serum HDL-cholesterol; TG, serum triglycerides; m. Chol, membrane cholesterol, HbA_1 , glycosylated haemoglobin A_1 .

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects	p value
rDPH	0.223 (0.220-0.226)	0.221 (0.219-0.224)	0.216 (0.216-0.220)	0.216 (0.213-0.221)	<0.001
rTMA-DPH	0.266 (0.264-0.268)	0.266 (0.264-0.268)	0.265 (0.263-0.268)	0.263 (0.262-0.266)	0.024
Basal Ca pump activity	2.09 (1.90-2.50)	1.66 (1.18-1.97)	1.77 (1.56-1.97)	1.57 (1.19-1.87)	0.001
Stim. Ca pump activity	5.53 (4.70-6.88)	4.19 (3.07-5.48)	5.45 (4.51-6.38)	5.37 (3.92-5.84)	0.028

Table 8.2b. Membrane anisotropy and calcium pump activity (μmol ATP/mg/h) in the four groups. Results are given as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test. Membrane fluidity is inversely related to membrane fluorescence anisotropy. *Abbreviations*: rDPH, DPH anisotropy; rTMA-DPH, TMA-DPH anisotropy; Ca, calcium; stim., calmodulin-stimulated.

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects
serum cholesterol versus				
(a) membrane cholesterol	0.6 (0.2 to 0.9)**	0.2 (-0.3 to 0.7)	0.5 (0.1 to 0.7)*	-0.1 (-0.5 to 0.4)
serum triglycerides versus				
(a) DPH anisotropy	-0.3 (-0.7 to 0.1)	-0.4 (-0.7 to 0.1)	-0.2 (-0.6 to 0.2)	-0.6 (-0.9 to -0.1)***
(b) TMA-DPH anisotropy	0.2 (-0.3 to 0.6)	-0.2 (-0.7 to 0.3)	-0.1 (-0.5 to 0.3)	-0.2 (-0.6 to 0.3)
membrane cholesterol versus				
(a) DPH anisotropy	-0.7 (-0.9 to -0.1)***	0 (-0.4 to 0.5)	-0.1 (-0.5 to 0.3)	0.3 (-0.2 to 0.6)
(b) TMA-DPH anisotropy	-0.1 (0.6 to 0.4)	0 (-0.5 to 0.5)	0.2 (-0.2 to 0.6)	0.1 (-0.4 to 0.5)

Table 8.3a. Correlation between fluorescence anisotropy, serum lipids and membrane cholesterol. Results are expressed as Spearman's rank correlation coefficient, r_S, (95% confidence intervals).* $p < 0.05$, ** $p < 0.01$, ***99 % confidence intervals, $p < 0.005$.

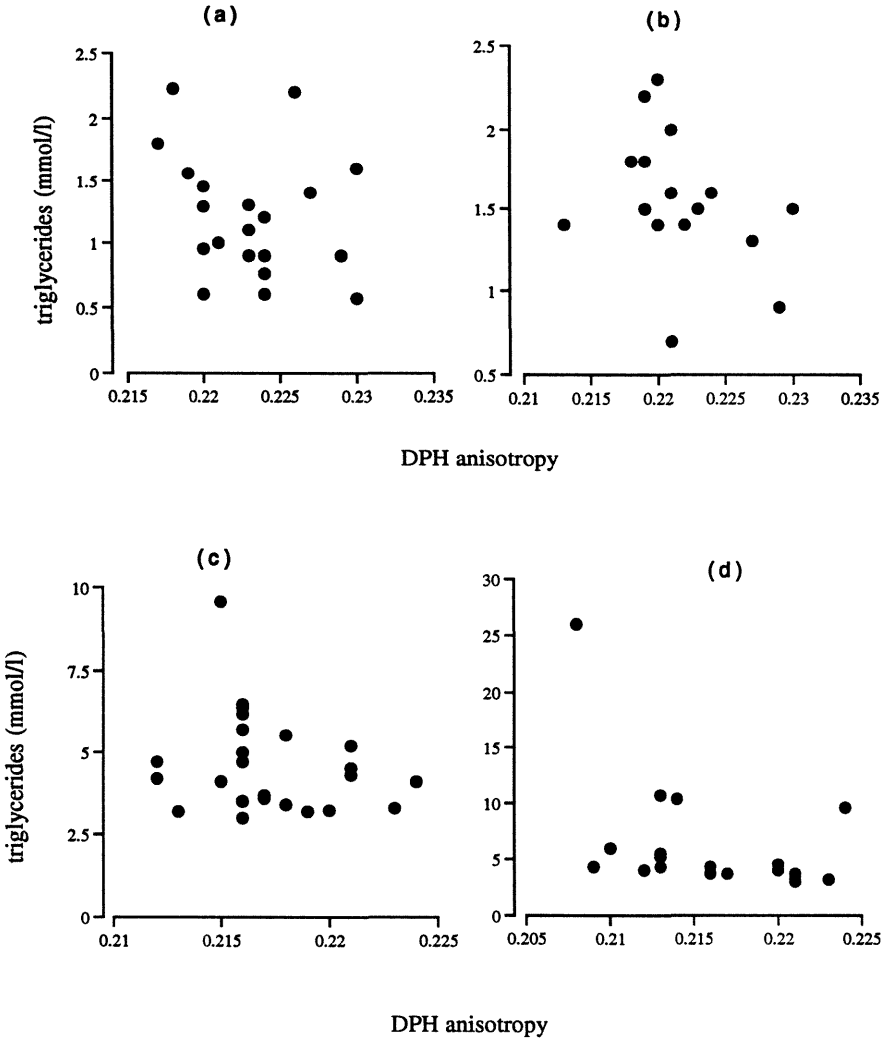

Figure 8.1. Relationship between serum triglycerides and DPH anisotropy. (a) normal controls, (b) normolipidaemic diabetic controls, (c) primary combined hyperlipidaemia, (d) hyperlipidaemic diabetic subjects. There was a consistent negative correlation between serum triglycerides and DPH anisotropy which reached statistical significance in (d), r_S=-0.6, $p < 0.005$. This relationship is difficult to see in panel (d) due to the compression of the data to include the single outlying point. The Spearman's rank correlation used to identify the relationship between parameters is not affected by the absolute values of outlying points.

(Table 8.2b) except in the diabetic controls, but only the difference in stimulated activity between the diabetic controls and the normal controls accounted for the overall significant difference in calmodulin-stimulated activity. Exogenous calmodulin stimulated the enzyme 2.5-fold in the normal controls, 2.6-fold in the diabetic controls, 3-fold in the primary combined hyperlipidaemic group and 3.4-fold in the hyperlipidaemic diabetic subjects. There were negative correlations between basal calcium pump activity and DPH anisotropy, $r_S = -0.5$, $p = 0.039$, in the diabetic controls (Table 8.3b), and between HbA_1 and TMA-DPH anisotropy, $r_S = -0.5$, $p = 0.01$, in hyperlipidaemic diabetic subjects (Table 8.3c).

Because the four groups were not well matched for sex, calcium pump activity was compared between the females and males within each group using the Mann-Whitney U-test. No significant differences in activity existed between the sexes (Table 8.3d).

Tables 8.4 - 8.6 give the results of subgroups analyses of the 4 main groups, using data from Tables 8.2a and 8.2b, to show the effects of membrane environment and/or diabetes on calcium pump activity. Because of the small sample sizes and the difficulty in power calculation for non-parametric tests (C. Jagger, personal communication) the possibility of a type II error exists in these comparisons and can only be minimized by very large sample sizes. The combined effects of diabetes and membrane environment on the calcium pump result in reduced basal calcium pump activity (Table 8.4a). Table 8.4b compares the direct effect of diabetes to that of the membrane environment. Both basal and calmodulin-stimulated calcium pump activities were similar in the two groups despite less favourable glycaemic control in the diabetic controls and a more fluid membrane hydrocarbon core in the primary combined hyperlipidaemia group.

Table 8.5 shows the effect of membrane environment on calcium pump activity. Basal calcium pump activity was reduced in the primary combined hyperlipidaemia group which had increased core membrane fluidity (Table 8.5a). In the two diabetic groups, despite increased membrane fluidity in the group with

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects
Basal activity versus				
(a) DPH anisotropy	0.1 (-0.4 to 0.5)	-0.5 (-0.8 to -0.3)*	-0.2 (-0.5 to 0.3)	0 (-0.5 to 0.4)
(b) TMA-DPH anisotropy	0.2 (-0.3 to 0.6)	-0.1 (-0.6 to 0.4)	0 (-0.4 to 0.4)	-0.1 (-0.5 to 0.3)
(c) serum triglycerides	-0.2 (-0.6 to 0.3)	0.2 (-0.3 to 0.6)	0.1 (-0.3 to 0.5)	0 (-0.4 to 0.5)
(d) membrane cholesterol	-0.3 (-0.7 to 0.3)	0 (-0.5 to 0.5)	-0.1 (-0.5 to 0.3)	-0.3 (-0.7 to 0.1)

Table 8.3b. Correlation between basal calcium pump activity and selected parameters. Results are expressed as Spearman's rank correlation coefficient, r_S, (95% confidence intervals).* $p < 0.05$.

	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects
HbA_1 versus			
(a) basal Ca pump activity	0.1 (-0.4 to 0.6)	0.3 (-0.2 to 0.6)	0.1 (-0.3 to 0.5)
(b) calmodulin stimulated Ca pump activity	0.3 (-0.2 to 0.7)	0 (-0.4 to 0.5)	0.1 (-0.4 to 0.5)
(c) DPH anisotropy	-0.2 (-0.7 to 0.3)	0.1 (-0.4 to 0.5)	-0.1 (-0.5 to 0.3)
(d) TMA-DPH anisotropy	-0.1 (-0.6 to 0.4)	0.4 (-0.1 to 0.7)	-0.5 (-0.8 to -0.1)*

Table 8.3c. Correlation between glycaemic control (HbA_1) and selected parameters. Results are expressed as Spearman's rank correlation coefficient, r_s, (95 % confidence intervals). *$p < 0.05$.

	normal controls	diabetic controls	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects
Basal activity	-0.19 (-0.72 to 0.10)	0.29 (-0.59 to 0.81)	0.02 (-0.44 to 0.32)	0.12 (-0.60 to 0.77)
Calmodulin-stimulated activity	-0.35 (-1.49 to 1.05)	0.18 (-1.77 to 1.97)	0.43 (-1.37 to 1.38)	0.41 (-1.01 to 2.95)

Table 8.3d. Comparison of calcium pump activity between females and males. Results are expressed as difference between female and male medians, μmol ATP/mg/h, (95 % confidence interval). Statistical analysis was by the Mann-Whitney U-test.

	normal controls	hyperlipidaemic diabetic subjects	p value
serum total cholesterol (mmol/l)	5.5 (4.8-5.8)	7.6 (7.0-9.0)	<0.05
serum triglycerides (mmol/l)	1.15 (0.90-1.53)	4.30 (3.70-5.72)	<0.05
membrane cholesterol (mmol/l of cells)	1.293 (1.208-1.456)	1.267 (1.190-1.397)	NS
DPH anisotropy	0.223 (0.220-0.226)	0.216 (0.213-0.221)	<0.05
TMA-DPH anisotropy	0.266 (0.264-0.268)	0.263 (0.262-0.266)	<0.05
Basal calcium pump activity (μmol ATP/mg/h)	2.09 (1.90-2.50)	1.57 (1.19-1.87)	<0.05
calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	5.53 (4.70-6.88)	5.37 (3.92-5.48)	NS

Table 8.4a. Effect of diabetes and membrane environment on red cell calcium pump activity: normal controls versus hyperlipidaemic diabetic subjects. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

	diabetic controls	primary combined hyperlipidaemia	p value
serum cholesterol (mmol/l)	5.0 (3.7-5.8)	8.2 (7.5-9.5)	<0.05
serum triglycerides (mmol/l)	1.50 (1.40-1.80)	4.25 (3.43-5.46)	<0.05
membrane cholesterol (mmol/l of cells)	1.107 (0.985-1.350)	1.327 (1.188-1.390)	NS
DPH anisotropy	0.221 (0.219-0.224)	0.216 (0.216-0.220)	<0.05
TMA-DPH anisotropy	0.266 (0.264-0.268)	0.265 (0.263-0.268)	NS
Basal calcium pump activity (μmol ATP/mg/h)	1.66 (1.18-1.97)	1.77 (1.56-1.97)	NS
Calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	4.19 (3.07-5.48)	5.45 (4.51-6.38)	NS
BMI (kg/m^2)	30.0 (27.2-34.1)	26.3 (23.6-27.7)	<0.05
HbA1 (%)	10.0 (8.2-11.2)	6.8 (6.4-7.5)	<0.05

Table 8.4b. Effect of diabetes and membrane environment on red cell calcium pump activity: diabetic controls versus primary combined hyperlipidaemia. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

	normal controls	primary combined hyperlipidaemia	p value
serum total cholesterol (mmol/l)	5.5 (4.8-5.8)	8.2 (7.5-9.5)	<0.05
serum triglycerides (mmol/l)	1.15 (0.90-1.53)	4.25 (3.43-5.46)	<0.05
membrane cholesterol (mmol/l of cells)	1.293 (1.208-1.456)	1.327 (1.188-1.390)	NS
DPH anisotropy	0.223 (0.220-0.226)	0.216 (0.216-0.220)	<0.05
TMA-DPH anisotropy	0.266 (0.264-0.268)	0.265 (0.263-0.268)	NS
Basal calcium pump activity (μmol ATP/mg/h)	2.09 (1.90-2.50)	1.77 (1.56-1.97)	<0.05
Calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	5.53 (4.70-6.88)	5.45 (4.51-6.38)	NS

Table 8.5a. Effect of membrane environment on red cell calcium pump activity: normal controls versus primary combined hyperlipidaemia. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

combined hyperlipidaemia, basal and calmodulin-stimulated calcium pump activities and glycaemic control were similar (Table 8.5b).

Table 8.6 shows the effect of diabetes on calcium pump activity. Despite similarities in serum lipids, membrane cholesterol and membrane fluidity between the two control groups, both basal and calmodulin-stimulated calcium pump activities were reduced in the diabetic controls (Table 8.6a). In the two hyperlipidaemic groups, serum lipids, membrane cholesterol, membrane fluidity and calcium pump activities were similar (Table 8.6b). In addition, as expected the group with primary combined hyperlipidaemia had better glycaemic control than the diabetic group.

8.4.4. Discussion

8.4.4.1. Statistical analyses

For reasons discussed in chapter 7, non-parametric statistics were employed in all analyses. Spearman's rank correlations were used to assess relationships between selected parameters in determining the relationship between serum lipids, membrane environment and membrane function. As also discussed in chapter 7, these correlations should be intepreted with caution due to multiple comparisons.

8.4.4.2. Serum lipids, membrane cholesterol and membrane fluidity

Shinitzky and Inbar (1976) demonstrated that incubating leukaemia cells with lipoprotein cholesterol *in vitro* increased membrane cholesterol content and they argued that this process could be conducted *in vivo* by lipoproteins provided that the plasma cholesterol levels were high enough. Cooper *et al.* (1974) and Jackson and Morgan (1982) have described a case of increased membrane cholesterol content in association with elevated serum cholesterol levels in liver cirrhosis and chronic cholestasis, respectively. In chapter 7 it was found that, contrary to these earlier studies, in moderately severe hypercholesterolaemia, membrane cholesterol levels

	diabetic controls	hyperlipidaemic diabetic subjects	p value
serum total cholesterol (mmol/l)	5.0 (3.7-5.8)	7.6 (7.0-9.0)	<0.05
serum triglycerides (mmol/l)	1.50 (1.40-1.80)	4.30 (3.70-5.72)	<0.05
membrane cholesterol (mmol/l of cells)	1.107 (0.985-1.350)	1.267 (1.190-1.397)	NS
DPH anisotropy	0.221 (0.219-0.224)	0.216 (0.213-0.221)	<0.05
TMA-DPH anisotropy	0.266 (0.264-0.268)	0.263 (0.262-0.266)	<0.05
Basal calcium pump activity (μmol ATP/mg/h)	1.66 (1.18-1.97)	1.57 (1.19-1.87)	NS
Calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	4.19 (3.07-5.48)	5.37 (3.92-5.84)	NS
HbA_1 (%)	10 (8.2-11.2)	8.6 (7.8-9.6)	NS

Table 8.5b. Effect of membrane environment on red cell calcium pump activity: diabetic controls versus hyperlipidaemic diabetic subjects. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

	normal controls	diabetic controls	p value
serum total cholesterol (mmol/l)	5.5 (4.8-5.8)	5.0 (3.7-5.8)	NS
serum triglycerides (mmol/l)	1.15 (0.90-1.53)	1.50 (1.40-1.80)	NS
membrane cholesterol (mmol/l of cells)	1.293 (1.208-1.456)	1.107 (0.985-1.350)	NS
DPH anisotropy	0.223 (0.220-0.226)	0.221 (0.219-0.224)	NS
TMA-DPH anisotropy	0.266 (0.264-0.268)	0.266 (0.264-0.268)	NS
Basal calcium pump activity (mol ATP/mg/h)	2.09 (1.90-2.50)	1.66 (1.18-1.97)	<0.05
Calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	5.53 (4.70-6.88)	4.19 (3.07-5.48)	<0.05
BMI (kg/m^2)	23.2 (21.8-28.6)	30.0 (27.2-34.1)	<0.05

Table 8.6a. Effect of diabetes on red cell calcium pump activity: normal versus diabetic controls. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

	primary combined hyperlipidaemia	hyperlipidaemic diabetic subjects	p value
serum total cholesterol (mmol/l)	8.2 (7.5-9.5)	7.6 (7.0-9.0)	NS
serum triglycerides (mmol/l)	4.25 (3.43-5.46)	4.30 (3.70-5.72)	NS
membrane cholesterol (mmol/l of cells)	1.327 (1.188-1.390)	1.267 (1.190-1.397)	NS
DPH anisotropy	0.216 (0.216-0.220)	0.216 (0.213-0.221)	NS
TMA-DPH anisotropy	0.265 (0.263-0.268)	0.263 (0.262-0.266)	NS
Basal calcium pump activity (μmol ATP/mg/h)	1.77 (1.56-1.97)	1.57 (1.19-1.87)	NS
Calmodulin-stimulated calcium pump activity (μmol ATP/mg/h)	5.45 (4.51-6.38)	5.37 (3.92-5.84)	NS
HbA_1 (%)	6.8 (6.4-7.5)	8.6 (7.8-9.6)	<0.05

Table 8.6b. Effect of diabetes on red cell calcium pump activity: primary combined hyperlipidaemia versus hyperlipidaemic diabetic subjects. Results are expressed as median (inter-quartile range). Statistical analysis was by the Kruskal-Wallis test for multiple comparisons (Siegel and Castellan Jr. 1988). *Abbreviations*: NS, non-significant.

and red cell membrane DPH and TMA-DPH anisotropies (and hence membrane fluidity) were similar to those in a normolipidaemic parallel group. In the present study membrane cholesterol levels were again similar in all 4 groups despite significant differences in serum cholesterol levels. As discussed in chapter 7, this is most likely due to homeoviscous adaptive mechanisms in the red cell membrane. Although membrane cholesterol levels were similar in the 4 groups, there was a weak positive correlation between serum and membrane cholesterol levels in the group with primary combined hyperlipidaemia, and a stronger positive correlation between the same parameters in the normal control group. No such relationship existed in the 2 diabetic groups. From *in vitro* studies a positive correlation between erum and membrane colesterol levels was expected in all groups (see section 7.1).

Membrane cholesterol levels reported for normolipidaemic subjects in chapter 7 (Table 7.2) do not agree with those of the normal controls in the present study (Table 8.2a) despite employing the same assay. The explanation for this is the choice of controls. In chapter 7 the selection was random, whereas in the present study selection was constrained by the need to match the groups. In addition, the sex ratios were different: M:F ratio of 1:3 in chapter 7, and 1:1 in the present study. Moreover, the membrane cholesterol values at 3 months (Table 7.4) were much nearer those in Table 8.2a. Furthermore, membrane cholesterol estimation in chapter 7 was done in one single run including patients and controls and that in the present study was done in another single run for all 4 groups, with a new standard curve for each run.

DPH anisotropy was decreased in the groups with combined hyperlipidaemia (Tables 8.2, 8.4, 8.5). Because membrane cholesterol levels were similar between the groups this is further evidence that serum triglycerides affect membrane fluidity more than serum cholesterol *in vivo*. The increase in membrane fluidity is predominantly in the hydrocarbon core of the membrane measured by DPH anisotropy, extending to the outer regions of the lipid bilayer measured by TMA-DPH anisotropy in the diabetic group with combined hyperlipidaemia. These

membrane fluidity changes could result from an altered membrane environment secondary to changes in the membrane fatty acid content in the hypertriglyceridaemic subjects. This remains to be investigated. As already discussed in chapter 7, there is also evidence that triglycerides lower the membrane cholesterol/phospholipid ratio thus increasing membrane fluidity (Aviram *et al.* 1988, Neerhout *et al.* 1968, Vakakis *et al.* 1983). With the wider range of triglycerides in the present study the negative correlation between DPH anisotropy and serum triglycerides noted in chapter 7 was present in the hyperlipidaemic diabetic group, r_S = -0.6, p = 0.0047, with a trend to a negative, although non-significant, correlation in the remaining 3 groups. The lack of a significant correlation between serum triglycerides and DPH anisotropy in the group with primary combined hyperlipidaemia is difficult to explain since the 2 hyperlipidaemic groups had similar triglyceride levels. In the normal control group, there was a positive correlation between serum and membrane cholesterol levels, and a strong negative correlation between membrane cholesterol and DPH anisotropy. This suggests that increasing the serum cholesterol concentration should increase both membrane cholesterol concentration and membrane hydrocarbon core fluidity. This is in contrast to *in vitro* fluorescence polarization studies with DPH and TMA-DPH which have shown that cholesterol restricts probe re-orientation in both the hydrocarbon core and the outer regions of the membrane (Shinitzky and Barenholz 1978, Straume and Litman 1987) and that an increase in membrane cholesterol content increases the order of the core substantially more than that of the interfacial region (Straume and Litman 1987).

Diabetes *per se* does not appear to affect membrane fluidity (Table 8.6a). Watala (1993) has reviewed extensively the literature on membrane fluidity in diabetes. Red cell membrane fluidity in type I diabetes, measured with DPH, was increased in 5 studies, decreased in 6 studies and unchanged in one study. Dowd *et al.* (1993a, 1994) also reported increased membrane fluidity in type I diabetes. Therefore, there are nearly as many studies showing increased red cell membrane

fluidity as there are reporting decreased membrane fluidity in type I diabetes. In the studies of Dowd *et al.* (1993a, 1994) membrane fluidity was unchanged using TMA-DPH.

Very few studies have been carried out on membrane fluidity in type II diabetes. In the review by Watala (1993) one study reported increased and one study reduced membrane fluidity. More recently Tong *et al.* (1993), using DPH, found no differences in fluidity in membrane ghosts and in intact red cells from 9 type II diabetic subjects and 6 controls. In the present study, there were no differences in DPH or TMA-DPH anisotropies between 16 normolipidaemic type II diabetic subjects and 20 normal control subjects.

Studies using pyrene fluorescence have shown no change in red cell membrane fluidity in type II diabetes (Caimi *et al.* 1990) and reduced membrane fluidity in type I diabetes (Bryszewska *et al.* 1986). On the other hand e.s.r. studies on red cells from type I diabetic subjects and DPH fluorescence polarization studies in platelets from both type I and type II diabetic subjects have consistently shown decreased membrane fluidity (reviewed by Watala, 1993). In mononuclear leucocytes from type II diabetic subjects, Tong *et al.* (1994) found a greater fluidity gradient with higher TMA-DPH anisotropy and lower DPH anisotropy than in control leucocytes. In the present study the fluidity gradients in the diabetic control and the normal control groups were not significantly different.

In theory, non-enzymatic glycosylation of haemoglobin should parallel that of membrane proteins, hence HbA_1 was used as a surrogate for membrane protein glycosylation in the present study. There was no correlation between HbA_1 and DPH and TMA-DPH fluorescence anisotropy in the diabetic controls and in the group with primary combined hyperlipidaemia, whereas a positive correlation between DPH fluorescence polarization and the extent of membrane protein glycation was described in red cells (Watala and Winocour 1992) and platelets (Winocour *et al.* 1992) from diabetic subjects. There was, however, a negative correlation between HbA_1 and TMA-DPH anisotropy, $r_S = -0.5$, $p = 0.01$, in the present hyperlipidaemic

diabetic group, but not in the diabetic control group - although they had similar HbA_1 values. In the study on platelet membranes (Winocour *et al.* 1992) total serum lipids and membrane cholesterol were also similar to those in an age and sex matched control group.

8.4.4.3. Calcium pump activity

Basal calcium pump activity was lower in the primary combined hyperlipidaemic group with increased DPH fluidity than in the control group most likely due to an effect of serum triglycerides on membrane environment. Similarly, the diabetic group with combined hyperlipidaemia had lower basal calcium pump activity than the diabetic control group due to an effect of serum triglycerides on membrane environment. However, calmodulin-stimulated calcium pump activity was higher in the diabetic group with combined hyperlipidaemia than in the diabetic control group. There are three possible explanations. *Firstly*, calmodulin may overcome the inhibitory effects of membrane environment on calcium pump activity. Adeoya *et al.* (1990) found a similar effect of calmodulin on the accumulation of calcium in inside-out vesicles (IOVs) from spontaneously hypertensive rats (SHR) and Wistar-Kyoto normotensive controls (WKYN). The temperature at which half-maximal basal calcium pump activity occurred was significantly different between the SHR and WKYN groups. This inter-group temperature difference was lost in the presence of calmodulin. They proposed that calmodulin stabilizes the Ca^{2+}-Mg^{2+}-ATPase enzyme such that the effects of membrane environment on enzyme activity are lost. Interestingly, in the same study no such temperature differences were observed using the same [γ-^{32}P]ATP hydrolysis techniques as were employed in the present study. Davis *et al.* (1985) found that oral administration of glucose significantly reduced basal calcium pump activity whereas overall calmodulin-stimulated activity was not reduced, suggesting again that calmodulin overcomes the inhibitory effects of glucose on calcium pump activity.

Secondly, there may have been a greater degree of glycosylation of membrane proteins in the diabetic controls than in the hyperlipidaemic diabetic subjects, although statistically glycaemic control was not significantly different between these two groups, median HbA_1 10% versus 8.6% respectively, and there was no correlation between HbA_1 and basal calcium pump activity.

Thirdly, in addition to the effects of calmodulin, the increased membrane fluidity in the hyperlipidaemic diabetic subjects could have increased the mobility of the Ca^{2+}-Mg^{2+}-ATPase enzyme thereby freeing it from the constraints encountered in the diabetic controls.

A negative correlation was found between basal calcium pump activity and DPH anisotropy, $r_S = -0.5$, $p = 0.034$, in the diabetic controls only but not in the groups with reduced DPH anisotropy.

In chapter 7 it was found that in primary hypercholesterolaemia with normal triglycerides membrane fluidity was similar to that in normolipidaemic volunteers. Bharaj *et al.* (1993) also found no differences in membrane fluidity and calcium pump activity in patients with familial hypercholesterolaemia in both red cell and platelet membranes. Taken together with the present findings, these results suggest that altered membrane fluidity secondary to an effect of high serum triglyceride levels reduces calcium pump activity. In the study of Taffet *et al.* (1993) in rat sarcoplasmic reticulum, dietary (n-3) fatty acids increased sarcoplasmic reticulum membrane (n-3) fatty acids and membrane fluidity and this was associated with a reduction in calcium pump activity despite the presence of the same number of calcium pump units. The fact that the number of calcium pump units was unchanged and that fatty acids can stimulate the enzyme (Schmalzing and Kutschera 1982) suggests that increased membrane fluidity was responsible for the reduction in calcium pump activity. Some of the effects of membrane lipids on calcium pump activity are mediated through the boundary lipids or phospholipid annulus surrounding the enzyme from which cholesterol is normally excluded (Warren *et al.* 1975). It is possible that hypertriglyceridaemia, either directly or via fatty acid

changes, increases the fluidity of the boundary lipids to a level above that necessary for optimal calcium pump activity resulting in the reduction in enzyme activity. Ray *et al.* (1969) found that triglycerides accounted for 3.9% of the total rat liver plasma membrane lipid content.

8.4.4.4. Membrane environment, diabetes and calcium pump activity

From the early studies on liposomes and isolated cell membranes, incorporation of cholesterol into the membrane was shown to alter membrane environment (i.e. membrane cholesterol, cholesterol/phospholipid ratio and membrane fluidity). Winocour *et al.* (1992) have also shown that in diabetes there is increased glycosylation of membrane proteins.

In the present study, basal calcium pump activity was reduced by 21% and calmodulin-stimulated activity by 24% in the diabetic control group, by 25% and 3%, respectively, in the hyperlipidaemic diabetic group and by 15% and 1%, respectively, in the group with primary combined hyperlipidaemia. A 60% reduction in basal calcium pump activity and a 20% reduction in calmodulin-stimulated calcium pump activity in red cell membranes from poorly controlled type I diabetic subjects compared to normal controls was reported in a previous study by Schaefer *et al.* (1987). In addition, partially purified calmodulin from diabetic patients was less effective at stimulating the enzyme than that from normal controls, suggesting that the reduction in enzyme activity was due to both an intrinsic calcium pump defect and diabetes-induced alterations in calmodulin. The reduction in both basal and calmodulin-stimulated calcium pump activities in the present study cannot be attributed to a modified calmodulin as endogenous calmodulin was removed from the red cell ghost membranes during preparation and exogenous calmodulin was used to stimulate the enzyme in all assays. This suggests that the reduction in calcium pump activity was due to either physical changes within the enzyme itself or to altered membrane environment.

A reduction in human red cell calcium pump activity in diabetes has been reported in other studies (Gonzalez Flecha *et al.* 1990, Gronda *et al.* 1986, Zemel *et al.* 1987). In addition, both the incubation of intact red cells or red cell membranes with glucose (Davis *et al.* 1985, Gonzalez Flecha *et al.* 1990, Gonzalez Flecha *et al.* 1993) and the oral or intravenous administration of glucose in man (Davis *et al.* 1985) cause a significant reduction in calcium pump activity. When red cell membranes were incubated with D-[6-^{3}H]glucose the reduction in calcium pump activity was concomitant with an increased incorporation of [6- ^{3}H]glucose into the calcium pump molecule, suggesting an increased glycosylation of the enzyme (Gonzalez Flecha *et al.* 1993). In the incubation experiments with glucose the reduction in enzyme activity, in conditions favouring non-enzymatic glycosylation, was proportional to the concentration of glucose used (Gonzalez Flecha *et al.* 1990). In addition, only the glycosylation of membrane inner-surface proteins (Gonzalez Flecha *et al.* 1990), perhaps the lysine residues near or at the active site (Filoteo *et al.* 1987), affected enzyme activity. Garner *et al.* (1990) have reported the glycosylation of the Na^{+}-K^{+}-ATPase enzyme. Glycosylation of the calcium pump protein (Davis *et al.* 1985, Gonzalez Flecha *et al.* 1990, Gonzalez Flecha *et al.* 1993, Schaefer *et al.* 1987) and/or of calmodulin (Schaefer *et al.* 1987) could, therefore, explain the observed reduction in calcium pump activity in some of these studies. In the study of Gonzalez Flecha *et al.* (1990) in poorly controlled type I diabetic patients there was a negative correlation between HbA_1 and calcium pump activity. As in the study by Schaefer *et al.* (1987), there was no relationship between calcium pump activity and glycaemic control in the present study. However, no correlation coefficients or confidence limits were quoted in the studies of Gonzalez Flecha *et al.* (1990) and Schaefer *et al.* (1987).

Neither serum and membrane lipids nor membrane fluidity were measured in the published studies on the calcium pump in diabetes. Since serum lipids are in dynamic equilibrium with membrane lipids (Shohet 1972, Shinitzky and Barenholz 1978), and membrane fluidity affects calcium pump activity (e.g. in the sarcoplasmic

reticulum, Bigelow and Thomas 1987), an alteration in membrane dynamics may have contributed to the altered calcium pump activity measured in these studies. In the present diabetic and normal control groups, with similar serum lipid levels, there were no differences in membrane cholesterol levels and membrane fluidity, suggesting that the reduction in calcium pump activity was due to changes in the physical properties of the enzyme itself.

The present study shows that both membrane environment and diabetes reduce basal calcium pump activity (Table 8.4a) and do so to the same extent (Table 8.4b). Furthermore, the combined effects of membrane environment and diabetes are similar to those of diabetes alone (Table 8.5b) and to those of membrane environment alone (Table 8.6b), i.e. the effects of membrane environment and diabetes are not additive.

8.5. GLYCOSYLATION OF THE RED CELL CALCIUM PUMP

8.5.1. Introduction

Glycosylation of proteins, where glucose non-enzymatically attaches to the amino groups of proteins, occurs in all individuals but when the proteins are exposed to high concentrations of glucose this process becomes augmented. Non-enzymatic glycosylation occurs in two stages, a reversible first stage leading to the formation of an Amadori product, and a slow and irreversible second stage leading to the formation of advanced glycosylation end products, AGEs (Jennings 1992, Monnier 1992):

glucose + NH_2-protein $\Leftrightarrow$ Schiff base $\Leftrightarrow$ Amadori product $\Rightarrow$ AGEs

AGEs accumulate on long-lived proteins such as collagen and capillary basement membranes. Glycosylation occurs in tissues as diverse as plasma proteins, haemoglobin and albumin (Bunn 1981) and in cell membranes including red cells (Bunn 1981, Gonzalez Flecha 1990, Miller *et al.* 1980, Watala 1988, Watala *et al.* 1985) and platelets (Cohen *et al.* 1989, Sampietro *et al.* 1986, Winocour *et al.* 1992).

The glycosylation of proteins modifies their properties. Thus, for example, the binding characteristics of albumin are altered, LDL-cholesterol becomes poorly recognized by fibroblasts and accumulates in macrophages (Jennings 1992), and calcium pump activity is reduced (Davis *et al.* 1985; Gonzalez Flecha *et al.* 1990, 1993). The reduction in calcium pump activity in poorly controlled type I diabetes in the study of Schaefer *et al.* (1987) was partly explained by non-enzymatic glycosylation of the regulator protein calmodulin. Using exogenous calmodulin in the last section of this study (section 8.4) calcium pump activity was shown to be reduced in the two diabetic groups which, despite differences in serum lipids, had

similar degrees of glycaemic control. It was argued that the reduction in enzyme activity in the diabetic controls was due to a direct effect of diabetes on the physical properties of the calcium pump. From the published studies this has been suggested to be a result of non-enzymatic glycosylation of the Ca^{2+}-Mg^{2+}-ATPase enzyme (Schaefer *et al.* 1987, Gonzalez Flecha *et al.* 1990, 1993).

8.5.2. Aims

The present study was initiated to investigate whether there were any differences in glycosylation of the calcium pump protein in the four study groups.

8.5.3. Materials and methods

8.5.3.1. Principles

The Western blotting techniques described in section 5.3.9 were employed. Glycosylation of the calcium pump should increase its molecular weight and hence reduce its electrophoretic mobility in an electric current.

8.5.3.2. Materials

(1) Sources of monoclonal mouse anti-Ca^{2+}-Mg^{2+}-ATPase antibody (R/4E3), mouse control antibody, other antibodies and the ECL reagents are given in section 5.3.9.

(2) A 20-track 4-12% SDS-polyacrylamide gel and Western blotting buffers were prepared as in section 5.3.9.

8.5.3.3. Methods

The stored surplus membranes (2 normal controls, 3 diabetic controls, 2 primary combined hyperlipidaemia and 2 hyperlipidaemic diabetic membranes) from section 8.4.1 were thawed on ice. Fifty micrograms (50 μg) of membrane protein were electrophoresed and blotted as described previously in the methods section 5.3.9 with

modifications after the nitrocellulose blotting stage as described in the legend to figure 8.2. The mobilities of the calcium pump proteins (M_r 138-140 kDa) in the four groups were compared.

8.5.4. Results

The Western blots are shown in Figure 8.2 (ECL film). There were no discernable differences in the mobility of the proteins in the region of the calcium pump (M_r 130-150 kDa) between the four groups. Two further bands were noted at 33 and 185 kDa using the R/4E3 antibody, while those at 38-63 kDa appeared in both the track probed with the test antibody and that probed with the control antibody.

8.5.5. Discussion

8.5.5.1. Assessment of glycosylation of the calcium pump

Having demonstrated differences in calcium pump activity between the four study groups and speculated that reduction in calcium pump activity in diabetes was due to alterations, most likely non-enzymatic glycosylation, in the enzyme itself it was decided to investigate this possibility using Western blotting techniques. Western blotting was chosen because it was easy to perform in the limited time available, there was expertise in the department and access to the relevant antibodies. Because there were no discernable differences in the mobility of the calcium pump (130-150 kDa band) between the groups, most likely due to insufficient sensitivity of the method to detect small changes in the carbohydrate content of the membrane proteins, studies to resolve questions of specificity of the anti-Ca^{2+}-Mg^{2+}-ATPase antibody used arising due to the recognition of bands at 33 and 185 kDa (such as pre-incubating the antibody with the Ca^{2+}-Mg^{2+}-ATPase enzyme) were felt to be inappropriate (see section 8.5.5.2). The R/4E3 anti- Ca^{2+}-Mg^{2+}-ATPase antibody, a gift from Dr East of the University of Southampton, was raised against purified human red cell calcium pump. There are no published studies on its specificity but it

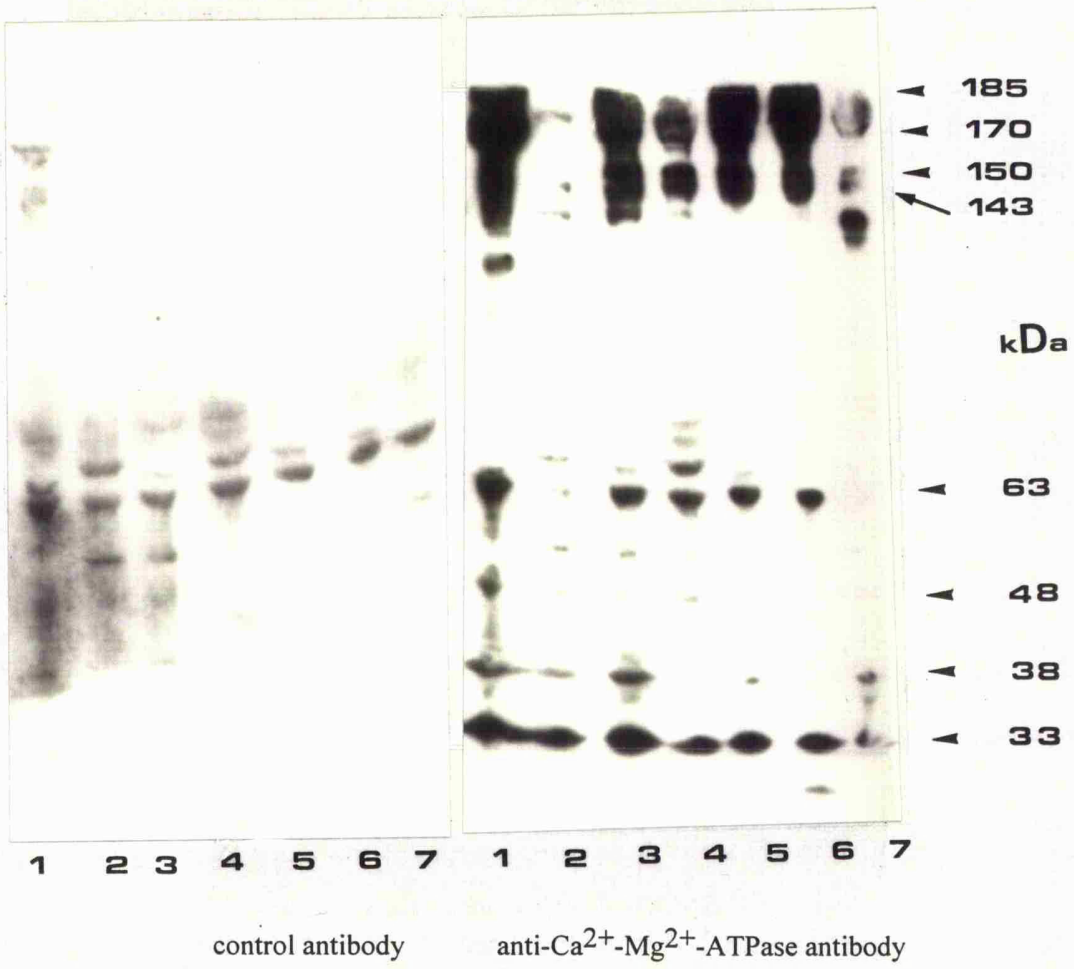

Figure 8.2. Effect of glycosylation on calcium pump mobility (representative sample from 9 membranes studied). There were no differences in the electrophoretic mobility of the Ca^{2+}-Mg^{2+}-ATPase enzyme (M_r 130-150) in the four groups. The lower molecular weight bands (38-63 kDa) are a result of non-specific staining as seen with the control antibody. *Tracks*: ***1*** - diabetic control, HbA_1 10.3%; ***2*** - hyperlipidaemic diabetic, HbA_1 10.3%; ***3*** - primary combined hyperlipidaemia, HbA_1 7.4%; ***4*** - diabetic control, HbA_1 7.5%; ***5*** - normal control; ***6*** - hyperlipidaemic diabetic, HbA_1 8.0%; ***7*** - primary combined hyperlipidaemia, HbA_1 6.4%. After nitrocellulose blotting the proteins were exposed to either the control or the study (R/4E3) antibody in a seperate container, but subsequent treatment of the membranes was identical.

was shown to recognize purified Ca^{2+}-Mg^{2+}-ATPase protein in ELISA and its specificity was confirmed by the demonstration of specific recognition of a 140 kDa band in Western blots of purified Ca^{2+}-Mg^{2+}-ATPase and of red cell ghost membrane proteins. This antibody has also been employed in Dr East's laboratory to identify novel clones encoding Ca^{2+}-Mg^{2+}-ATPase sequences (M. East, personal communication).

A 4-12% gradient gel was used for these initial experiments to determine which bands could be recognised by the test antibody because of its ability to resolve bands in the 20-200 kDa range. Had there been differences in enzyme mobility in the gels a 5-6% acrylamide gel would then have been used such that the relevant bands would have migrated towards the centre of the gel, although the bands could have become less distinct in the lower percentage gel.

8.5.5.2. Interpretation of results

Multiple bands were demonstrated on the gels. The 38-63 kDa bands also appeared in the control tracks probed with non-immune serum and were, therefore, due to non-specific adsorption of antibodies. The 33kDa band most likely represented a proteolytic fragment of a higher molecular weight protein whose epitope is recognised by the monoclonal antibody. Protease inhibitors have been shown to reduce calcium pump activity (Madden *et al.* 1981). Because the same membrane samples analyzed by Western blotting were used for enzyme activity experiments no protease inhibitors were included in the preparation and storage of these membrane suspensions. Storage could have led to protein degradation causing loss of signal as seen in lanes 2 and 7 in the present Western blots.

The nature of the 185 kDa band was not resolved by this study, but it could be a result of non-covalent association of proteins (multiple protein aggregates) due to residual secondary structure of the proteins in SDS. Since initial experiments did not allow resolution of higher molecular weight glycosylated species of the Ca^{2+}-Mg^{2+}-ATPase (130-150 kDa) protein in samples from diabetic patients it was

decided not to pursue Western blot analysis. For this reason the identity of the 33 kDa and 185 kDa immunoreactive species was not studied further.

Another possible way of identifying the calcium pump protein band would be to expose the nitrocellulose paper, after the proteins have been blotted onto it, to [γ-^{32}P]ATP in the absence of magnesium. Because the phosphorylated enzyme intermediate requires Mg^{2+} and ATP to dephosphorylate, the phosphorylated enzyme intermediate can be identified when the labelled nitrocellulose paper is exposed to an Xray film. However, this would require reactivation of at least a proportion of the Ca^{2+}-Mg^{2+}-ATPase protein on the nitrocellulose paper.

The problems encountered herein could be solved in future studies by employing isoelectric focusing before blotting the membrane proteins, although the resolution may still be a problem if there is a spectrum of glycosylation products. Colorimetric methods (Kennedy *et al.* 1980, Murtiashaw *et al.* 1983, Pecoraro *et al.* 1979) may be more sensitive for assessing the extent of glycosylation of the calcium pump protein. Other methods that have been employed for this purpose include radioimmunoassay (Javid *et al.* 1978) and chromatography and electrophoresis (Spicer *et al.* 1978).

8.6. SUMMARY

(1) Basal calcium pump activity is reduced to a similar level in normolipidaemic type II diabetes, primary combined hyperlipidaemia and in combined hyperlipidaemia secondary to type II diabetes. Calmodulin-stimulated calcium pump activity is reduced in normolipidaemic diabetic control subjects only.

(2) Calmodulin overcomes the inhibitory effects of membrane environment.

(3) Core membrane fluidity was increased in the groups with combined hyperlipidaemia, with a negative correlation between serum triglycerides and DPH anisotropy.

(4) Reduced calcium pump activity in combined hyperlipidaemia appeared to be secondary to the effect(s) of serum triglycerides. The mechanism in diabetes does not appear to involve altered membrane dynamics but could involve non-enzymatic glycosylation of the calcium pump protein.

(5). Higher glycosylation states could not be defined with the Western blotting methods used.

Chapter 9

GENERAL DISCUSSION

9.1. SUMMARY OF MAJOR FINDINGS

The findings in chapters 7 and 8 suggest that serum triglyceride levels affect the physical properties of the red cell membrane. Despite differences in serum cholesterol levels, membrane cholesterol levels were similar in the two groups studied at baseline in chaper 7, and in all the four groups in chapter 8. Membrane fluidities were also similar, except in the groups with combined hyperlipidaemia where increased membrane fluidity appeared to be related to hypertriglyceridaemia. Indeed where correlations existed, there was a consistent inverse relationship between serum triglyceride levels and the measured DPH anisotropy. Although there was no direct relationship between serum and membrane cholesterol levels a reduction in serum cholesterol, with an associated increase in serum triglycerides, was accompanied by an increase in membrane fluidity. Diabetes *per se* had no effect on membrane fluidity.

Calcium pump activity was reduced in diabetes and in primary combined hyperlipidaemia, an effect which was overcome by calmodulin except in the diabetic control group only.

9.1.1. Membrane cholesterol content

It has always been assumed, according to the membrane hypothesis derived essentially from *in vitro* studies, that serum and membrane cholesterol levels are in dynamic equilibrium *in vivo*. The present results, which are in agreement with those of Neerhout (1968), indicate that this view is simplistic: the red cell is more dynamic and is able to maintain its membrane cholesterol levels within narrow limits, a form of homeoviscous adaptation, perhaps in an attempt to maintain constant membrane fluidity. Thus, despite different serum cholesterol levels in the groups studied in chapters 7 and 8, membrane cholesterol levels were similar. In particular, in the group with isolated hypercholesterolaemia, membrane fluidity was also similar to that in the control group. In 6 of the 8 groups studied, there was no correlation

between serum and membrane cholesterol levels. According to the membrane hypothesis (see preface), a positive correlation between serum and membrane cholesterol levels (as in the normal control and primary combined hyperlipidaemic groups in chapter 8) was expected. It is possible that there is a membrane cholesterol level above which no further serum cholesterol can incorporate into the membrane bilayer leading to the apparent lack of correlation between serum and membrane cholesterol levels. This is analogous to the relationship between membrane microviscosity and the cholesterol/phospholipid mole ratio discussed in section 3.3.1. In all the 8 groups studied there was no correlation between membrane cholesterol and TMA-DPH fluorescence anisotropy. TMA-DPH anisotropy is inversely related to the fluidity of the outer regions of the membrane bilayer where membrane cholesterol is located. There was a discrepancy between the *in vivo* findings of this study and what was predicted from previous *in vitro* studies. Hence, a conclusion of this study is that caution should be exercised in extrapolating *in vitro* findings to *in vivo* states.

Interestingly, although there were no differences in baseline membrane cholesterol levels between the two groups in chapter 7, when serum cholesterol was reduced by 13% there was a 12% reduction in membrane cholesterol content with an associated 9% rise in serum triglycerides. Homeoadaptive processes that maintain membrane fluidity within a narrow range were proposed in chapter 7. It is possible that these mechanisms have noticeable effects on membrane fluidity after a longer period of time than the 3 months in the present study.

9.1.2. Emerging role of triglycerides

There were consistent negative correlations between serum triglycerides and DPH fluorescence anisotropy. Notably DPH anisotropy was decreased in the groups with combined hyperlipidaemia. This suggests increased hydrocarbon core fluidity in the membrane bilayer associated with serum triglycerides. The indirect evidence from the present studies and from published studies in primary hypercholesterolaemia

(Bharaj *et al.* 1993, 1994; Dowd *et al.* 1993b; Muller *et al.* 1990) suggests that serum triglycerides may be more important than serum cholesterol in the modification of membrane fluidity. In addition, calcium pump activity was reduced in the groups with hypertriglyceridaemia and increased membrane fluidity (chapter 8) but not in the groups with isolated hypercholesterolaemia. This suggests that serum triglycerides may also be important in regulating membrane function (see section 9.1.3.)

Further support for the effect of serum triglycerides on membrane function comes from studies on other membrane transport processes. There is a consistent positive correlation between sodium-lithium countertransport (SLC) activity and serum triglycerides (Adragna *et al.* 1985, Brent *et al.* 1989, Carr *et al.* 1990, Corrocher *et al.* 1985, Dowd *et al.* 1993b, Hespel *et al.* 1988, Hunt *et al.* 1986), which may be mediated through an increased membrane fluidity (Dowd *et al.* 1993b) secondary to hypertriglyceridaemia (Brent *et al.* 1989, Carr *et al.* 1991). There is also a positive correlation between serum triglycerides and Na^+-K^+ cotransport (Hajem *et al.* 1990, Hespel *et al.* 1988), passive Na^+ permeability (Hajem *et al.* 1990), passive lithium (Li^+) permeability and Li^+-K^+ cotransport (Hunt *et al.* 1986) which, again, may be mediated through increased membrane fluidity (Wiley and Cooper 1975).

In a wider clinical context there is growing evidence that triglycerides are aetiologically important in coronary heart disease (CHD) (Assman and Brewer 1991; Assman and Schulte 1992; Austin *et al.* 1991; Castelli 1986, 1992; Gotto 1992; Gotto *et al.* 1991; Packard 1994; Sempos *et al.* 1993; Simons 1992), including diabetic CHD (Fontbonne *et al.* 1989, Janka 1985, Laakso *et al.* 1988, Merrin *et al.* 1992, West *et al.* 1983).

9.1.3. Relationship between membrane structure and function

The present studies have shown that serum lipids affect membrane structure and function. Membrane structure was represented by membrane cholesterol and membrane fluidity measured by the two probes DPH, which labels the membrane

hydrocarbon core, and TMA-DPH, the cationic derivative of DPH which labels the outer regions of the membrane i.e. the lipid-water interface. (Fluidity is inversely related to anisotropy). Calcium pump (Ca^{2+}-Mg^{2+}-ATPase enzyme) activity was used as a measure of membrane function for two reasons: *(1)* there was local expertise in the assessment of the function of this transmembrane protein, and *(2)* previous experiments in the department had shown its importance in hypertension, suggesting a possible role for this enzyme in vascular disease.

Membrane structure, as defined herein, in hypercholesterolaemia was not different from controls (chapter 7). In combined hyperlipidaemia (chapter 8) membrane fluidity was increased and there was an associated decrease in calcium pump activity i.e. membrane structure and function were altered. Bharaj *et al.* found no difference in membrane fluidity (1993, 1994) and calcium pump activity (1993) in familial hypercholesterolaemia and normolipidaemic controls using both platelet and red cell membranes. Together with the present findings this suggests that impairment of calcium pump activity was secondary to the effect of serum triglycerides and not related to serum cholesterol levels. The negative correlation between DPH anisotropy and basal calcium pump activity in the diabetic controls in chapter 8 would suggest that increased membrane fluidity increases calcium pump activity, but the 2 groups with hypertriglyceridaemia had lower basal enzyme activity. This implies, as discussed in chapter 8, that there is an optimal fluidity above which calcium pump activity declines. The present study (chapter 8) is the first in the literature to suggest a modulating effect of serum triglycerides on calcium pump activity.

9.1.4. Diabetes and membrane function

The relationship between non-insulin treated type II diabetes mellitus, membrane environment and membrane function has been partially elucidated. As in the studies of Caimi *et al.* (1990) and Tong *et al.* (1993), membrane fluidity was no different to controls in the present study (chapter 8), although the reported associations in the

literature have been contradictory (reviewed by Watala 1993). Despite similar membrane fluidities between normal and diabetic control subjects both basal and calmodulin-stimulated calcium pump activities were reduced in the diabetic controls. These results are in agreement with those of Zemel *et al.* (1987) in hypertensive type II diabetic subjects although membrane environment was not assessed in that study. This suggests a direct effect of diabetes on the calcium pump protein rather than a response to altered membrane environment in this condition. From the published literature glycosylation of the enzyme was felt to be the most likely explanation. Attempts to detect differences in glycosylation of the enzyme using Western blotting techniques were unsuccessful for the reasons discussed in chapter 8. The mechanism of inhibition of the calcium pump in non-insulin treated type II diabetes, therefore, remains unexplained but is likely to be due to non-enzymatic glycosylation.

It is arguable whether the present results in type II diabetes can be extrapolated to type I diabetes, but a review of the literature suggests that the same controversies in type II diabetes rage in type I diabetes regarding membrane fluidity (reviewed by Watala 1993) and that calcium pump activity is also reduced in the latter (Schaefer *et al.* 1987).

It is possible, but unproven, that impaired membrane function can lead to cardiovascular disease. There is an increased cardiovascular risk in diabetes mellitus (Gibbs 1993, Jensen *et al.* 1987, Krolewski *et al.* 1987, Laws *et al.* 1993, Manson *et al.* 1991, Sosenko *et al.* 1993, Uusitupa *et al.* 1993). Reaven (1988) described 'syndrome X', characterized by hypertriglyceridaemia, low HDL, hypertension, central obesity and insulin resistance, which, together with increased LDL and smoking, accounts for the increase in cardiovascular risk. Hypertriglyceridaemia (Asplund-Carlson *et al.* 1993; Hamsten *et al.* 1985, 1987; Mehta *et al.* 1987) and hyperinsulinaemia (Juhan *et al.* 1991) are associated with raised plasma plasminogen activator inhibitor-1 (PAI-1) activity, combinations which have been described in survivors of myocardial infarction (Hamsten *et al.* 1985). The diabetic groups in the present study (chapter 8) were obese and, due to NIDDM, insulin resistant (although

this was not formally assessed), but their blood pressure and HDL profiles were similar to those of the other 2 groups. Because the present study was not designed to assess cardiovascular risk factors in general no further information regarding 'syndrome X' can be extrapolated from the data.

9.2. UNEXPLAINED RESULTS

There were also a number of unexpected results in the present studies. Thus, the positive correlation between serum triglycerides and TMA-DPH anisotropy in the normolipidaemic group in chapter 7 and, in chapter 8, the negative correlation between HbA_1 and TMA-DPH anisotropy in the hyperlipidaemic diabetic subjects, the negative correlation between membrane cholesterol and DPH anisotropy but no correlation between membrane cholesterol and TMA-DPH anisotropy, and the negative correlation between basal calcium pump activity and DPH anisotropy in the diabetic controls only, are difficult to explain. On the other hand, the lack of correlations between parameters does not mean that a relationship does not exist. The number of subjects in each group is an important factor that could have influenced the correlations. Furthermore, in the present work non-parametric statistical tests were employed. These tests, which are applied to non-normally distributed data, are less powerful than their parametric equivalents. The non-parametric tests were employed due to the distribution of the data and also to avoid making too many assumptions such that inappropriate conclusions were drawn.

9.3. LIPIDS, CALCIUM AND ATHEROSCLEROSIS:- POSSIBLE MECHANISMS

At a cellular level, impaired calcium pump activity as described in chapter 8 could lead to a sustained increase in intracellular calcium, endothelial dysfunction and eventually to cell death (Weistein and Heider 1987). In addition, LDL atherogenicity

is increased in hyperlipidaemia (Kugiyawa *et al.* 1990, Mangin *et al.* 1993, Ross 1993, Steinberg *et al.* 1989, Yanagisawa *et al.* 1988) and in diabetes (Stiko-Ram *et al.* 1990). The resulting pro-proliferative and pro-thrombotic effects (Nakaki *et al.* 1989, Takuwa *et al.* 1989) of hyperlipidaemia and/or diabetes could lead to the initiation and, in the presence of abnormal rheology, to the progression of atheroma.

The progression from endothelial dysfunction to the formation of a calcified lipid-laden fibrous plaque occurs through various calcium-regulated processes (Weinstein and Heider 1987), hence calcium could play an important role in the pathogenesis of atheroma. This is supported by evidence from animal studies that calcium antagonists retard atherogenesis (Henry 1985, Weinstein and Heider 1987). Raised intracellular calcium activates cytoplasmic transglutaminase which catalyses the irreversible cross-linking of membrane proteins including band 3 and spectrin, leading to abnormal morphology and reduced deformability of the red cell (Rice-Evans and Chapman 1981). In addition to altered red cell rheology (Isogai *et al.* 1981, MacRury *et al.* 1993, Schmid-Schonbein and Volger 1976, Weed *et al.* 1969), platelet reactivity (Shattil *et al.* 1975, Stuart *et al.* 1980) and endothelial contraction are enhanced. Therefore, the reduction in calcium pump activity in diabetes and in hyperlipidaemia described in chapter 8 may raise intracellular calcium concentration and cause rheological abnormalities that, in addition to initiating atheroma, can exacerbate pre-existing atheroma.

9.4. SUMMARY

What emerges from chapters 7 and 8 is that, because membranes can maintain their cholesterol content within narrow limits, serum triglycerides may be more important than serum cholesterol in the modification of both membrane fluidity and function. This is supported by studies in sodium-lithium countertransport (SLC) and other membrane transport systems. The mechanism of impaired membrane function in diabetes remains unexplained.

The impaired calcium pump activity secondary to diabetes mellitus and/or hyperlipidaemia described in chapter 8 may contribute to endothelial dysfunction and to abnormal rheology, processes which are central to atherogenesis.

Chapter 10

CONCLUSIONS AND PROPOSED FURTHER STUDIES

10.1. CONCLUSIONS

10.1.1. Membrane fluidity

(1) Despite the differences in serum cholesterol levels, membrane cholesterol levels were similar in the various groups suggesting that the red cell membrane maintains its cholesterol levels within a narrow range. However, a reduction in serum cholesterol at 3 months was accompanied by a reduction in membrane cholesterol content and, with a concomitant rise in serum triglycerides, an increased membrane fluidity. This confirms the hypothesis that serum lipids affect the physical properties of the red cell plasma membrane.

(2) Membrane fluidity was similar between the primary hypercholesterolaemic and normolipidaemic groups. In the groups with combined hyperlipidaemia the fluidity of the hydrocarbon core of the membrane was increased. There was no consistent correlation between membrane cholesterol and fluorescence anisotropy but a consistent negative correlation existed between serum triglycerides and DPH anisotropy. This suggests that serum triglycerides affect membrane fluidity more than serum cholesterol.

(3) Both DPH and TMA-DPH anisotropies were similar in the normal and the normolipidaemic diabetic control groups suggesting that type II diabetes *per se* does not affect membrane fluidity.

10.1.2. Calcium pump activity

(1) Both type II diabetes and combined hyperlipidaemia reduce basal calcium pump activity to the same extent. Combined hyperlipidaemia appears to reduce calcium pump activity through an altered membrane environment secondary to the

effects of hypertriglyceridaemia. The mechanism in type II diabetes remains unresolved but is likely to involve a combination of non-enzymatic glycosylation of the calcium pump protein and altered membrane environment secondary to lipid abnormalities, if present.

(2) Calmodulin overcomes the inhibitory effects of membrane environment on calcium pump activity.

10.2. SUGGESTED FURTHER STUDIES

(1) The present findings suggest that the red cell membrane maintains its cholesterol content within narrow limits by adaptive mechanisms. However, a reduction in serum cholesterol at 3 months resulted in a fall in membrane cholesterol content and increased membrane fluidity. If the theory of membrane homeoviscous adaptation is correct, membrane cholesterol and membrane fluidity measured at, say, 12 months on treatment should have returned to baseline values.

(2) The reduction in basal calcium pump activity in combined hyperlipidaemia appeared to be through an altered membrane environment secondary to the effect of hypertriglyceridaemia. Measuring calcium pump activity before and after the normalization of serum triglycerides can confirm this triglyceride effect. Similarly, in the diabetic subjects, measuring calcium pump activity and the degree of glycosylation of the calcium pump protein using colorimetric methods before and after improved glycaemic control would assess the effect of hyperglycaemia on enzyme activity.

(3) A definitive assessment of the roles of membrane environment and enzyme glycosylation can be afforded by detergent solubilization of the enzyme and reconstitution into phospholipid liposomes. The inhibitory effects of membrane

environment should be absent in the solubilized enzyme whereas the effects of enzyme glycosylation should persist.

(4) To define any higher glycosylation states of the calcium pump protein it would be necessary to deglycosylate the purified enzyme and analyse its carbohydrate composition.

(5) Finally, the kinetics of enzyme inhibition (reduced calcium affinity and/or reduced maximum velocity) needs to be determined.

Chapter 11

APPENDICES

11.1. STANDARD CURVE FOR THE DETERMINATION OF MEMBRANE PROTEIN CONCENTRATION

tube number	1	2	3	4	5	
concentration	0	50	100	150	200	μg BSA
	0	0.1	0.2	0.3	0.4	ml BSA standard
	0.5	0.4	0.3	0.2	0.1	ml deionised water

11.2. CALCULATION OF CALCIUM PUMP ACTIVITY

The released radioactivity (disintegrations per min, dpm) is corrected for spontaneous ATP hydrolysis by subtracting the value of the reagent blank.

Let radioactivity in 5 μl labelled ATP = T dpm,

Giving an activity of $T/5$ dpm/μl , or $T \times 10^6/5$ dpm/l

Stock solution has an ATP concentration of 30 mmol/l.

Therefore, 30 mmol ATP have an activity of $T \times 10^6/5$ dpm,

or $(T \times 10^6)/(30 \times 5)$ dpm/mmol,

or $(T \times 10^3)/(30 \times 5)$ dpm/μmol

If 0.3 ml have Y dpm, the number of μmol of ATP hydrolysed in time t hours is

$$\frac{Y \times 30 \times 5}{T \times 10^3}$$

But the reaction mixture has a volume of 1 ml, hence

Number of μmol ATP hydrolysed in time t hours is

$$\frac{Y \times 30 \times 5}{T \times 10^3 \times 0.3}$$

If the membrane protein concentration is p mg/ml, the

Protein content in 10 μl of sample is 10 x p mg/1,000.

Therefore the amount of ATP hydrolysed in 1 ml of reaction mixture is

$$\frac{Y \times 30 \times 5\ \mu\text{mol ATP}}{T \times 10^3 \times 0.3} \times \frac{1}{t\ \text{hrs}} \times \frac{1{,}000}{10 \times p\ \text{mg}}$$

If the efficiency of the extraction is E%,

At 100% efficiency the amount of ATP hydrolysed is,

$$\frac{Y \times 30 \times 5 \times 1{,}000 \times 100}{T \times 10^3 \times 0.3 \times t \times 10 \times p \times E}\ \mu\text{mol ATP hydrolysed/mg/h}$$

or $$\frac{Y \times 5{,}000}{T \times t \times p \times E}\ \mu\text{mol ATP hydrolysed/mg/h} \quad \text{.....(11.1)}$$

where T = radioactivity (dpm) in 5 μl of labelled ATP

Y = radioactivity (dpm) in 0.3 ml aliquot

t = time of reaction in hours

p = membrane protein concentration in mg/ml

E = efficiency of the extraction procedure (%), given by

$$\frac{H}{T \times 0.3} \times 100\% \quad \text{.....(11.2)}$$

where H = radioactivity (dpm) in 0.3 ml aliquot from hydrolysis tube.

Calcium pump activity becomes

$$\frac{Y \times 5{,}000 \times T \times 0.3}{T \times t \times p \times H \times 100} \quad \mu\text{mol ATP hydrolysed/mg/h}$$

or

$$\frac{15 \times Y}{H \times p \times t} \quad \mu\text{mol ATP hydrolysed/mg/h.....(11.3).}$$

11.3. WESTERN BLOTTING TECHNIQUES

11.3.1. Western blotting reagents

(1) 100 ml solution 30A:- 30% acrylamide and 0.8% bisacrylamide

(2) 100 ml solution 45A:- 45% acrylamide and 1.2% bisacrylamide

(3) 1 l solution B x 1:- 25 mM Tris-HCl (3.03g trizma base), 192 mM glycine and 0.1% sodium dodecylsulphate (SDS), pH 8.3

(4) 3 l blotting buffer:- 43.2 g glycine (192 mM) and 9.08 g trizma base (25 mM), pH 8.7, to 2.7 l with deionised water. The solution was degased on ice and 300 ml of methanol (1%, v/v) added to a final volume of 3 l.

(5) 1 l solution C:- 250 mM Tris-HCl, pH 6.8

(6) 1 l solution D:- 750 mM Tris-HCl, pH 8.8

(7) Solution E:- 1% sodium dodecylsulphate (SDS)

(8) 10 ml solution F:- 1% ammonium persulphate, prepared just before use.

(9) 100 ml solution H x 3:- 187 mM Tris-HCl, 6 % SDS, 657 mM sucrose, pH 6.8, and a minute quantity of bromophenol blue dye

(10) 1 l block buffer (5% milk solution (Marvel™)) - 10 ml of 1M Tris (10 mM final concentration), 0.9% NaCl, 0.05% Tween 20, 50 g dried milk, pH 7.5

(11) 500 ml overlay buffer:- 5 ml of 1M Tris (final concentration 10 mM), 0.9% NaCl, 0.05% Tween 20, pH 7.5

(12) 1 l sarcosyl buffer:- 10 ml of 1M Tris (10 mM final concentration), 1 M NaCl, 0.25% sarcosine, 0.25% nonidet p40, pH 7.5

(13) Temed:- N,N,N'N'-tetramethylethylene diamine

(14) 1 l Coomassie staining medium:- 250 ml isopropanol, 100 ml acetic acid, 650 ml deionised water, 140 mg Coomassie G250 and 140 mg Coomassie R250

(15) 10 ml ECL detection reagents 1 and 2, (1:1 ratio)

(16) Antibodies:- monoclonal mouse anti-Ca^{2+}-Mg^{2+}-ATPase antibody (R/4E3, gift from Dr M. East, Southampton University), biotinylated rabbit anti-mouse antibody (Dako, High Wycombe, England), streptavidin-biotinylated horseradish peroxidase (HRP, Amersham, England); control antibody was anti-[α_2-subunit of voltage-sensitive skeletal muscle calcium channel] antibody

(17) Amidoblack stain:- 0.1% amidoblack (w/v), and methanol, acetic acid and deionised water in the ratio 45:10:45

(18) Amidoblack destaining solution:- 90% methanol, 2% acetic acid and 8% deionised water (v/v)

(19) Gel destaining solution:- 10% acetic acid.

11.3.2. Preparation of 4-12% gradient gel and 4% stacking gel

	4 % gel	12 % gel	4 % stacking gel
solution C	-	-	5 ml
solution D	7.5 ml	7.5 ml	-
solution 30A	2.0 ml	-	1.33 ml
solution 45A	-	4.0 ml	-
solution E	1.5 ml	1.5 ml	1.0 ml
solution F	0.75 ml	0.75 ml	0.5 ml
deionised water	3.3 ml	1.26 ml	2.17 ml
temed	10 µl	10 µl	50 µl

11.3.3. High and low molecular weight standards (Sigmamarkers, lyophilized)

Protein	*molecular weight (kDa)*
carbonic anhydrase, bovine erythrocytes	29
glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	36
ovalbumin, chicken egg	45
albumin, bovine serum	66
phosphorylase b, rabbit muscle	97
galactosidase, E. coli	116
myosin, rabbit muscle	205

11.3.4. Blotting of proteins onto nitrocellulose paper

The stacking gel was removed and the proteins transferred from the main gel onto nitrocellulose paper in a Western blot sandwich made up, from positive to negative, of plastic plate, scotch pad, 3 mm Whatman filter paper, nitrocellulose paper (taking care to align the standard track with that on the gel), gel, 3 mm Whatman filter paper, scotch pad and plastic plate (Figure 11.1).

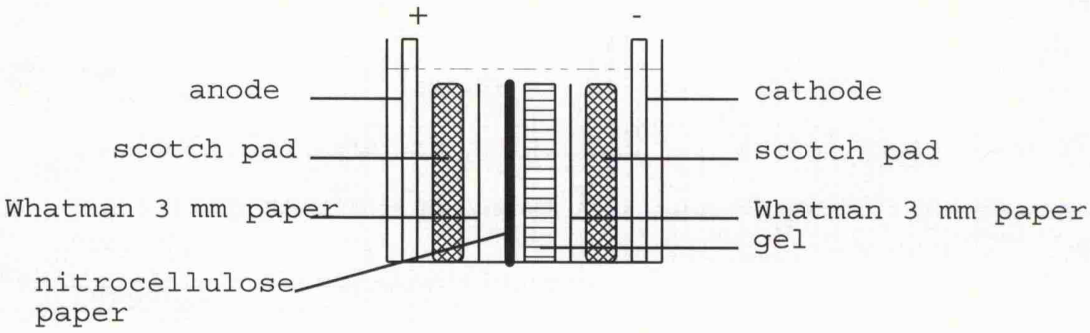

Figure 11.1. Western blot sandwich.

This sandwich was put into a blotting tank cooled externally with ice and internally with a cycled water cooling system, and the proteins were blotted onto nitrocellulose paper at 80 volts for 2.5 hours.

The apparatus was disassembled carefully and the nitrocellulose paper separated from the gel. Protein transfer was checked by staining the gel at room temperature with Coomassie blue for one hour and destaining in 10% acetic acid over 24 hours to remove non-specific stains. The 1.5 cm x 15 cm standard track was stained with amidoblack for 2 mins and destained for 5 mins.

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