VESICULATION AND RAFTS IN
STOMATOCYTIC RED CELLS

by

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ABSTRACT

This thesis describes work on ATP-dependent endocytic vesiculation and sphingomyelin and cholesterol-rich lipid rafts in the membrane of normal red cells and those with 'hereditary stomatocytosis' (HSt) syndromes, a set of rare congenital human haemolytic anaemias which show a 'leak' to the univalent cations Na⁺ and K⁺ and in some cases the deficiency of a 32 kDa raft protein, 'stomatin'. The work explores the idea that the pathophysiology of these diseases may be secondary to an abnormality in cellular processes of vesiculation based on membrane rafts.

It was found that an in vitro process of ATP-dependent vesiculation, which occurs in normal red cell membranes, was completely absent in two pedigrees with overhydrated HSt (OHSt), the most severe version of these diseases. Both this defect and the cation leak were corrected by the cross-linking reagent, dimethyl adipimidate. In eleven other HSt pedigrees with different variants of the stomatocytosis syndromes ATP-dependent endocytic vesiculation was present.

It was determined that lipid rafts existed in the normal erythrocyte membrane and that stomatin was partially associated with the rafts. Rafts were found to exist in the OHSt membrane in spite of the lack of stomatin. The degree
of raft association of various membrane proteins, was investigated and the proportion of raft associated actin was found to be significantly reduced in OHSt cells.

It was found that in normal red cell membranes, the process of ATP-dependent vesiculation could be inhibited by incubating erythrocyte ghosts with an antibody to stomatin. The raft disrupters nystatin, filipin, digitonin, saponin and methyl-β-cyclodextrin were also found to inhibit this process. Further work exploring the mechanism of ATP-dependent endocytosis was performed.

This work is consistent with the suggestion that the basic defect in the leaky OHSt cell may lie in a raft-based process of endocytosis, which may involve the stomatin protein.
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ABBREVIATION LIST

A = Absorbance

Å = Angstrom (10nm)

AChE = Acetylcholinesterase (E.C. 3.1.1.7)

ADP = Adenosine diphosphate

APS = Ammonium persulphate

ATP = Adenosine 5'-triphosphate

AT\(^{32}\)P = Radiolabelled ATP

ATP\(_\gamma\)S = ATP gamma S

Aqu-1 = Aquaporin-1

BSA = Bovine serum albumin

CA = Carbonic anhydrase

CaATP = Ca\(^{2+}\) complexed to ATP

cGMP = Cyclic guanosine monophosphate

\(^{14}\)CDMA = Radiolabelled DMA

CaMgATP = Ca\(^{2+}\), Mg\(^{2+}\) and ATP complex

CHC = Cryohydrocytosis

CPDA = citrate phosphate dextrose adenine
dH₂O = Distilled water

DFP = Diisopropyl fluoro-phosphate

DHSt = Dehydrated hereditary stomatocytosis

DIG = Detergent insoluble glycolipid enriched complex

DMA = Dimethyl adipimidate

DMF = Dimethyl formamide

DMP = Dimethyl pimelimidate

DMSO = Dimethyl sulphoxide

DTNB = 5,5'-Dithio-bis(2-Nitrobenzoic acid)

DTT = Dithiothreitol

EC₅₀ = concentration causing half maximal effect

ECL = Enhanced chemiluminescence

EDTA = Ethylenediamine tetra-acetic acid

EM = Electron microscopy

ENaC = Amiloride sensitive sodium channel

EV = Endocytic vesiculation

Fab = Fragment antigen binding

Fc = Fragment crystallisable

FP1 = Familial pseudohyperkalaemia type 1 (Edinburgh)
FP2 = Familial pseudohyperkalaemia type 2 (Chiswick)

GLUT-1 = Glucose transporter 1

GPA = Glycophorin A

GPA = Glycophorin C

GPI = Glycosylphosphatidylinositol

GSL = Glycosphingolipid

GST = Glutathione S-transferase

Hb = Haemoglobin

HbA = Normal haemoglobin

HbS = Haemoglobin S in sickle cells

HBSS = Hanks' balanced salt solution

HflKC = Complex of the 2 bacterial proteins HflK and HflC

HMW = High molecular weight

HPCHA = High phosphatidylcholine haemolytic anaemia (same as DHSt)

HRP = Horseradish peroxidase

HSt = Hereditary stomatocytosis

IC\textsubscript{50} = Concentration causing half maximal inhibition

IEF = Isoelectric focusing

IgG = Immunoglobulin G
IP$_3$ = Inositol triphosphate

ITP = Inosine 5'-triphosphate

kDa = kilo Daltons

$k_i$ = Inhibition constant

$L_0$ = Liquid ordered/ membranes in crystalline state

$L_c$ = Liquid disordered/ fluid phase membranes

LMW = low molecular weight

MALDI-TOF = Matrix assisted laser desorption/ ionisation -Time of flight

MBCD = Methyl-beta-cyclodextrin

MBS = MES-buffered saline

MCHC = Mean cell haemoglobin concentration

MCV = Mean cell volume

MDCK = Mardin-Darby canine kidney

MES = 4-morpholine ethanesulphonic acid

MgATP = Magnesium ATP complex

MHCII = Major histocompatibility type II antigens

mks = Markers

MOPS = 3-[N-Morpholino]propanesulphonic acid

MRF alpha = Membrane folate receptor alpha
NEM = N-ethylmaleimide

NMDG = N-methyl-D-Glucamine

obr = ouabain+bumetanide-resistant

OD = optical density

OHSt = Overhydrated hereditary stomatocytosis

P₁ = Inorganic phosphate

PBS = Phosphate-buffered saline

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

PKC = Protein kinase C

PLAP = Placental alkaline phosphatase

ppm = parts per million

PS = Phosphatidylserine

PVDF = Immobilon P poly (vinylidene difluoride)

RT = Room temperature

SCP2 = Sterol carrier protein 2

SDS = Sodium dodecyl sulphate

SDS-PAGE = SDS-polyacrylamide gel electrophoresis

SLP-2 = Stomatin-like protein-2
SS = Homozygous sickle cell anaemia

TBS = Tris-buffered saline

TEMED = N,N,N',N'-tetramethylethylenediamine

TLCK = N-α-p-tosyl-L-leucine chloromethyl ketone

v/v = Volume/ volume

w/v = Weight/ volume
ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

The work to be described in this thesis examines the erythrocyte membrane in a group of dominantly inherited haemolytic anaemias collectively known as the hereditary stomatocytoses. Patients with these diseases have two striking abnormalities with their erythrocytes: a 'stomatocytic morphology' (which implies an expansion of the inner leaflet of the lipid bilayer) and a membrane leak to the univalent cations Na\(^+\) and K\(^+\). The reason(s) for this shape and this leak is unknown. The work described in this thesis concentrates on two interrelated areas; a possible defect in endocytic vesiculation of the erythrocyte membrane and an examination of lipid rafts in normal and patient erythrocyte membranes.

Here, an overview of the erythrocyte membrane will be provided. What is known about the role of the stomatin protein that is implicated in some of these conditions also will be reviewed. Background information on endocytic vesiculation in erythrocyte ghosts and on lipid rafts will also be provided.

1.1 THE RED CELL

The human red cell is generated from stem cells in the bone marrow via erythropoiesis, the erythrocyte lineage being controlled by erythropoietin secreted by the kidney. The spleen removes senescent cells from the circulation. The main
function of the red cell is the transport of oxygen to the tissues via the carrier molecule haemoglobin and the removal of carbon dioxide.

1.2 THE RED CELL MEMBRANE

The red cell membrane is a flexible structure with multiple functions. It will be described in some depth because if any of these functions fail and the membrane permeability barrier is breached, haemolysis can occur (Lux and Palek, 1995).

1.2.1 Function

The principal function of the membrane is to form a barrier between the haemoglobin (Hb) molecules within the cell and the surrounding plasma, shielding the Hb from oxidative stresses in the plasma and enclosing within the cell reducing systems that maintain the integrity of the Hb. The membrane must undertake transport activities to supply the intracellular contents with necessary substrates, and must allow the transfer of bicarbonate and chloride anions in and out of the cell as part of the respiratory cycle. The membrane must allow the transport of iron atoms in response to erythropoietin. Since the impermeant intracellular contents of the red cell attract water by osmosis, tending to make the cell burst, the membrane has a ‘pump-leak’ system to control intracellular Na⁺ and K⁺ content and cell volume. The membrane must have strength and flexibility
(necessary for negotiation of capillaries) and prevention of adhesion to the endothelium (Lux and Palek, 1995).

1.2.2 Structure

The red cell membrane is composed of a lipid bilayer studded by and supported by membrane proteins. These proteins may either span the bilayer (which are termed 'integral') or they may be 'peripheral', which means they do not penetrate the bilayer, but interact with integral proteins and lipids at the membrane surface (Lux and Palek, 1995). In the erythrocyte, the major peripheral proteins are all located at the cytoplasmic surface and include the structural proteins (such as spectrin and actin) and enzymes (such as glyceraldehyde-3-phosphate dehydrogenase) (Lux and Palek, 1995). The membrane skeleton (cytoskeleton) interacts with the integral membrane proteins and the lipid bilayer itself and gives the membrane its strength and integrity (Tse and Lux, 1999). An excellent detailed review of the components of the red cell membrane is available which discusses the structure of normal membranes, though emphasises aspects that are most involved in haemolytic diseases (Lux and Palek, 1995). Here, a brief account will be given.

Lipids make up approximately 45% of the red cell membrane and the remainder is made up of proteins and glycoproteins (Fairbanks et al., 1971). The
predominant lipids are phospholipids and unesterified cholesterol and there are also small amounts of glycolipids (Sweeley and Dawson, 1969).

The red cell cytoskeleton traditionally refers to the insoluble protein-containing residue remaining after Triton X-100 (or other nonionic detergent) extraction of red cells (Sheetz, 1979) or their ghosts (Yu et al., 1973). Components of the cytoskeleton include alpha and beta spectrin, ankyrin, protein 4.1 and actin and these are anchored to the membrane at multiple sites (Tse and Lux, 1999).

The major proteins of the red cell membrane can be separated on SDS gels (Figure 1.1). They have been extensively reviewed (Bennett, 1989; Bennett, 1990; Alper, 1991; Bennett, 1992; Chasis and Mohandas, 1992; Cartron et al., 1993; Cohen et al., 1993; Conboy, 1993; Gallagher and Forget, 1993; Peters and Lux, 1993; Tanner, 1993; Winkelmann and Forget, 1993; Stewart, 1997). The membrane also contains hundreds of minor proteins, which have received less attention (see Cartron and Agre 1993; Redman and Marsh 1993; Rosse 1993 for reviews).

Figure 1.2 shows a schematic model of the red cell membrane that has been taken from Lux and Palek (1995). This diagram has been built on over the years as more information is found and it must be stressed that it can only be
considered as a working model since new information about its organisation is still to be worked out.
Proteins of purified normal human red cell membrane, electrophoresed in the SDS gel system and stained by Coomassie Blue. Numbers such as ‘4.1’ refer the conventional numbering system of Fairbanks et al. (1971).

Gel by kind courtesy of Mrs. MC Chetty.
Schematic model of the red cell membrane. The diagram shows how the proteins are currently believed to be inserted in the red cell membrane. Numbers such as '4.1' refer to the conventional numbering system of Fairbanks et al. (1971).

GPA = glycophorin A, GPC = glycophorin C.

OHSt blood showing the morphology of stomatocytes. In this blood film, the stomatocytes (cup-shaped cells) can be distinguished from the discocytes (disc shaped cells as found in normal blood).
Spectrin is a fibrous polypeptide, made up of the isoforms α and β spectrin which form flexible rod like heterodimers which are joined end to end to form a tetramer (Tse and Lux, 1999). This has binding sites for spectrin and other proteins (Tse and Lux, 1999). Ankyrin (also known as band 2.1, though there are also isoforms 2.2, 2.3 and 2.6 (Lux and Palek, 1995)) attaches spectrin to the inner membrane surface (Bennett and Stenbuck, 1979; Luna et al., 1979; Yu and Goodman, 1979). Ankyrin is bound to the anion exchanger protein (also known as band 3) (Bennett and Stenbuck, 1980) and there is some evidence (Korsgren and Cohen, 1988; Lux and Palek, 1995) that pallidin (band 4.2) stabilises this link. Spectrin is associated with actin (Karinch et al., 1990). Band 4.1 and adducin are involved in stabilisation of this bond (Gardner and Bennett, 1987).

Actin subunits form into microfilaments and these consist of filamentous actin (F actin) and tropomyosin (Fowler and Bennett, 1984). There is evidence (Siegel and Branton, 1985) that dematin (also known as band 4.9) binds to actin and bundles actin filaments into cables.

Both lipids and proteins are asymmetrically distributed across the bilayer (Bergelson and Barsukov, 1977; Op den Kamp, 1979; Etemadi, 1980). This is termed trans asymmetry and there is evidence that an aminophospholipid flipase (also known as the aminophospholipid translocase) is responsible (Devaux, 1991;
Devaux, 1992). This protein produces and maintains the asymmetry by translocation of lipids from the outer to the inner bilayer (Zachowski et al., 1986; Connor and Schroit, 1987).

Protein kinases phosphorylate almost all the membrane skeletal proteins (Lux and Palek, 1995). Spectrin, ankyrin, band 3, protein 4.1 and dematin are phosphorylated by caesin kinases. Spectrin, ankyrin, adducin, protein 4.1 and dematin are phosphorylated by protein kinase A. Protein kinase C phosphorylates adducin, 4.1 and dematin and tyrosine kinases phosphorylate band 3 and band 4.1 (Cohen and Gascard, 1992). In all cases studied, phosphorylation inhibits interactions between the membrane and the proteins (Lux and Palek, 1995).

1.2.3 Fluxes and channels

The normal intracellular \([\text{Na}^+]\) is 5-11 mmol/(l cells) and \([\text{K}^+]\) is 88-105 mmol/(l cells) in the red cell (Stewart and Turner, 1999). There are specialised transporters, which regulate movement of ions across the membrane in the form of channels, leak pathways, pumps and exchangers (Lux and Palek, 1995). In normal cells monovalent and divalent cations do not easily permeate the lipid bilayer and rely on these transporters for movement across the red cell membrane (Lux and Palek, 1995). Hence, ion concentrations in normal red cells are under strict control.
The red cell membrane is permeable to water, partly due to the specialised red cell membrane protein aquaporin-1 (also known as chip 28) which is a water channel protein accelerating the osmotic flow of water (Zeidel et al., 1992; Nielsen et al., 1993) and also due to the features common to many types of cell membrane which cause local discontinuities in the lipid bilayer. These include small cavities in the bilayer, lipids with large polar head groups forming micelles when dispersed with water, and integral membrane proteins (Lux and Palek, 1995).

Water will move depending on ion concentrations (by osmosis). Therefore ion concentrations in the cell govern the hydration state of the cell. In cells with a higher than normal concentration of ions, more water will diffuse into the cell causing cell swelling. This cell would be termed overhydrated. If the ion concentration was lower than normal, the reverse would occur, leading to a dehydrated cell.
1.3 THE HEREDITARY STOMATOCYTOSES

These are a group of dominantly inherited haemolytic anaemias characterised by a leak to Na\(^+\) and K\(^+\) in the erythrocyte plasma membrane (Dacie, 1985; Stewart and Turner, 1999; Delaunay et al., 1999b). They are very heterogeneous with about 10 different forms with wide variation in severity of anaemia. The different variants relevant to this thesis are described in chapter 2. They range from the original, very severely haemolytic form (Lock et al., 1961) to mild asymptomatic conditions which present with pseudohyperkalaemia (a temperature-dependent K\(^+\) loss from erythrocytes on cooling to room temperature causes an artefactual pseudohyperkalaemia).

Stomatocytes are a feature of hereditary stomatocytosis (the name was coined to describe the erythrocyte morphology in the first case (Lock et al., 1961)). They are swollen, cup-shaped red cells (Figure 1.3) with a markedly increased permeability to the cations Na\(^+\) and K\(^+\) that occur in the blood of individuals with hereditary stomatocytosis. However, occasionally they are found in normal controls (Stewart and Turner, 1999).

In all forms of the disease there appears to be a leaky channel through which Na\(^+\) and K\(^+\) pass, though it has not been characterised. The abnormality is within the ‘passive leak’ component of Na\(^+\) and K\(^+\) transport across the
erythrocyte membrane. This is the component of the K⁺ flux that is resistant to the
two inhibitors ouabain and bumetanide (Stewart and Turner, 1999). The
commoner inherited haemolytic anaemia 'hereditary spherocytosis' is in some
respects superficially similar, but shows a different erythrocyte morphology, and
does not show a major cation leak (Stewart and Turner, 1999). The actual
families to whose red cells will be the subject of this thesis will be considered in
Chapter 2, below.

1.4 THE STOMATIN PROTEIN

In 1982 an American group reported that a 32kDa protein, later called 'stomatin' was absent from the membrane in the most severe form of hereditary
stomatocytoses known as overhydrated hereditary stomatocytosis (OHSt) (Lande
et al., 1982; Eber et al., 1989; Stewart et al., 1992). Figure 1.4 shows this
deficiency (courtesy of GW Stewart).
Figure 1.4 Coomassie stained 2D gels of normal and OHSt red cell membrane proteins illustrating the absence of a 32kDa protein in OHSt red cell membranes (Courtesy of G. Stewart).

Coomassie stained 2D gels of normal and OHSt red cell membrane proteins. In this Coomassie stain, it can be seen that a 32kDa protein is present in the normal red cell membranes (left panel), but is absent from the OHSt membranes (right panel). Arrows indicate direction of IEF (horizontal dimension) and SDS electrophoresis (vertical). IEF = isoelectric focusing.

Figure 1.5 Immunocytochemical staining of OHSt cord blood film with an anti-stomatin antibody (Courtesy of B. Fricke and M. v During)

Immunocytochemical staining of OHSt blood with anti-stomatin. In this blood film, the stomatin is stained brown. The cord blood contains a lot of very immature cells (normoblasts) which are brown, indicating that it is probably the young cells which are stomatin positive.
The gene for this protein (EPB72) has been isolated and cloned (Hiebl-Dirschmied et al., 1991; Stewart et al., 1992) and is found on chromosome 9 (Gallagher and Forget, 1995). However, no mutation of this gene is found in these diseases (Wang et al. 1992; GW Stewart, AC Argent, JF Ajetunmobi, MC Chetty, unpublished).

Recent immunocytochemical studies in this laboratory, in collaboration with a German laboratory, indicate that, in fact, the protein is not completely absent: some protein is present in a minority of the red cells in this condition (Figure 1.5). These cells turn out to be younger cells (B Fricke, M v During, GW Stewart, MC Chetty, personal communication) as shown by anti-stomatin staining of OHSF cord blood (Figure 1.5). Immunocytochemical studies of other tissues from these patients (platelets, lymphocytes, neutrophils, brain, spleen, liver, lung, kidney and endothelial cells) show normal stomatin. (B Fricke, M v During, GW Stewart, MC Chetty, personal communication). However, its deficiency in this disease is striking and must somehow be related to the pathophysiology.

The amino acid sequence of stomatin suggests that it is tethered to the membrane by 2 hydrophobic spanning regions near its N-terminus and has its C-terminus in the cytoplasm (Figure 1.6) (Hiebl-Dirschmied et al., 1991; Stewart et al., 1992).
The stomatin protein is very strongly membrane attached, probably via two hydrophobic membrane spanning sequences. The stomatin protein is shown in red and the lipid bilayer is shown in green.
Stomatin has been linked by sequence homology with three functionally informative homologues in the simple nematode *Caenorhabditis elegans* (Huang et al., 1995). *Mec-2* is strongly homologous to stomatin and is one of 18 'mec' genes, mutations in all of which cause mechano-insensitivity (inability to perceive a touch stimulus). Amongst this group are also *mechs*-4 and 10, which code for subunits of an ion channel, homologous to the amiloride-sensitive Na\(^+\) channel (ENaC) in mammals (Chalfie et al., 1993).

*Unc-24* is another stomatin homologue: recessive mutations cause an ataxic motor phenotype (Barnes et al., 1996). This protein shows sequence-homology to stomatin only at the N-terminus. Its C-terminus is homologous to the 'non-specific lipid transfer protein' or 'sterol carrier protein 2' (SCP2). SCP2 is a soluble intracellular protein (unlike stomatin, which is membrane-bound) that mediates transfer of lipids between separate bilayers (Wirtz, 1991a; Wirtz, 1991b; Wirtz, 1997). *Unc-24* would be expected to be tethered to the membrane and non-soluble due to the stomatin-like N-terminus. Soluble lipid transfer proteins might be expected to shuttle lipids between membrane compartments such as plasma membrane and endoplasmic reticulum: but a *tethered* lipid transfer protein could move lipids only between adjacent areas of the plasma membrane, or between the plasma membrane and abutting vesicles in the cytosol.
The chimaeric homologue *unc-24* led to the investigation as to whether the C-terminus of stomatin, which does not show any sequence homology to *unc-24*, had lipid transfer activity. Unpublished preliminary data in our laboratory (courtesy of Dr. MM Ho) has implicated stomatin as a lipid transfer protein. A fluorescent lipid transfer assay was used to investigate the lipid transfer activity of the bacterially expressed stomatin-fusion protein D13-7. This contains the C-terminus of stomatin with an accidental mutation in which threonine\textsubscript{225} is replaced by an alanine.

The principles of this lipid transfer assay are as follows: a pool of donor vesicles containing both fluorescent lipids and quencher lipids (resulting in all the fluorescence being quenched) are mixed with a larger pool of acceptor vesicles containing neither fluorescent lipids or quenchers. If a lipid transfer protein is added to the system it acts as a shuttle between the vesicles. If it picks up a fluorescent lipid from a donor vesicle and moves it to an acceptor, fluorescence is no longer quenched and an increase in fluorescence is seen.

Using this assay, D13-7 was found to transfer phosphatidylethanolamine, phosphatidylyserine and phosphatidylcholine, but not sphingomyelin (Ho, Stewart, unpublished). However, the wild-type protein (threonine\textsubscript{225} rather than alanine) did not show this positive result. Nevertheless, this could provide a clue as to the
role of the protein. The fluorescent assay demands continuous random activity whereas the wild-type protein could work in a more controlled fashion as suggested in the scheme shown in Figure 1.7. If the role of stomatin does involve transfer of lipids this may well involve a raft-related structure (see section 1.6).

A third *C. elegans* homologue, *unc*-1, exists and mutations cause variations in anaesthetic sensitivity (Rajaram et al., 1999). *Unc*-1 appears to show interactions with a degenerin ion channel (Rajaram et al., 1999) which are related to the ENaCs (Chalfie et al., 1993).

The gene for stomatin is widely expressed in tissues throughout the human and the animal kingdom. Wider sequence homology searches indicate that it could be the prototype of a superfamily, which includes bacterial proteins and the raft protein flotillin (Tavernarakis et al., 1999). It appears that these proteins are generally involved in the regulation of protein turnover, for example, prohibitin (Dell'Orco et al., 1996) and HflKC (Kihara et al., 1996). These observations suggest that stomatin may have a role in the control of protein turnover, via rafts: this idea might be pertinent to the pathophysiology of the stomatocytosis.
Figure 1.7 Scheme suggesting how stomatin might work as a lipid transfer protein

Possible mechanism for lipid transfer protein tethered at its N-terminus. The C-terminus might work in a controlled fashion picking up a lipid from one part of the membrane and moving it to another.
1.5 ENDOCYTIC VESICULATION IN RED CELL GHOSTS

Part of the work in this thesis examines a process of ATP (adenosine triphosphate)-dependent endocytic vesiculation in erythrocyte ghosts from patients with hereditary stomatocytosis. In this section, the concept and background of endocytic vesiculation in erythrocyte ghosts will be discussed in normal cells. Then evidence implicating a disruption of endocytic vesiculation in the pathology of hereditary stomatocytosis will be presented.

1.5.1 Endocytic vesiculation in normal erythrocyte ghosts

There are many forms of vesiculation in erythrocytes (see table 1.1) and this thesis focuses on a specific form of ATP-dependent endocytic vesiculation which occurs in a broken red cell population in vitro. Endocytic vesiculation is not generally considered a special feature of erythrocytes. It refers to the process by which eukaryotic cells internalise extracellular fluid, macromolecules and particles into membrane-bound vesicles. It involves invagination of the plasma membrane and release of free vesicles into the cytoplasm. In contrast, exocytic vesiculation (exocytosis) involves the budding off of vesicles into the extracellular fluid.
Table 1.1 Table showing some of the different methods of inducing erythrocytes to vesiculate.

<table>
<thead>
<tr>
<th>Type of vesiculation</th>
<th>Details/ methods</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endocytic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Energy dependent</td>
<td>Incubation of ghosts with ATP, Mg²⁺ and Ca²⁺</td>
<td>(Penniston and Green, 1968; Jarrett and Penniston, 1976; Birchmeier and Singer, 1977a; Hardy and Schrier, 1978; Hayashi et al., 1978; Birchmeier et al., 1979; Schrier et al., 1986a; Schrier et al., 1986b; Muller et al., 1994)</td>
</tr>
<tr>
<td>2. Nonenergized (All methods believed to work by stripping the cytoskeleton)</td>
<td>(a) Hypotonic lysis to form haemoglobin-free ghosts results in spontaneously formed inside out vesicles after 20 minutes.</td>
<td>(Kant and Steck, 1972; Schrier et al., 1986a; Schrier et al., 1986b; Lew et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Incubation of ghosts with (b) trypsin or (c) EDTA.</td>
<td>(Hardy and Schrier, 1978; Schrier et al., 1986a)</td>
</tr>
<tr>
<td>3. Drug-induced</td>
<td>Addition of amphiphatic cationic drugs (primaquine, chlorpromazine, vinblastine) to intact erythrocytes induces stomatocytic shape changes followed by endocytic vacuoles. All require ATP and are inhibited by sodium vanadate and sodium fluoride.</td>
<td>(Schrier et al., 1986a; Schrier et al., 1986b; Thatte and Schrier, 1988; Schrier et al., 1992a; Johnson et al., 1994)</td>
</tr>
<tr>
<td>4. Sphingomyelinase C induced</td>
<td>Incubation of intact erythrocytes with sphingomyelinase C causes breakdown of sphingomyelin (which faces externally) and stomatocytic shape changes followed by endocytic vesiculation.</td>
<td>(Wilbers et al., 1979; Allan and Walkin, 1988)</td>
</tr>
<tr>
<td>Type of vesiculation</td>
<td>Details/ methods</td>
<td>References</td>
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<td>---------------------------</td>
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</tr>
<tr>
<td>5. Spontaneous</td>
<td>Seen in neonatal erythrocytes. Induced by incubation. Not proceeded by stomatocytic shape change and not inhibited by vanadate.</td>
<td>(Colin and Schrier, 1991)</td>
</tr>
<tr>
<td>6. Receptor-mediated</td>
<td>Transferrin receptor-mediated, only occurs in motile R1 reticulocytes (neonatal) and not adults. Not proceeded by stomatocytosis. Inhibited by sodium cyanide.</td>
<td>(Blight and Morgan, 1987; Thatte and Schrier, 1988; Colin and Schrier, 1991)</td>
</tr>
<tr>
<td>Exocytic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. ATP-depletion</td>
<td>Incubation for 4-6 hours in absence of ATP results in exocytosis</td>
<td>(Lutz et al., 1977; Muller et al., 1981; Butikofer et al., 1987; Butikofer et al., 1989; Civenni et al., 1998)</td>
</tr>
<tr>
<td>8. Calcium loading</td>
<td>Incubation with Ca ionophore</td>
<td>(Allan et al., 1980; Butikofer et al., 1989; Civenni et al., 1998)</td>
</tr>
<tr>
<td>9. By altering relative area of inner and outer leaflet of lipid bilayer</td>
<td>(a) Dimyristoylphosphatidylcholine (DMPC)-induced: incubation of intact erythrocytes with sonicated suspensions of DMPC causes echinocytosis followed by release of vesicles. (Interestingly, was found to be reduced in DHSf (Butikofer et al., 1989a).)</td>
<td>(Ott et al., 1981; Butikofer and Ott, 1985; Butikofer et al., 1987; Butikofer et al., 1989a)</td>
</tr>
<tr>
<td></td>
<td>(b) Merocyanine 540-induced: When added to ghosts causes echinocytosis followed by microvesiculation.</td>
<td>(Allan et al., 1989)</td>
</tr>
</tbody>
</table>
The process of budding and vesiculation of erythrocyte membrane is a critical biological function at a number of stages in the lifespan of an erythroid cell. Iron is transported into the erythroblast for the synthesis of haemoglobin during differentiation by receptor-mediated endocytosis of vesicles containing complexed transferrin receptor, transferrin and iron (Ciechanover et al., 1983). The reticulocyte membrane is remodelled by endocytic and exocytic vesiculation later in erythroid terminal differentiation (Gasko and Danon, 1974). During this period of reticulocyte maturation a number of integral membrane proteins, including the transferrin receptor, are lost from the cell surface (Horton, 1983). However, the amount of endocytic vesiculation occurring in mature erythrocytes is small, so most investigators have chosen to study a related process in erythrocyte ghosts.

In the present work an in vitro process of ATP-dependent endocytic vesiculation which occurs in erythrocyte ghosts was studied. Erythrocyte ghosts are the red blood cell membrane (a broken cell population) and have been used as a means of exploring characteristics of an accessible plasma membrane. Although this method of studying vesiculation in broken cells is unphysiological the actual physiological role of this process of ATP-dependent endocytic vesiculation is unknown, although intracellular vesicles are seen in human red cells (Lew et al.,
1985). However, it is important to distinguish between this *in vitro* process and real physiological events that occur in the whole, natural cell.

Erythrocyte ghosts are made by lysis of erythrocytes followed by washing so only the membranes are kept and the haemoglobin containing cytoplasm is removed. Under certain conditions they can be resealed containing different contents in their cytoplasm. The first observations of endocytic vesiculation in erythrocyte ghosts as distinguished from intact erythrocytes involved a model system for studying 'energisation' of the plasma membranes by ATP. When ATP was resealed within erythrocyte ghosts, endocytosis began leading to vacuole formation (Penniston and Green, 1968). Further studies indicated that the specific substrate for endocytosis in resealed human erythrocyte ghosts is MgATP (Ben-Bassat et al., 1972). Moreover it was found that there is a strict requirement for Mg$^{2+}$ and low concentrations of Ca$^{2+}$ (Schrier et al., 1973).

Various methods were developed to measure this process in ghosts including phase contrast microscopy (Ben-Bassat et al., 1972) and scanning electron microscopy (Sheetz and Singer, 1977). Under appropriate conditions the ghosts prepared from normal disk-shaped intact cells took on a highly crenated shape. Incubation at 37°C in the presence of MgATP in isotonic buffer caused conversion from the crenated shape to the disk shape and eventually to the cup
shape (Sheetz and Singer, 1977). The problem with microscopy is that even with electron microscopy sections true vesicles are indistinguishable from artefacts resulting from occasional membrane infoldings (Lew et al., 1985).

Jarett and Penniston (1976) used an acetylcholinesterase (AChE) (E.C.3.1.1.7) assay (Ellman et al., 1961) to study endocytosis in erythrocyte ghosts. This is a fast, easy, inexpensive and quantitative assay to measure endocytic vesiculation in erythrocytes. The assay utilised the AChE of the erythrocyte membrane. This is a surface enzyme located only on the outside of the erythrocyte membrane, which disappears as the membrane is internalised. Endocytic vesiculation was shown by a loss of cell surface AChE activity that occurs when vacuoles form making the AChE on the vacuole surface inaccessible to exogenous substrate. This was compared to the methods of phase contrast microscopy and electron microscopy and showed that all three methods gave a quantitative measure of the percentage of total membrane area taken in as vesicles: The electron microscopy determined 27.7% +/- 2.1% (standard error of the mean, n=23) of the membrane to be taken up as vesicles. The percentage loss of AChE activity was 25.1% +/- 1.2% (n=2). Eighty percent of the membranes contained vesicles when observed under the phase contrast microscope (Jarrett and Penniston, 1976).
Reduction in AChE activity was shown to be due to membrane barrier effects rather than inactivation as shown by recovery of AChE activity in the presence of 0.5% Triton X-100. Under the phase contrast microscope it was observed that at this concentration of Triton the solution was clear with no intact membranes and only a few particles.

1.5.2 ATP-dependent endocytic vesiculation in patients with hereditary stomatocytosis and the corrective action of dimethyl adipimidate (DMA)

Certain evidence points to the possibility that certain HSt kindreds may have a defect in endocytic vesiculation of their red cell membrane which can be corrected along with the ion leak if cells are first treated with the bifunctional crosslinking agent DMA (Mentzer et al., 1978). The work described in this thesis has extended this observation to all of the available patients with hereditary stomatocytosis in the UK.

It has previously been found in OHSt that the increased cation permeability resulting in the red cell morphology could be corrected in vitro by DMA at concentrations of 1mM (Mentzer et al., 1976; Schroter and Ungefehr, 1976; Mentzer et al., 1978). When the blood was treated with DMA the ouabain-associated potassium loss was reduced from 15 to 1.7 meq per litre of red cells per hour and the sodium gain reduced from 22 to 2.5 meq per litre of red cells per
hour. The previously abnormal red cell volume, cation concentration and deformability were also corrected and the cells no longer appeared to be stomatocytic. The survival time of stomatocytes treated with DMA and infused into rats was double that of untreated stomatocytes (Mentzer et al., 1976).

Mentzer et al. (1978) found that ATP-dependent endocytic vesiculation in resealed red ghosts in OHSt was strikingly impaired but became normal if cells were treated with 2mM DMA prior to making ghosts. At this concentration, DMA had no effect on endocytic vesiculation, cation concentration, red cell volume or deformability in control cells or cells from a patient with cryohydrocytosis.

Further, Ca^{2+} influx in intact erythrocytes, Ca^{2+} influx in red ghosts and Ca^{2+} retention in red ghosts were normal and were not influenced by DMA. Neither did DMA improve protein kinase-mediated phosphorylation of membrane proteins by ATP (which is only 20-40% of normal control values). ^14C-DMA was reacted with stomatocytes and afterwards radiolabel was found to be associated with phosphatidylserine and phosphatidylethanolamine and was also widely distributed amongst membrane proteins (Mentzer et al., 1978).
1.5.3 Mechanism of action of DMA

Figure 1.8 shows the structures of DMA and dimethyl pimedilate (DMP). They are homobifunctional imidoesters which possess two identical groups which can react with primary amine groups to form stable covalent bonds (Hand and Jencks, 1962; Mattson et al., 1993). They are water soluble and membrane permeable and are available with varying distances between the groups for different crosslinking needs (Pepinsky et al., 1980). DMA has a maximal crosslinking dimension of 7.3-9 Å (Mentzer et al., 1978) and crosslinks free amino groups (particularly lysines) in polypeptides (Niehaus and Wold, 1970) to form stable covalent bonds. It is a promiscuous reagent, reacting with a variety of cellular components.

Figure 1.8 Structures of dimethyl adipimidate (DMA) and dimethyl pimedilate (DMP)

Structures of DMA and DMP. These bifunctional imidoesters have 2 reactive groups and differ only in the length of their carbon chain. (Structures taken from Pierce catalogue).
The mechanism by which DMA interacts with the stomatocytic erythrocytes is not fully understood. Various evidence suggests that it works by crosslinking of the membrane causing some kind of stabilization so that cation alterations are prevented. Given that it is a crosslinker, the likelihood must be that it works by bridging adjacent lysine groups but it is not clear which protein or proteins these are on. It was found by Mentzer et al. (1978), that crosslinking was barely detectable with DMA concentrations of 1mM which corrects the cation permeability of stomatocytosis (Mentzer et al., 1978). This suggests that either it is only a minor component in the membrane that is crosslinked, present in small quantities, or intramolecular, rather than intermolecular crosslinking is responsible for its effects. This may explain why other crosslinkers with the same reactive groups and only differing in carbon chain length (either longer or shorter than DMA) were less effective or totally without effect (Mentzer et al., 1978).

Neihaus and Wold (1970) have demonstrated that DMA concentrations as low as 0.08mM cause covalent crosslinking of lysine residues in both the membrane and haemoglobin. It has been found that DMA-treated erythrocytes are resistant to osmotic lysis but maintain their responsiveness to gramicidin-induced $K^+$ permeability (Krinsky et al., 1974). This suggests that the DMA causes a
physiological stabilization of the membrane. It has been found to increase the mechanical resistance of red blood cells (Kaske et al., 1976).

Lubin et al. (1975) investigated DMA as an antisickling agent. Like cyanate, another inhibitor of sickling (Gillette et al., 1973), DMA alters oxygen affinity. However they found that unlike cyanate it inhibited sickling in completely deoxygenated erythrocytes. This indicates that factors other than altered oxygen affinity must also be involved. It also blocked the usual K⁺ loss and increase in viscosity which accompany sickling. It was reported to have few deleterious effects in vitro (Gillette et al., 1973). It has been suggested (Lubin et al., 1975) that DMA might have a direct effect on the membrane or membrane–haemoglobin interaction which stabilizes the cell in the discoid configuration and prevents cation alterations.

In chronic myeloid leukaemia the proportion of spectrin dimers compared with tetramers in erythrocyte membranes is increased when compared with controls. Crosslinking of these erythrocytes with DMA has been shown to significantly modify the organisation of not only spectrin, but also other cytoskeletal proteins such as ankyrin, band 4.2 and band 5 (actin) (Basu et al., 1990).
1.5.4 Endocytic vesiculation and action of DMA as an anti-sickling agent in sickle cell anaemia

Several haemolytic anaemias, including sickle cell anaemia, haemoglobin H disease and hereditary spherocytosis have shown spontaneous red cell membrane exo- or endocytic vesiculation in vivo (Allan et al., 1981; Wagner et al., 1986).

Evidence suggests that an abnormal endocytic process occurs in sickle cell disease (Williamson et al., 1990). This is believed to give rise to vesicles expressing a CaATPase (Lew et al., 1985; Williamson et al., 1990; Williamson et al., 1992) and these vesicles are thought to cause the dehydration which causes the clinical effects of the disease (Lew et al., 1985). Although DMA has been found to display antisickling effects in these cells (see section 1.5.3 and Lubin et al., 1975; Guis et al., 1984; Pennathur-Das et al., 1984; Gibson et al., 2000), to date the effects of DMA on these vesicles has not been considered. The evidence is presented in more detail in chapter 5.

There are certain parallels between sickle cell disease and HSt: both have leaky cells giving rise to a haemolytic anaemia; both have a vesiculation abnormality and DMA has positive effects on cells from both conditions. This has led to the consideration that perhaps they share a comparable pathology resulting from a defect in membrane vesiculation. This is considered in chapter 5.
1.5.5 Machinery involved in endocytic vesiculation in erythrocyte ghosts

Despite much work, the mechanism of ATP-dependent endocytic vesiculation in erythrocyte ghosts is not fully understood and has been further investigated here (Chapter 9). Two possible roles for ATP have been suggested (Birchmeier and Singer, 1977a) based on the fact that cleavage of the terminal phosphate bond of ATP is required for the erythrocyte ghost shape changes to occur (Sheetz and Singer, 1977). The first is as a substrate for an ATPase, as with an actinomyosin-like contractile activity, or with an ion transport activity. The second possible role which Birchmeier and Singer (1977) present evidence to favour is as a substrate for a protein or lipid kinase, that results in the phosphorylation of one or more components critical to the shape changes.

The biochemical effects of the action of ATP were examined. It was shown that the spectrin component 2 of the erythrocyte membrane is the only membrane protein that is significantly phosphorylated under the conditions where shape changes are produced. This activity was demonstrated in erythrocyte ghosts prepared from intact cells prelabelled with $^{32}$P (radiolabelled phosphate). The results suggested that the erythrocyte membrane possesses kinase and phosphatase activities that produce phosphorylation and dephosphorylation of a specific site on spectrin component molecules. It was suggested that these induce
a rearrangement of the membrane structure leading to invagination and, subsequently, to vesiculation (Birchmeier and Singer, 1977a).

Other evidence suggests that an ATPase is responsible: Schrier et al. (1975) found that the energy for endocytosis in erythrocyte ghosts appears to be derived from MgATP and that membrane internalisation is preceded by activation of a membrane associated CaMgATPase and by the active efflux of Ca\(^{2+}\). They found that ruthenium red, a specific inhibitor of CaMgATPase and Ca\(^{2+}\) transport, inhibited ATP-dependent endocytic vesiculation in a concentration related manner. Although ruthenium red is supposedly specific for the CaMgATPase (Schrier et al., 1975) a variety of ATPases are known to be present in the erythrocyte membrane (Ca, Mg, NaK, the aminophospholipid flipase and myosin) and conceivably these could all be possible targets of its action.

Devaux (1991) suggested the following hypothesis to explain the process of ATP-dependent endocytic vesiculation. He suggested that a net redistribution of phospholipids from the outer to the inner monolayer cause an increase in the inner membrane surface compared to the outer, resulting in stomatocytic shapes and vesiculation. The role of the aminophospholipid flipase has been stressed (Devaux, 2000) which is responsible for the transport of phosphatidylserine and phosphatidylethanolamine from the outer to the inner leaflet of the plasma.
membrane. Stimulation of the aminophospholipid flipase was found to provoke endocytic-like vesicles in erythrocytes and stimulate endocytosis in K562 cells.

Muller et al. (1994) found evidence to support this hypothesis by investigating the influence of various spin-labelled phospholipid analogues (which are known for their selective localisation either in the outer or inner leaflet of the plasma membrane) on the ATP-dependent endocytic vesiculation of human erythrocyte ghosts by monitoring AChE activity. They found that the incorporation of lipid-like molecules into the outer leaflet decreased the extent of vesiculation while the accumulation of those substances in the inner leaflet did not.

The aminophospholipid flipase has not been isolated from erythrocytes but a 120kDa Mg\(^{2+}\)-requiring ATPase of unknown function that is present in the erythrocyte membrane (inhibited by vanadate, fluoride and Ca\(^{2+}\) and which requires phosphatidylserine to be active), has been suggested as a possible candidate (Morrot et al., 1990). The aminophospholipid flipase is an ATP-dependent transport system (Devaux, 1991; Devaux, 1992) requiring MgATP at the inner membrane surface (Seigneuret and Devaux, 1984), being inhibited by vanadate (50\(\mu\)M), free Ca\(^{2+}\) (over 0.2\(\mu\)M) (Zachowski et al., 1986; Comfurius et
Various compounds have been used to study the mechanism of ATP-dependent endocytic vesiculation. Schrier et al. (1986b) looked at vanadate and found it to inhibit ATP-dependent endocytic vesiculation in erythrocyte ghosts. Its target of inhibition was likely to be ATP-hydrolysing power producers (i.e. pumps, ATPases, most likely the aminophospholipid flipase). It also inhibits tyrosine phosphatases (Tsiani et al., 1998; Sims et al., 2000), but this action seems to be unlikely here.

1.6 LIPID RAFTS

1.6.1 Evidence for the existence of lipid rafts and their isolation

The lipid raft hypothesis was formulated more than 10 years ago and originated from studies on epithelial cell polarity (Simons and van Meer, 1988). The apical membrane of Mardin-Darby canine kidney (MDCK) cells is enriched in glycosphingolipids and sphingomyelin; and glucosylceramide (a glycosphingolipid) was found to be preferentially transported to the apical membrane in these cells (Simons and van Meer, 1988). To explain this it was postulated that microdomains exist, containing dynamic assemblies of cholesterol and sphingolipids in the exoplasmic leaflet of the bilayer and that these were
sorting stations for proteins destined for the apical membrane (Simons and van Meer, 1988).

There is now much evidence for the existence of lipid rafts, believed to be membrane microdomains, formed by the dynamic clustering of glycosphingolipids and cholesterol that are insoluble in nonionic detergents and have a low buoyant density. They associate with GPI-anchored proteins in the outer leaflet and acylated proteins in the inner leaflet of the lipid bilayer. Due to the high melting point of the lipids contained in rafts, they are found in a more rigid liquid crystalline state ($L_o$), surrounded by fluid liquid phase membranes ($L_c$) (Brown and London, 1998).

Prior to this, it had been thought that detergent insolubility of certain membrane associated proteins was due to them being associated with the cytoskeleton (Streuli et al., 1981; Kim and Campbell, 1983; Davies et al., 1984; Hoessli and Rungger-Brandle, 1985). However, Hooper and Turner (1988) observed that GPI-anchored proteins were resistant to nonionic detergents such as Triton X-100. A GPI-linked protein cannot be directly anchored to the cytoskeleton, because neither the peptide nor the lipid membrane anchor penetrate all the way through the membrane. The cytoskeleton is inside and does not penetrate the membrane either.
This insolubility in nonionic detergents in the cold where they form glycolipid enriched complexes has now become well established (Hooper and Bashir, 1991; Brown and Rose, 1992) and is crucial because it allows a way of isolating and characterising them. Due to their high lipid content, the resulting low-density, detergent-insoluble glycolipid-enriched domains (DIGs) can be isolated by flotation through a sucrose density gradient. This allows any associated proteins to be identified and allows DIGs to be distinguished from other detergent-insoluble complexes.

It has been questioned whether rafts exist in vivo or are simply an experimental artefact. A key issue regarding their methods of isolation is whether the 'rafts' exist prior to detergent treatment, or whether the insoluble components which are isolated in this manner were once independent and randomly dispersed in the membrane (Brown, 1998). Detergent-free methods of isolating the rafts have helped to resolve these issues (Schnitzer et al., 1995b; Stan et al., 1997) though achieve varying degrees of enrichment (Brown, 1998). However, techniques of single particle trafficking (Pralle et al., 2000), fluorescence polarisation (Varma and Mayor, 1998) and protein crosslinking (Friedrichson and Kurzchalia, 1998; Kasahara and Sanai, 2000) indicate that GPI-anchored proteins do cluster into discrete domains and also allows direct visualisation of the rafts.
The size of the rafts observed in these studies range from 70-370nm (Hooper, 1999).

1.6.2 Content of rafts

There are hundreds of lipid species of various classes in mammalian plasma membranes. Glycerophospholipids (phosphatidylcholine and phosphatidylethanolamine are examples) make up the fluid part of the membrane. Lipid rafts, enriched in sphingolipids and cholesterol, bind proteins. These domains are less fluid than the rest of the membrane (Brown and London, 1998).

One of the most important properties of lipid rafts is that they can include or exclude proteins to different extents. GPI-anchored proteins, transmembrane proteins and doubly acylated tyrosine kinases of the Src family are associated with rafts and are incorporated into the DIGs (Simons and Ikonen, 1997). Various assays or markers are typically used to identify rafts. Alkaline phosphatase (EC 3.1.3.1) is a GPI anchored protein associated with the raft fraction (Parkin et al., 1999a). Flotillin is a 48kDa non-GPI-anchored membrane protein, first isolated from lung tissue, which is strongly associated with rafts (Bickel et al., 1997; Parkin et al., 1999a; Edgar and Polak, 2001). In some tissues, caveolin can act as a raft marker but it is not always present.
1.6.3 Function

Lipid rafts have been proposed to function as moving platforms for the attachment of proteins when membranes are moved around inside the cell, during signal transduction (Simons and Ikonen, 1997; Hooper, 1999) and endocytosis (Simons and Ikonen, 1997; Simons and Toomre, 2000). Several signalling molecules partition into the DIG fraction (Anderson, 1993; Lisanti et al., 1994; Parton and Simons, 1995; Hooper, 1999). If GPI-anchored proteins are clustered together, they can activate signalling pathways due to being able to communicate with each other. Palmitoylation (the addition of palmitic acid to a cysteine) of proteins increases the affinity of certain proteins for rafts. Palmitic acid is a fatty acid that sits in the lipid bilayer, so palmitoylation of a protein facilitates that protein to sit in the bilayer. Crosslinking can have the same effect (Simons and Ikonen, 1997).

1.6.4 Caveolae

Caveolae are 50-100nm flask-shaped invaginations associated with, or in the vicinity of, the plasma membrane devoid of clathrin coats and present in many cell types (Parton, 1996; Anderson, 1998). They contain clusters of glycosphingolipids (GSLs) (Tran et al., 1987) and require cholesterol to function (Rothberg et al., 1990). They have a high level of the protein caveolin and are
formed from lipid rafts by polymerisation of caveolins (Kurzchalia et al., 1992; Rothberg et al., 1992; Hooper, 1999). These are hairpin-like palmitoylated integral membrane proteins that tightly bind cholesterol (Dupree et al., 1993; Murata et al., 1995; Parton, 1996).

Like rafts, caveolae are insoluble in nonionic detergents and have low density due to a high lipid content (Simons and Ikonen, 1997). They are implicated in endocytosis (Tran et al., 1987), transcytosis (Ghitescu et al., 1986; Anderson, 1993) and signal transduction (Sargiacoma et al., 1993; Simons and Ikonen, 1997; Hooper, 1999). However, these events do occur in cell types lacking caveolin, for example signal transduction in T lymphocytes (Fra et al., 1994) and endocytosis by the apical membrane of enterocytes (Danielsen and van Deurs, 1995).

The possibility that caveolae and rafts are synonymous was considered, but in 1995 morphologically distinct caveolae were separated from rafts (Schnitzer et al., 1995a; Schnitzer et al., 1995b; Schnitzer et al., 1995c). The caveolae contained caveolin, ganglioside GM1, Ca$^{2+}$-ATPase, the inositol 1,4,5-triphosphate receptor (Schnitzer et al., 1995c) and proteins involved in vesicle budding, docking and fusion (Schnitzer et al., 1995a). However, the GPI-anchored proteins were excluded from the caveolae and were found to be
concentrated in the DIGs (Schnitzer et al., 1995b). This demonstrated that rafts can exist without caveolin (and therefore caveolae) implicating caveolae as a subset of rafts.

1.6.5 Lipid raft disrupters

There are some well-known inhibitors of raft formation, notably nystatin and methyl-β cyclodextrin (MBCD) (Hooper, 1999). Because lipid rafts are enriched in cholesterol and sphingolipids, disrupting or altering the level of these lipids in the cell membrane can be used to disrupt lipid rafts. This enables their role in biological processes to be studied. Agents that have been shown to disrupt lipid rafts are shown in Table 1.2 taken from Hooper (1999).
### Table 1.2 Agents which have been shown to disrupt lipid rafts (taken from Hooper (1999)).

<table>
<thead>
<tr>
<th>Action</th>
<th>Compound</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition of cellular biosynthesis</td>
<td>Compactin</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor</td>
<td>(Rothberg et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Lovastatin</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor</td>
<td>(Taraboulos et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Squalestatin</td>
<td>Squalene synthase inhibitor</td>
<td>(Stulnig et al., 1997)</td>
</tr>
<tr>
<td>Oxidation of cholesterol to cholestenone</td>
<td>Cholesterol oxidase</td>
<td>Enzyme</td>
<td>(Smart et al., 1994)</td>
</tr>
<tr>
<td>Cholesterol binding agents</td>
<td>Digitonin</td>
<td>Cardiac glycoside</td>
<td>(Rothberg et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Filipin</td>
<td>Polyene antibiotic</td>
<td>(Rothberg et al., 1990);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Schnitzer et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>Methyl-β-cyclodextrin</td>
<td>Glucopyranoside cyclic oligomers</td>
<td>(Klein et al., 1995);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Ilangumaran and Hoessli, 1998)</td>
</tr>
<tr>
<td></td>
<td>Nystatin</td>
<td>Polyene antibiotic</td>
<td>(Rothberg et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Saponin</td>
<td>Sapogenin glycoside</td>
<td>(Cemeus et al., 1993)</td>
</tr>
<tr>
<td></td>
<td>Streptolysin O</td>
<td>Thiol-activated cytolysin</td>
<td>(Xie and Low, 1995)</td>
</tr>
<tr>
<td>Inhibition of cellular sphingolipid biosynthesis</td>
<td>Fumonisin B₁</td>
<td>Mycotoxin, ceramide synthase inhibitor</td>
<td>(Stevens and Tang, 1997)</td>
</tr>
<tr>
<td></td>
<td>D-threo-1-phenyl-2-decanoylamino-3- morpholino-1-propanol.HCl</td>
<td>Glucosylceramide synthase inhibitor</td>
<td>(Sheets et al., 1997)</td>
</tr>
</tbody>
</table>
Cholesterol on its own does not confer detergent-insolubility. Glycosphingolipids are insoluble by themselves and sphingomyelin is resistant to detergent extraction in the presence of cholesterol (Schroeder et al., 1994). On the basis of these and other observations it has been proposed that the plasma membrane of eukaryotic cells would contain enough sphingolipids to form detergent-insoluble domains when cholesterol is present, but not in the absence of sterols.

1.6.6 Are there rafts in erythrocytes?

At the time the work described in this thesis was performed very little was known about the possible existence of rafts in erythrocytes: the presence of membrane microdomains resistant to extraction by nonionic detergents at 4° C has been previously identified in erythrocytes at 31% w/v sucrose (Civenni et al., 1998). The ability of exogenously-added GPI-anchored molecules (AChE, variant surface glycoprotein (VSG) and procyclin) to colocalize with the endogenous GPI-anchored proteins in these detergent-insoluble complexes was examined. It was found that although they were incorporated into the membrane, unlike the endogenous GPI-anchored proteins which were detergent-insoluble, the exogenous molecules were completely solubilized. This shows that it is not the GPI-anchored proteins themselves, but the way they are arranged in the
membrane that conferred this insolubility. In addition, the exogenously added GPI-anchored proteins were not released from erythrocytes along with the endogenous GPI-anchored proteins during exocytic vesiculation. These data suggest that they are not colocalizing with the endogenous GPI-anchored proteins into the specific microdomains (Civenni et al., 1998). This suggests that there may well be lipid rafts present in erythrocytes and an aim of this thesis was to investigate this further.
1.7 HYPOTHESES

At the outset of the work to be described here, the following principal hypotheses were advanced:

i. That the apparent lipid transfer activity of the C-terminus of stomatin might be involved in the lateral movement of lipid across the membrane.

ii. That the previously noted abnormality in ATP-dependent vesiculation was important in the pathophysiology of the hereditary stomatocytoses, possibly because in normal cells an ion channel is regulated by turnover of a membrane protein via such a process.

iii. That these processes of lateral movement of lipids and vesiculation might be related.
CHAPTER 2

HEREDITARY STOMATOCYTOSIS PATIENTS

The hereditary stomatocytoses encompass a group of heterogeneous diseases. In this chapter the different clinical variants dealt with in this thesis will be described. This work has dealt with 8 variants, 13 pedigrees and 17 patients in total. They are presented in table 2.1 and table 2.2 and the family trees are illustrated in Figure 2.1. This chapter also deals with the way they are diagnosed, the bizarre temperature effects they show, gene mapping and clinical problems encountered.
Table 2.1 Table showing pedigrees studied in this thesis

<table>
<thead>
<tr>
<th>Family Label</th>
<th>Key Members</th>
<th>Location</th>
<th>Diagnosis</th>
<th>References</th>
</tr>
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<td>A</td>
<td>A-II-1</td>
<td>Brighton</td>
<td>Overhydrated hereditary stomatocytosis</td>
<td>(Meadow, 1967; Stewart et al., 1992)</td>
</tr>
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<td>Manchester</td>
<td>Overhydrated hereditary stomatocytosis</td>
<td>(Lock et al., 1961; Stewart et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>B-III-2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C-II-3</td>
<td>Harrow</td>
<td>Overhydrated hereditary stomatocytosis</td>
<td>unpublished</td>
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<td>D-II-2</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>(Carella et al., 1998)</td>
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</tr>
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<td>Family Label</td>
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<td>Location</td>
<td>Diagnosis</td>
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<td>Cryohydrocytosis</td>
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<td>N-III-7</td>
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<td>Familial pseudohyperkalaemia</td>
<td>(Stewart et al., 1979; Stewart and Ellory, 1985)</td>
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</tbody>
</table>
Figure 2.1 Family trees.
Closed symbols denote affected individuals; open symbols, non-affected; grey, status unknown. Family members under study arrowed. Where both parents are unaffected, but their offspring is this denotes a spontaneous mutation.

Family A, Brighton

Family B, Manchester

Family C, Harrow

Family D, Uxbridge, Omagh

Family E, Stondon Massey

Family F, Watford

Family G, Watford

Family H, Bushey, Hertfordshire

Family I, Hurstpierpoint, Sussex
Figure 2.1 continued...

Family F, Hemel.
Figure 2.1 continued...

Family J, Blackburn

Family K, Woking

Family M, Edinburgh

Family L, Chiswick
Table 2.2. Thirteen pedigrees of the leaky hereditary stomatocytosis syndromes showing eight distinguishable variants. Distinction depends on the severity of the ion leak (which roughly equates with the intracellular Na/K abnormality, not shown); the temperature dependence of the leak flux; the presence or absence of the stomatin protein; and the effect of DMA on the leak.

<table>
<thead>
<tr>
<th>Pedigree</th>
<th>Family label</th>
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<th>MCHC</th>
<th>Leak rate</th>
<th>Tmprtr</th>
<th>Stomat</th>
<th>Lipid</th>
<th>Mapping</th>
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<td>10-20</td>
<td>28-30</td>
<td>x40</td>
<td>MT</td>
<td>↓</td>
<td>N</td>
<td>na</td>
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<td>B</td>
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<td>9-11</td>
<td>10-20</td>
<td>28-30</td>
<td>x40</td>
<td>MT</td>
<td>↓</td>
<td>N</td>
<td>na</td>
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<td>9-11</td>
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<tr>
<td>Watford</td>
<td>G</td>
<td>CHC</td>
<td>12-14</td>
<td>5-10</td>
<td>32-35</td>
<td>x4</td>
<td>U</td>
<td>+</td>
<td>↑ether</td>
<td>not 16</td>
</tr>
<tr>
<td>Bushey</td>
<td>H</td>
<td>CHC</td>
<td>12-14</td>
<td>5-10</td>
<td>32-35</td>
<td>x4</td>
<td>U</td>
<td>+</td>
<td>↑ether</td>
<td>not 16</td>
</tr>
<tr>
<td>Hurstpierpoint</td>
<td>I</td>
<td>CHC</td>
<td>12-14</td>
<td>5-10</td>
<td>32-35</td>
<td>x4</td>
<td>U</td>
<td>+</td>
<td>N</td>
<td>not 16</td>
</tr>
<tr>
<td>Blackburn</td>
<td>J</td>
<td>Blackburn</td>
<td>12-14</td>
<td>5-10</td>
<td>32-35</td>
<td>x4</td>
<td>SS</td>
<td>+</td>
<td>N</td>
<td>not 16</td>
</tr>
<tr>
<td>Woking</td>
<td>K</td>
<td>Woking</td>
<td>12-14</td>
<td>5-10</td>
<td>32-35</td>
<td>x4</td>
<td>P</td>
<td>+</td>
<td>N</td>
<td>na</td>
</tr>
<tr>
<td>Chiswick</td>
<td>L</td>
<td>FP Chiswick</td>
<td>N</td>
<td>1</td>
<td>33</td>
<td>x1.5</td>
<td>Shoulder</td>
<td>+</td>
<td>N</td>
<td>na</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>M</td>
<td>FP</td>
<td>N</td>
<td>2.5</td>
<td>36</td>
<td>x1.1</td>
<td>SS</td>
<td>+</td>
<td>N</td>
<td>16</td>
</tr>
</tbody>
</table>

Key: OHSt, overhydrated HSt; DHSt, dehydrated HSt; CHC, cryohydrocytosis; FP, familial pseudohyperkalaemia; Retic, reticulocyte count; effect DMA, effect of dimethyl adipimidate on both leak and vesiculation; Leak rate, multiple of normal K leak rate at 37°C; Tmprtr; temperature dependence of leak rate; stomatin, presence or relative absence of stomatin in red cells; EV, presence or absence of ATP-induced endocytosis in broken red cell membranes; Lipid, lipid composition of membrane. N, normal; nt, not tested, na, not applicable (pedigree too small); MT, monotonic; U, U-shaped with minimum at 23°C; P, parallel to normal with minimum at 8°C; SS, shallow slope with low Q10 in interval 37-20°C; shoulder, minimum at 25°C, maximum at 12°C; ↑PC, increased phosphatidylcholine content of membrane; ↑ether, increased ether lipid content of membrane.
2.1 CLINICAL VARIANTS

Kindreds can be distinguished by a series of diagnostic features, including the severity of the cation leak, the temperature dependence of the leak, the presence or absence of stomatin, the phospholipid content of the membrane and the degree of hydration of the cell. At present by these means eight variants in the UK can be distinguished (Table 2.2). The variants to which we have access, and which will be the main focus of the work in this thesis are: overhydrated HSt (OHS\textsubscript{t}), dehydrated HSt (DHS\textsubscript{t}), cryohydrocytosis (CHC), familial pseudohyperkalaemia Edinburgh type 1 (FP\textsubscript{1}) and familial pseudohyperkalaemia Falkirk/ Chiswick type 2 (FP\textsubscript{2}), HSt Woking (Jarvis et al., 2001) and HSt Blackburn (Stewart and Turner, 1999).

2.1.1 Overhydrated hereditary stomatocytosis (OHSt)

This is the most severe condition and will form the main focus of this thesis. These patients have an extraordinarily high passive leak to Na\textsuperscript{+} and K\textsuperscript{+}, with a flux rate 20-40 times normal (Zarkowsky et al., 1968). The influx of Na\textsuperscript{+} exceeds the efflux of K\textsuperscript{+}. The NaK pump is stimulated by the high intracellular [Na\textsuperscript{+}] but cannot compensate for it (Morlé et al., 1989). This leads to grossly abnormal intracellular cation concentrations causing the cells to gain water and swell. The
swollen cells are osmotically fragile (Lux and Palek, 1995). Patients are chronically jaundiced. Fortunately the condition is rare.

The integral membrane protein ‘stomatin’ is deficient from their cells (Lande et al., 1982; Eber et al., 1989; Hiebl-Dirschmied et al., 1991; Stewart et al., 1992). However, the protein was purified from a normal control and the gene sequenced and was found to be normal in the patients (Wang et al., 1992).

During this work, the OHSt patients have been referred to as Brighton, Manchester and Harrow. Unless it is stated otherwise, the patient Manchester always refers to B-III-1 (see table 2.1 or figure 2.1).

2.1.2 Dehydrated hereditary stomatocytosis (DHSt)

This is the most common of the conditions. It is a haematologically milder condition than OHSt. The fluxes to Na\(^+\) and K\(^+\) are only 2-3 times normal (Stewart and Turner, 1999). The dehydration is probably due to the fact that the increase in permeability to K\(^+\) exceeds the augmentation in Na\(^+\). Monovalent cation pump activity is stimulated by the increased intracellular [Na\(^+\)] but is unable to compensate for K\(^+\) loss. Cation and water depletion occur leading to dehydration of the cells (Joiner et al., 1986).

The stomatin protein is not absent from the erythrocyte membranes from these patients (Stewart and Turner, 1999). Their erythrocyte membranes show an
excess of phosphatidylcholine (PC) and the condition is identical to that described previously as a high phosphatidylcholine haemolytic anaemia (HPCHA) (Clark et al., 1993).

Some European cases of DHSt have been associated with a transient, self limiting, perinatal oedema (Entazami et al., 1996) (Grootenboer et al., 1998). All DHSt cases tested so far map to a locus on chromosome 16q23-qter (Carella et al., 1998).

2.1.3 Cryohydrocytosis (CHC)

This is the next most prevalent condition in the UK. It is characterised by a mild anaemia, a reticulocytosis of about 5% and normal or close to normal levels of haemoglobin (Miller et al., 1965). When fresh, the red cells display mild dehydration with a high mean cell haemoglobin concentration (MCHC). If they are left to stand at room temperature, they begin to lose K⁺ and gain Na⁺ and become overhydrated with a low MCHC. If they are left to stand in the cold, these changes become very marked, with Na⁺ entry predominating over K⁺ loss (Coles et al., 1999a), leading to marked lysis if left overnight in EDTA or heparin. Storage at low temperatures leads to an increase in intracellular [Na⁺], an increase in cell hydration (Coles et al., 1999a) and a subsequent rise in the mean red cell volume (MCV) after which lysis occurs.
Autohaemolysis on cold storage is particularly high and some of the patients present with artefactually high plasma \([K^+]\) readings due to the loss of \(K^+\) into the plasma from red cells during storage. Because the storage falsely suggests that the patients are hyperkalaemic, this phenomenon is termed 'pseudohyperkalaemia'. Patients are recalled to hospitals by anxious physicians after routine plasma electrolyte estimations and are retested and found to be normal when a repeat urgent analysis is performed. These effects can all be attributed to the bizarre temperature dependence (section 2.4 and Figure 2.2) of the \(Na^+/K^+\) leak (Stewart and Turner, 1999).

2.1.4 Familial pseudohyperkalaemia (a heterogeneous group)

Familial pseudohyperkalaemia (FP1 and FP2) is characterised by erythrocyte \(K^+\) loss which is exaggerated in the cold. It is very mild with the haematology and the leak at 37°C being virtually normal. Therefore, patients have only mildly abnormal cells and little or no haemolysis or anaemia (Stewart et al., 1979; Stewart and Ellory, 1985). The Edinburgh family (FP1) has a mildly dehydrated condition (Stewart et al., 1979; Stewart, 1993) and maps to chromosome 16 along with DHSt (Iolascon et al., 1999). The Chiswick and Falkirk pedigrees (FP2) (Haines et al., 2001a) show a similar combination of virtually normal haematology and temperature dependent pseudohyperkalaemia, but are different
from the Edinburgh pedigree (FP1) by virtue of a shoulder-shaped temperature
dependence in the 'passive leak' to K.

2.1.5 HSt Woking

This is a unique pedigree and is the second most severe after OHSt (Jarvis et al.,
2001). The intracellular \([Na]^+\) and \([K]^+\) and the 'leak' \(K^+\) influxes are more
abnormal than DHSt, while the temperature dependence of the flux is different
from Blackburn (see below) and cryohydrocytosis.

2.1.6 HSt Blackburn

This is another unique kindred, with an almost normal level of haemoglobin, 5-
8% reticulocytosis, an identical flux to cryohydrocytosis (5-6 times normal)
showing a shallow slope temperature abnormality and frank anaemia (Coles et al.,
1999b). This condition can be differentiated from HSt Woking and
cryohydrocytosis by virtue of the temperature dependence of the cation leak,
which shows a 'shallow slope' abnormality.

2.2 CLINICAL DIAGNOSIS

A blood film is a vital clue due to the presence of stomatocytes. All families go
through stages where they are diagnosed as 'atypical hereditary spherocytosis'.
This is a dangerous diagnosis due to the thrombotic complications after
splenectomy in OHSt and DHSt (Stewart et al., 1996). Splenectomy failure in an
affected family member can be a useful distinguishing feature from hereditary spherocytosis. Variable hyperkalaemia is marked in certain cases (Coles et al., 1999a; Coles et al., 1999b) and suggests leaky cells but is not always present, for example in dehydrated HSt.

Once the diagnosis is suspected, it can be confirmed by measurement of isotopic flux rates. However, in the frankly haemolytic cases, a simple measure of intracellular [Na⁺] and [K⁺] on cells washed in cold isotonic Tris-buffered magnesium chloride suffices.

2.3 CLINICAL PROBLEMS

2.3.1 Thrombosis after splenectomy

Treatment for certain cases of HSt used to be splenectomy. However within the last decade, it has emerged that worldwide, thrombotic problems are not uncommon in OHSt and DHSt cases. Prior splenectomy was the common link in these cases. Within pedigrees, adults who had been splenectomized developed one or more thrombotic problems, including deep and superficial vein thromboses, portal vein thromboses, arterial thromboses and abdominal pain (Inoue et al., 1989; Stewart et al., 1996; Smith and Segel, 1997; Perel et al., 1999; Delaunay et al., 1999b; Grootenboer et al., 2000). Splenectomy in cryohydrocytosis cases does not appear to be associated with thrombotic...
complications (Coles et al., 1999a). No patients with familial pseudohyperkalaemia have ever been splenectomized (Stewart and Turner, 1999) although the original proband of the Edinburgh pedigree has recently suffered a deep vein thrombosis (GW Stewart, personal communication). Correct diagnosis is thus very important. It is important for cases not to be misdiagnosed as hereditary spherocytosis and splenectomized (Schroter and Eber, 1989; Delaunay, 1999a; Bolton-Maggs, 2000).

2.3.2 Perinatal oedema

Two cases of perinatal oedema have been reported (Entazami et al., 1996; Grootenboer et al., 1998) associated with DHSt. In both cases, though severe it spontaneously resolved. Transfusions did not cause resolution of the oedema implying that the haemolytic anaemia was not the cause.

2.3.3 Iron overload

Iron overload has been problematic in the Denver DHSt pedigree (Lane et al., 1990; Stewart et al., 1996). However, although iron saturations can be high in these cases, severe iron related damage has not yet been reported in the UK.
2.3.4 Pseudohyperkalaemia

In particular, the cryohydrocytosis and familial pseudohyperkalaemia variants present with high plasma \([K^+]\) readings due to \(K^+\) loss from their cells on storage. There is no abnormality in the \(K^+\) handling in their body and it is purely artefactual.

2.3.5 Pseudomacrocytosis

The mean cell volume (MCV) can also be reported to be high. In cryohydrocytosis, the cells can swell on storage before the blood count is performed. Physicians need to be alerted to this in order to avoid unnecessary questions about alcohol intake or unnecessary tests for vitamin B12 and folate.

2.3.6 Tiredness

Patients complain of tiredness out of proportion to their anaemia. It has been suggested that these cells may be less efficient than normals in the respiratory cycle. Due to the difficulties in investigating fatigue, the cause is not understood and more physiological studies are required.

2.4 TEMPERATURE EFFECTS

An important diagnostic route for these pedigrees is the temperature dependence on the ouabain+bumetanide-resistant (obr) potassium influx (leak) since it can be used to distinguish between different kindreds. Miller, who described the first
cryohydrocytosis phenotype, made the first temperature observation in
stomatocytosis (Miller et al., 1965). Since then, much work has been performed
in our laboratory to investigate a number of families with different temperature
related effects. A study of the dependence of the ouabain + bumetanide resistant
(обр) K\(^+\) fluxes in these conditions was performed and is shown in Figure 2.2
taken from data from the Stewart laboratory (Stewart and Turner, 1999).
Figure 2.2 Temperature dependence of 'passive leak' K fluxes in different variants of the hereditary stomatocytosis syndromes
In normal cells, the temperature dependence is not trivial, showing a minimum at about 10°C (Stewart et al., 1980). OHSt does not show a minimum, giving a monotonic curve. The DHSt curve is parallel to normal, but shifted up the y-axis. By contrast, the cryohydrocytosis curve shows a minimum at 20°C with the flux at 0°C exceeding the flux at 37°C. A shallow slope variation in which the flux shows a lower $Q_{10}$ than normal is seen in the FP1 (Edinburgh) and HSt Blackburn cases. The Woking case shows an identical flux to cryohydrocytosis and Blackburn at 37°C, but the temperature dependence is much steeper (Stewart and Turner, 1999).

2.5 GENE MAPPING

The pedigrees in the most severe of these conditions, OHSt, are too small for mapping. DHSt and FP1 (Edinburgh) map to a locus on chromosome 16 (Carella et al., 1999); HSt Blackburn maps to another, as yet undefined, locus (Carella et al., 1999) and cryohydrocytosis maps to yet another locus (A Iolascon, M Carella, GW Stewart, personal communication). No gene has yet been identified at any locus.
CHAPTER 3

MATERIALS AND METHODS

3.1 REAGENTS

All reagents were purchased from Sigma-Aldrich [Dorset, UK] or BDH Laboratory Supplies [Poole, UK] unless stated otherwise and were of the highest grade available.

3.2 BUFFERS

Buffers were autoclaved after being made up to the compositions shown in Table 3.1. All pHs were at room temperature unless otherwise stated.
Table 3.1 Composition of buffers.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase buffer/substrate</td>
<td>50mM glycine, 1mM MgCl₂·6H₂O, 4.4mM p-nitrophenylphosphate, pH 10.5</td>
</tr>
<tr>
<td>Blotting buffer</td>
<td>1 vol. Laemmli buffer, 2 vol. methanol, 7 vol. dH₂O</td>
</tr>
<tr>
<td>Equilibration buffer</td>
<td>0.025M sodium acetate, 20mM cysteine, 10mM EDTA, 0.02% sodium azide, pH to 6.2 with NaOH and acetic acid</td>
</tr>
<tr>
<td>Hanks' buffered saline solution (HBSS) Purchased from Life Technologies</td>
<td>0.185gL⁻¹CaCl₂·2H₂O, 0.4gL⁻¹KCl, 0.06gL⁻¹KH₂PO₄, 0.1gL⁻¹MgCl₂·6H₂O, 0.1gL⁻¹MgSO₄·7H₂O, 8gL⁻¹NaCl, 0.35gL⁻¹NaHCO₃, 0.048gL⁻¹Na₂HPO₄, 1gL⁻¹D-Glucose, 0.01gL⁻¹Phenol Red c</td>
</tr>
<tr>
<td>Laemmli running buffer</td>
<td>3g Tris, 14.4g Glycine, 1g SDS per litre pH 8.9</td>
</tr>
<tr>
<td>Laemmli sample buffer</td>
<td>62.5mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 2.5% (v/v) glycerol, 2.5mM dithiothreitol (DTT), trace of bromophenol blue (BPB)</td>
</tr>
<tr>
<td>MES-buffered saline</td>
<td>25mM MES, 0.15M NaCl, pH 6.5</td>
</tr>
<tr>
<td>MOPS cell wash</td>
<td>146mM NaCl, 20mM MOPS-NMDG pH 7.4</td>
</tr>
<tr>
<td>MOPS lysis buffer</td>
<td>10mM MOPS-NMDG pH 7.4, 2.5mM MgCl₂, 1mM CaCl₂</td>
</tr>
<tr>
<td>0.1M Phosphate buffer pH 8 for acetylcholinesterase assay</td>
<td>186.4ml 0.2M Na₂HPO₄, 13.6ml 0.2M NaH₂PO₄, 200ml dH₂O</td>
</tr>
<tr>
<td>Phosphate buffer for ghosts</td>
<td>5mM Na₂HPO₄ made pH 8 with 5M NaOH</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS)</td>
<td>0.0015M NaH₂PO₄, 0.02M Na₂HPO₄, 0.15M NaCl, pH 7.4</td>
</tr>
<tr>
<td>Tris buffer</td>
<td>TrisCl pH to 7.4 with HCl</td>
</tr>
<tr>
<td>Tris buffered saline (TBS)</td>
<td>146mM NaCl, 20mM TrisCl pH 7.4</td>
</tr>
<tr>
<td>Tris lysis buffer</td>
<td>10mM TrisCl pH 7.4, 2.5mM MgCl₂, 1mM CaCl₂</td>
</tr>
</tbody>
</table>
3.3 SPECIFIC REAGENTS

Table 3.2 lists the specific reagents and their sources if not from Sigma-Aldrich.

It also contains information as to how the reagents were made up.

Table 3.2 Table of specific reagents: Source and how stock solutions are made up.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source if not from Sigma</th>
<th>Concentration and solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For protein determination</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bicinchoninic acid solution (BCA)</td>
<td></td>
<td>4% (w/v)</td>
</tr>
<tr>
<td>4% (w/v) CuSO₄·5H₂O</td>
<td></td>
<td>4% (w/v)</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td></td>
<td>standard solution (1mg/ml).</td>
</tr>
<tr>
<td>acrylamide</td>
<td>Design a gel</td>
<td>30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide stock solution.</td>
</tr>
<tr>
<td><strong>Imidoesters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMA (dimethyl adipimidate dihydrochloride)</td>
<td>Pierce [Cheshire, UK]</td>
<td>2mM in HBSS</td>
</tr>
<tr>
<td>DMP (dimethyl pimelimidate dihydrochloride)</td>
<td></td>
<td>2mM in HBSS</td>
</tr>
<tr>
<td><strong>Raft disrupters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saponin</td>
<td>dissolved in Tris lysis buffer at a concentration of 5mg/ml</td>
<td></td>
</tr>
<tr>
<td>Digitonin</td>
<td>dissolved in Tris lysis buffer at a concentration of 768mg/ml</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 continued

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source if not from Sigma</th>
<th>Concentration and solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filipin</td>
<td></td>
<td>50mg/ml (76.5mM) in ethanol then further diluted in TBS so final stock solution was 0.5mg/ml</td>
</tr>
<tr>
<td>MBCD</td>
<td></td>
<td>dissolved in Tris lysis buffer at a concentration of 100mM</td>
</tr>
<tr>
<td>Nystatin</td>
<td></td>
<td>5mg/ml stock made up in methanol and spun to clear</td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leupeptin</td>
<td></td>
<td>1M stock solution in 10mM Tris pH 7.4</td>
</tr>
<tr>
<td>Benzamidine</td>
<td></td>
<td>1M stock solution in 10mM Tris pH 7.4</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td></td>
<td>1mg/ml stock solution in methanol</td>
</tr>
<tr>
<td>DFP</td>
<td></td>
<td>0.5M stock solution in dry isopropanol</td>
</tr>
<tr>
<td>TLCK</td>
<td></td>
<td>10mg/ml in dH₂O</td>
</tr>
<tr>
<td>DTT</td>
<td></td>
<td>1M stock in dH₂O</td>
</tr>
<tr>
<td><strong>Protein kinase inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Calbiochem [San Diego, California, USA]</td>
<td>1μg/μl (2.14mM) stock solution made up in DMSO</td>
</tr>
<tr>
<td>A3 hydrochloride</td>
<td>Calbiochem</td>
<td>2mM stock made up in DMSO</td>
</tr>
<tr>
<td>H9</td>
<td>Calbiochem</td>
<td>2mM stock made up in DMSO</td>
</tr>
<tr>
<td>Genestein</td>
<td>Calbiochem</td>
<td>2mM stock made up in DMSO</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>Calbiochem</td>
<td>2mM stock made up in DMSO</td>
</tr>
</tbody>
</table>
Table 3.2 continued

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source if not from Sigma</th>
<th>Concentration and solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine 5(^\prime)-triphosphate (ATP) disodium salt</td>
<td>Boehringer-Mannheim GmbH</td>
<td>50mM stock pH 7.4 Tris lysis buffer adjust pH with Tris dissolved in Tris lysis buffer. MOPS was used in place of Tris for DMA and membranes experiment.</td>
</tr>
<tr>
<td>Tris ATP</td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td>ATP(_\gamma)S</td>
<td>Boehringer-Mannheim GmbH</td>
<td>as above</td>
</tr>
<tr>
<td>Inosine 5(^\prime)-triphosphate (ITP) trisodium salt</td>
<td></td>
<td>as above</td>
</tr>
<tr>
<td><strong>Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D13-7</td>
<td>Made by Dr MM Ho and Dr R Graham, [Rayne Institute, University College London]</td>
<td>500(\mu)g/ml</td>
</tr>
<tr>
<td>120-45 GST</td>
<td>as above</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 continued

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Source if not from Sigma</th>
<th>Concentration and solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Molecular weight markers</td>
<td>Amersham Pharmacia Biotech [Buckinghamshire, UK]</td>
<td></td>
</tr>
<tr>
<td>(high and low molecular weights)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sodium orthovanadate</td>
<td>Gift from Prof. Anthony Segal [Rayne Institute, University College London]</td>
<td>2mM in dH₂O pH10</td>
</tr>
<tr>
<td>N-ethylmaleimide (NEM)</td>
<td></td>
<td>200mM in ethanol</td>
</tr>
</tbody>
</table>
3.4 ANTIBODIES AND PROTEINS

Table 3.3 provides information about antibodies used.

### Table 3.3 Table detailing the antibodies used in this thesis

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Type</th>
<th>Specificity</th>
<th>Purchased/ gift from</th>
<th>Supplied as (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary antibodies</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-actin (C-11)</td>
<td>goat</td>
<td>Polyclonal IgG</td>
<td>C-terminus of human actin</td>
<td>Santa Cruz Biotechnology [Santa Cruz, California, USA]</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>anti-annexin V</td>
<td>goat</td>
<td>Polyclonal IgG</td>
<td>C-terminus of annexin V</td>
<td>Santa Cruz</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>anti-annexin VI</td>
<td>rabbit</td>
<td></td>
<td></td>
<td>Prof. Anthony Segal [Rayne Institute, University College London]</td>
<td></td>
</tr>
<tr>
<td>anti-aquaporin-1 (L-19)</td>
<td>goat</td>
<td>Polyclonal IgG</td>
<td>C-terminus of human aquaporin-1</td>
<td>Santa Cruz</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>anti-band 2.1 (ankyrin)</td>
<td>rabbit</td>
<td>Polyclonal IgG</td>
<td>human red cell band 2.1</td>
<td>J Pinder (Fordham) [MRC Muscle and Cell Motility Unit, Randall Centre for Molecular Mechanisms of Cell Function, King's College London]</td>
<td></td>
</tr>
<tr>
<td>anti-band 3</td>
<td>rabbit</td>
<td>Polyclonal IgG</td>
<td>human red cell band 3</td>
<td>J Pinder</td>
<td></td>
</tr>
<tr>
<td>anti-band 4.1</td>
<td>rabbit</td>
<td>Polyclonal IgG</td>
<td>human red cell band 4.1</td>
<td>J Pinder</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Species</td>
<td>Type</td>
<td>Specificity</td>
<td>Purchased/ gift from</td>
<td>Supplied as (if known)</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>---------</td>
<td>-----------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------------------------------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>anti-clathrin heavy chain (C-20)</td>
<td>goat</td>
<td>Polyclonal IgG</td>
<td>C-terminus of human clathrin heavy chain</td>
<td>Santa Cruz</td>
<td>200μg/ml</td>
</tr>
<tr>
<td>anti-flotillin</td>
<td>mouse</td>
<td>Monoclonal IgG</td>
<td>reactive against human, rat and mouse flotillin</td>
<td>Transduction Laboratories [Exeter, UK]</td>
<td>250μg/ml</td>
</tr>
<tr>
<td>anti-glycophorin A</td>
<td>mouse</td>
<td>Monoclonal IgG</td>
<td>human red cell glycophorin A</td>
<td>J Pinder</td>
<td></td>
</tr>
<tr>
<td>anti-GST (glutathione S transferase)</td>
<td>rabbit</td>
<td>Polyclonal IgG</td>
<td>human GST expressed from plasmid pGEX-2T</td>
<td>Dr DL Rimm, [Department of Pathology, Yale University School of Medicine]</td>
<td></td>
</tr>
<tr>
<td>anti-spectrin α</td>
<td>rabbit</td>
<td>Polyclonal IgG</td>
<td>human red cell α spectrin</td>
<td>J Pinder</td>
<td></td>
</tr>
<tr>
<td>anti-stomatin (see below)</td>
<td>rabbit</td>
<td>Polyclonal IgG, affinity purified against maltose binding protein fusion protein containing C-terminus stomatin (amino acids 144-288)</td>
<td>D13-7 (GST bacterially expressed fusion protein containing C-terminus of human stomatin, amino acids 144-288)</td>
<td>Dr MM Ho and Dr R Graham</td>
<td>300μg/ml</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

<table>
<thead>
<tr>
<th>Name</th>
<th>Species</th>
<th>Type</th>
<th>Specificity</th>
<th>Purchased/ gift from</th>
<th>Supplied as (if known)</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-goat</td>
<td>donkey</td>
<td>Polyclonal IgG, HRP-conjugate</td>
<td>goat IgG</td>
<td>Santa Cruz</td>
<td>100μg/ml</td>
</tr>
<tr>
<td>anti-mouse</td>
<td>sheep</td>
<td>IgG, HRP-conjugate</td>
<td>mouse IgG</td>
<td>Amersham Pharmacia Biotech [Buckinghamshire, UK]</td>
<td>500μg/ml</td>
</tr>
<tr>
<td>anti-rabbit</td>
<td>donkey</td>
<td>IgG, HRP-conjugate</td>
<td>rabbit IgG</td>
<td>Amersham</td>
<td>500μg/ml</td>
</tr>
</tbody>
</table>
3.4.1 Anti-stomatin antibody

The rabbit polyclonal antibody was raised by making serial injections of a GST fusion protein containing amino acids 141-288 (the C-terminal portion) of the full stomatin protein. After high titres were achieved, the serum was harvested and affinity purified against a column to which the same stomatin domain fused with a maltose binding protein fusion partner was bound (Coles et al., 1999b). I am grateful to Dr. MM Ho, Dr. R Graham and Mrs. MC Chetty for supplying the antibody.

3.4.2 Anti-stomatin Fab fragments

Fab fragments are the univalent fragments of antibody molecules. Firstly, 2ml protein G beads (IgG Fc receptor Type III) with a binding capacity of 29mg IgG were washed three times in PBS. The resulting packed resin was added to 2ml of anti-stomatin rabbit serum which had a protein concentration of 12.6mg/ml. This was incubated for 15 minutes at room temperature to allow the IgG to bind, pelleted by centrifugation and the supernatant aspirated. The bound IgG was eluted from the resin by temporary acidification with 100mM glycine-Cl, pH 2.7 (3ml) and the resin vortexed for 30 seconds before peletting by centrifugation. The supernatant was collected and immediately neutralised with 1M Tris base.

The elution step was repeated three times and the neutralised supernatant was
dialysed overnight at 4°C against 500ml PBS containing 0.02% sodium azide.

The original serum and the eluates were loaded onto a gel and subjected to SDS-PAGE to confirm the presence of IgG molecules.

Papain was used to cleave the IgG molecules into Fab and Fc fragments. The conditions for the incubation of papain were determined empirically. The pooled eluates (4 ml) were incubated with 150μl packed washed papain at 37°C for 3 hours. The eluate before and after papain treatment was subjected to SDS-PAGE with reducing and non-reducing sample buffer (not containing DTT) and stained with Coomassie blue. This confirmed the presence of Fab and Fc fragments in the eluate after treatment with papain. Fc fragments were removed by addition of iodoacetamide to a final volume of 75mM.

The resulting Fab fragments were subjected to a column containing D13-7 kindly donated by Dr R Graham to isolate anti-stomatin Fabs and was eluted off with Tris and glycine. Anti-stomatin activity was confirmed by probing of Western blots of membranes, which gave a strong signal at a dilution of 1:100.

3.4.3 Stomatin protein

D13-7 is a GST (glutathione-S-transferase)-stomatin fusion protein containing amino acids 144-288 (end) of the mature stomatin protein i.e. the C-terminus. It differs from wild-type stomatin by one amino acid: Thr255 is changed to an
alanine (Stewart et al., 1992). 120-45 GST is exactly the same as D13-7 except
that there is threonine at 255, as in the normal sequence (Stewart et al., 1992).

3.5 GENERAL METHODS

3.5.1. Blood collection

Patient and control blood was collected into citrate phosphate dextrose adenine
(CPDA) for cryohydrocytosis patients and heparin for all other patients. Patients
had not been transfused in the past 6 months prior to having their blood taken for
experimental purposes. Experiments were performed within 2 days of blood
collection. A control sample collected into the same anti-coagulant was always
taken at the same time.

3.5.2. Protein gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was
performed using the method of Laemmli (1970). Mini-gels were cast using
Hoefer Mighty Small II multi gel caster. Gels varied in percentage of
polyacrylamide according to requirement and the precise concentrations are given
in the Figure legends. The resolving gels were made up according to table 3.4 for
the percentages indicated. Gradient gels were made up according to table 3.5 (one
mini gel) and were poured using a gradient maker (Hoefer SG50) and pump
(Miniplus 2 Gilson) to provide a continuous 7 to 17% gradient. The solutions
were mixed before adding TEMED and were immediately poured. The gels were overlayed with water-saturated butanol and left to set. After setting, the butanol was washed off for 10 minutes under running water and the stacking gel was poured on top. Table 3.6 shows the composition of a single 4% stacking gel.
### Table 3.4 Composition of 100ml of resolving gel vol. (ml)

<table>
<thead>
<tr>
<th></th>
<th>7%</th>
<th>9%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris 1.5M pH 8.8</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>23.3</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>Ammonium</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>persulphate (APS) (1.5%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>49</td>
<td>41.5</td>
<td>31.5</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### Table 3.5 Composition of one 7-17% gradient mini gel vol. (ml)

<table>
<thead>
<tr>
<th></th>
<th>17</th>
<th>7%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 8.9</td>
<td>1.39</td>
<td>1.39</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>2.1</td>
<td>0.88</td>
</tr>
<tr>
<td>APS (1.5%)</td>
<td>0.22</td>
<td>0.1</td>
</tr>
<tr>
<td>water</td>
<td>0</td>
<td>1.36</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.037</td>
<td>0.037</td>
</tr>
<tr>
<td>sucrose</td>
<td>0.37g</td>
<td>0</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.003</td>
<td>0.003</td>
</tr>
</tbody>
</table>

### Table 3.6 Composition a single 4% stacking gel vol. (ml)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Vol. (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris pH 6.9</td>
<td>0.625</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>0.5</td>
</tr>
<tr>
<td>1.5% APS</td>
<td>0.25</td>
</tr>
<tr>
<td>water</td>
<td>3.8</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.05</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005</td>
</tr>
</tbody>
</table>
3.5.3. Loading of gels

Samples were electrophoresed alongside high and/or low molecular weight markers on polyacrylamide gels (acrylamide from Design a gel).

3.5.4. Staining of gels

*Coomassie blue staining*

Coomassie brilliant blue R250 (0.2%), methanol (45%) and acetic acid (10%) were mixed for 2 hours before filtration. This filtrate was used to stain gels. Gels were incubated in excess quantities of stain overnight and destained in a solution of 25% methanol and 10% ethanol until the background was clear.

*Silver staining*

Gels were fixed overnight in 50% methanol, 12% acetic acid. This step was followed by 3x 20-minute washes in 50% ethanol. Gels were pretreated for 1 minute in a solution containing 0.2g/l sodium thiosulphate, followed by 3x 20-second rinses in water. They were then stained with 0.1% silver nitrate for 20 minutes. After rinsing twice in water for 20 seconds, gels were developed with a solution composed of 3% (w/v) sodium carbonate, 0.00925% formaldehyde and 2mg/l sodium thiosulphate until the bands were visible. The longer a gel was left to develop, the more likely that low abundance bands became visible. However, if gels were left too long, levels of background staining, as other parts of the gel
become exposed, could become excessive. Following development, gels were rinsed twice for 2 minutes in water before development was stopped by incubation in 50% methanol, 12% acetic acid for 10 minutes. Gels were then washed for 20 minutes in 50% methanol before reswelling and storing in 5% methanol. Gels were scanned, dried or digested for matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (see section 3.12.6).

3.5.5. Drying of gels

After staining, gels were either digested for MALDI-TOF analysis or were dried. Before drying, gels were treated for 2 hours in 4% (v/v) methanol and 5% (v/v) glycerol. The gels were then sandwiched in cellophane drying film (Amersham Pharmacia Biotech), assembled in a drying frame (Amersham Pharmacia Biotech) and left to dry at room temperature overnight.

3.5.6. Western blotting protocol

Immunoblotting was conducted with a Transblot SD-Semi Dry Transfer Cell (Biorad) electroblotter using Immobilon P poly (vinylidene difluoride) (PVDF) membranes (Hybond™-P Amersham Pharmacia Biotech [Buckinghamshire, UK]) prewetted in methanol. Western blot transfer was performed at 100mA per minigel for 1 hour. Membranes were then blocked for 1 hour in 5% Marvel dried
skimmed milk and 2% bovine serum albumin in PBS with 0.05% (w/v) Tween 20. This block was also present during the primary and secondary antibody incubations. Antibody dilutions were determined empirically and membranes were incubated in them overnight at 4°C. The primary antibodies mouse anti-human flotillin-1, goat anti-human actin, goat anti-annexin V, goat anti-aquaporin-1, goat anti-human clathrin heavy chain and mouse anti-glycophorin A were all used at 1:500. Rabbit anti-annexin VI was used at 1:1000. Rabbit anti-stomatin was used at 1:20000. Anti-band 3, band 4.1, ankyrin (band 2.1), spectrin α (all rabbit antibodies) were used at 1:750. After the primary antibody incubation, two 5-minute washes and two 20-minute washes were performed in PBS containing 0.05% Tween 20. The membranes were incubated with secondary antibodies for 1 hour at room temperature (anti-mouse (1:2000), anti-rabbit (1:2000) and goat (1:2000)). This was followed by two 5 minute washes and two 20 minute washes in PBS containing 0.05% Tween 20. Enhanced chemiluminescence was used as the detection system (ECL Amersham Pharmacia Biotech [Buckinghamshire, UK]) and blots were exposed to Hyperfilm™. Films were developed in a Velopex MD2000 automated developer (Medivance) or by hand.
3.5.7. Scanning of blots and gels

Gels and blots were scanned using a Linocolour 1200 scanner using Linocolor Elite 6.0 or Adobe Photoshop 5 software.

3.5.8. Methods for the measurement of acetylcholinesterase (AChE)

Endocytic vesiculation was measured using AChE as a red cell surface marker based on Ellman's method (Ellman et al., 1961) as used by others (Jarrett and Penniston, 1976; Allan and Walkin, 1988). AChE is a GPI-anchored protein and hence can also be used as a marker of rafts (Salzer and Prohaska, 2001).

The method used was adapted from others who used AChE as a marker for endocytosis and was initially set up to be performed using cuvettes and a spectrophotometer. This was then adapted to a plate reader method. In the initial assay, plastic cuvettes containing 3mls of 83.3μM DTNB, 125μM sodium bicarbonate and 0.1M phosphate buffer, pH 8, mixed immediately before the assay, were prepared containing cuvette stirrers. To this was added 4μl ghost suspension, which was allowed to warm for 2 minutes to 37°C and mix in a double-beam recording spectrophotometer (Shimadzu model UV-3000). A cuvette containing vehicle without ghosts was used as the blank. The settings used were as follows: λs = 5, scan speed = 10, absorbance measured at 412nm (wavelength), optical density (OD) = 0-0.5. The spectrophotometer was zeroed.
and 20μl of 0.075M acetylthiocholine iodide was added to the cuvette (final concentration = 0.5mM). The change in absorbance was measured against time.

The gradient of the first 4 minutes of the straight line was measured. The gradient obtained corresponded to the AChE activity and this was measured both for the ATP and ITP condition. The percentage loss of AChE activity in the presence of ATP compared with that in the presence of ITP (control) was calculated and endocytic vesiculation was estimated using the equation:

\[
\text{Endocytic vesiculation} = (1 - \frac{\Delta A (\text{ATP})}{\Delta A (\text{ITP})}) \times 100\%
\]

where \(\Delta A\) = change in absorbance.

**Use of Triton X-100**

Many of the original reports (Jarrett and Penniston, 1976; Birchmeier et al., 1979; Allan and Walkin, 1988) of the AChE assay used Triton X-100 to lyse the cells to determine total AChE. It was found here that Triton inhibited AChE activity (not shown). Other detergents (saponin, nonidet P40, Brij-96) were tested in an attempt to minimise interference with the assay, but none of the detergents tested was satisfactory.

**96 well plate method**

The technique was adapted to a 96-well method. This was advantageous since it allowed experiments with numerous conditions to be performed rapidly. Samples
(10µl) were pipetted into a 96 well plate in quadruplicates. Eight wells contained 10µl vehicle without ghosts (Tris lysis buffer) and were used as a baseline.

Immediately, a solution containing final concentrations of 83.3µM DTNB, 125µM sodium bicarbonate and 0.5mM acetylthiocholine iodide in 0.1M phosphate buffer pH 8 was mixed and 200µl of this mixture was pipetted into each well. The plate was placed in the plate reader [Anthos labtech instruments] and set to shake hard at the beginning and every 2 minutes and to read the absorbance at 405nm every 2 minutes for 30 minutes. Gradients for the change in absorbance over time for each well were calculated. Quadruplicate samples were averaged (the SEM typically ranging from 1-2% of this mean) and the mean baseline gradient subtracted to obtain AChE activity.

3.5.9. Measurement of alkaline phosphatase activity

Alkaline phosphatase activity was measured as described by Parkin et al. (1999b). A standard working solution of p-nitrophenol was made up by diluting 20µl of 10mM stock to 1ml with distilled water. Zero (blank), 20, 40, 60 and 100µl aliquots of the standards were pipetted into individual wells in a 96-well microtitre plate and the volumes made up to 100µl with distilled water. Unknown samples (100µl) were also pipetted into the microtitre plate. Samples were then incubated in alkaline phosphate buffer/substrate (100µl) (see table 3.1 for
composition) at 37°C until the absorbance (at 405nm) of the samples fell within the calibration range of the standard wells.

3.5.10. Determination of protein concentration using the Micro bicinchoninic acid (BCA) method

The BCA and 4% (w/v) CuSO$_4$·5H$_2$O solutions were mixed at a 50:1 ratio to give the working protein reagent. BSA standards (0 (blank), 2, 4, 6, 8, and 10μg) and 10μl of the sample were pipetted into separate wells of a 96-well microtitre plate. Then 200μl of the working protein reagent was added to each well and plates were then incubated at 37°C for 30 minutes. Absorbance was determined at a wavelength of 570nm.

3.5.11. Measurement of ouabain+bumetanide-resistant K$^+$ influx

Ouabain+bumetanide-resistant K$^+$ influxes (flux) measurements were performed by Mrs. MC Chetty and Miss. H Jarvis using methods used previously in this laboratory (Coles et al., 1999a; Coles et al., 1999b).

3.6 PREPARATION OF PHOSPHATE-FREE GHOSTS

Patient and control blood was washed three times in TBS and resuspended at a haematocrit of 50%. Phosphate-free ghosts were prepared by a method adapted from Pennistion and Green (1968). One volume of the cell suspensions was lysed in 20 volumes of cold Tris lysis buffer and incubated on ice. This was left at 2°C
for 10 minutes before the ghosts were pelleted by centrifugation (35 minutes, 20,000g, 2°C) using the 12158 rotor in the 3K30 sigma centrifuge. The supernatant was removed and 1 volume of packed ghosts was resuspended in 50 volumes of Tris lysis buffer.

3.7 INDUCTION OF ATP-DEPENDENT ENDOCYTIC VESICULATION

Identical aliquots of very well mixed ghost suspension from each of the conditions were added to make final concentrations of 0-12.5mM ATP or ITP (control). In some cases only one concentration of nucleotide, 5mM (which was found to cause maximal endocytic vesiculation), was assayed. Suspensions were mixed and incubated on ice for 10 minutes to allow the ATP or ITP to penetrate the ghosts before they were resealed by warming to 37°C and incubating for 35 minutes. ATP and ITP will chelate Mg^{2+} and hence reduce the concentration of free, ionised Mg^{2+} in solution. ITP was used to control for any effects due to the reduction of the Mg^{2+} concentration. Endocytic vesiculation was measured by reduction in AChE activity as described in section 3.5.8.

3.8 TRANSMISSION ELECTRON MICROSCOPY (EM)

Ghosts were fixed by resuspension at 10% haematocrit in 2% glutaraldehyde and 50mM Na-cocodylate pH 7.4. Transmission EM was performed by Dr. GV
Landon and Dr. PS Rowley [Department of Histopathology, University College London].

3.9 INCUBATION OF WHOLE ERYTHROCYTES WITH CHEMICALS

3.9.1. DMA and DMP

This method was modified from Mentzer et al. (1978): erythrocytes were washed three times in PBS before resuspension in 0-6.3mM DMA or sucrose in Hanks' balanced salt solution (HBSS) at a haematocrit of 5%. The cells were incubated at 37°C for 30 minutes and then washed 3 times in TBS to stop the reaction (due to the reaction being quenched by a primary-amine-containing solution). The final pellet was diluted to 50% haematocrit in TBS and one aliquot was used to prepare ghosts for measurement of ATP-dependent endocytic vesiculation whilst another was used for the measurement of the ouabain+bumetanide-resistant K⁺ influx.

3.9.2. Raft disrupters

Normal erythrocytes were washed three times in TBS before resuspension at 2% haematocrit in TBS with compounds known to disrupt lipid rafts (Hooper, 1999). Agents used were: MBCD (10mM, 37°C, 30 minutes), nystatin (0-5μg/ml, room temperature in the dark, 30 minutes), filipin (7.5μg/ml, 37°C, 30 minutes) for the endocytosis assays and filipin (0-100μg/ml, 37°C, 30 minutes) or nystatin (0-500ng/ml, room temperature in the dark, 30 minutes) for measurement of
ouabain-bumetanide-resistant K\(^+\) influx. After incubation, treated and untreated (containing the vehicle compounds were dissolved in) erythrocytes were washed three times in TBS and phosphate-free ghosts were prepared and ATP-dependent endocytic vesiculation was measured using 0-12.5mM ATP for experiments with a constant concentration of raft disrupter and 5mM ATP for experiments with variable concentrations of raft disrupter. An aliquot of blood was used to measure ouabain-bumetanide-resistant K\(^+\) influx for each condition in the nystatin and filipin experiments.

3.10 METHODS TO MEASURE THE EFFECT OF INCUBATING GHOSTS WITH VARIOUS CHEMICALS ON ATP-DEPENDENT ENDOCYTIC VESICULATION IN ERYTHROCYTE GHOSTS.

Packed ghosts were diluted 1:50 in Tris lysis buffer. Various reagents were added at 4\(^\circ\)C and ATP dependent endocytic vesiculation was measured.

3.10.1. Antibody experiments

Phosphate-free ghosts were prepared as described in section 3.5. The supernatant was removed and 1 volume of packed ghosts was resuspended in 50 volumes of Tris lysis buffer. Antibodies were added as shown in table 3.3 and left to incubate on ice for 30 minutes before the addition of ATP or ITP. ATP-dependent endocytic vesiculation was measured from 0-12.5mM ATP.
Table 3.7 Antibodies incubated with the ghosts

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Highest concentration tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-stomatin</td>
<td>1.5μg/ml (1:200 dilution)</td>
</tr>
<tr>
<td>anti-GST</td>
<td>1.5μg/ml (1:200 dilution)</td>
</tr>
<tr>
<td>anti-flotillin-1</td>
<td>1.25μg/ml (1:200 dilution)</td>
</tr>
<tr>
<td>anti-annexin VI</td>
<td>1μg/ml (1:200 dilution)</td>
</tr>
<tr>
<td>anti-aquaporin-1</td>
<td>1μg/ml (1:200 dilution)</td>
</tr>
<tr>
<td>anti-spectrin α</td>
<td>unknown (1:200 dilution)</td>
</tr>
<tr>
<td>anti-actin</td>
<td>1μg/ml (1:200 dilution)</td>
</tr>
</tbody>
</table>

After initial studies showing that ATP-dependent endocytic vesiculation was inhibited by anti-stomatin antibody at dilutions in excess of a 1: 2000 dilution (150ng/ml), anti-stomatin antibody was added to the ghosts in concentrations ranging from 0 to 0.148ng/ml to find a concentration which inhibited endocytic vesiculation but did not saturate. This was found to be 63ng/ml. Controls were without antibody and in the presence of an anti-GST rabbit “isotype control” antibody (1.5μg/ml).

Blocking with stomatin protein

Stomatin protein in the form of D13-7 was added using concentrations ranging from 0 to 2.5μg/ml. This was incubated for 10 minutes on ice before addition of
63ng/ml anti-stomatin antibody to each condition. Controls were no antibody or 63ng/ml rabbit GST antibody. Antibodies were incubated on ice for a further 30 minutes before addition of ATP or ITP.

3.10.2. Raft disrupters

Ghosts were incubated for 30 minutes on ice with the following raft disrupters in separate experiments: digitonin (0-80μM), saponin (0-625μg/ml), MBCD (0-12.5mM), filipin (0-250μg/ml). ATP or ITP was added and ATP-dependent endocytic vesiculation was measured.

3.10.3. Other compounds

The effects of protein kinase inhibitors (1.07mM staurosporine, 10μM A3 hydrochloride, 10μM H9, 10μM genestein, 10μM wortmannin) 1mM sodium orthovanadate, and 1mM N-ethyl maleimide (NEM) on endocytic vesiculation were investigated using fixed concentrations of these compounds. These chemicals were added to the ghosts and ATP or ITP was immediately added and ATP-dependent endocytic vesiculation was measured. Compounds, which showed a significant effect, were investigated further. These were NEM, staurosporine and sodium orthovanadate.
3.10.4. ATP γS

ATP γS (5mM) was used in place of ATP in order to determine if cleavage of the phosphate bond is necessary for ATP-dependent endocytic vesiculation to occur.

3.11 METHODS TO PREPARE RAFTS IN ERYTHROCYTES

3.11.1. Washing the blood

Fresh heparinised blood was taken from controls and patients and was washed three times in PBS. The buffy coat was removed and discarded. Packed red cells were resuspended in 10 volumes of MBS.

3.11.2. Preparation of membranes

Membranes could not be made by the normal method, which uses EDTA, since this is thought to disrupt the rafts (Parkin et al., 1996a; Parkin et al., 1996b). Therefore, membranes were made by lysing 1 volume of packed red cells in 100 volume of hypotonic phosphate buffer (5mM Na₂HPO₄, 5mM NaH₂PO₄, pH 8 with NaOH) and centrifuging 3-4 times at 12,000g at 4°C taking off as much of the supernatant as possible each time. The pellet was resuspended in MBS and a protein assay was performed. Membranes were used at a protein concentration of 0.4mg/ml.
3.11.3. Sucrose gradients for detergent based isolation of DIGs

The methods used were adapted from the Hooper laboratory (Parkin et al., 1999b) and were performed at 0°C or on ice unless stated otherwise. Conditions were optimised for raft separation in erythrocytes or erythrocyte membranes. This is described in chapter 7. After optimisation of conditions, rafts were prepared as follows:

One volume of erythrocytes at 10% haematocrit were added to one volume of 1% Triton X-100 in MBS (or one volume of membranes at a protein concentration of 0.4mg/ml were added to one volume of 0.5% Triton X-100 in MBS), mixed and left to stand on ice for 10 minutes. This was followed by 30 second sonication, split into six 5 second bursts at an amplitude of 9 microns on a ‘Soniprep’ 150 sonicator. The resulting suspension was then added to two volumes of 90% sucrose in MBS to a final sucrose concentration of 45%. A discontinuous sucrose gradient was prepared in MBS: In 12ml Beckman ultracentrifuge tubes, solutions were layered so that 4mls of the solubilized sample in 45% sucrose was at the bottom, 4 mls of 39% sucrose in the middle and 4 mls of 5% sucrose at the top of the tube. The ultracentrifuge tubes were placed in a Beckman SW41 rotor and centrifuged at 140,000g for 18 hours (Beckman Optima™ LE-80K) at 0°C. This was repeated 3 times for each sample.
In certain experiments (all experiments described in chapters 7 and 8, but not those in chapter 6), protease inhibitors were employed to prevent protein degradation (see Chapter 9, section 9.4.1). Protease inhibitors were added throughout the experiment to the final concentrations of: TLCK 10μg/ml, DTT 1mM, leupeptin 10μg/ml, aprotonin 10μg/ml, benzamidine hydrochloride 1mM, pepstatin A 1μg/ml, DFP 0.5mM. Since the work described in chapter 8 coincided with experiments presented in chapter 7, protease inhibitors were also added in experiments performed here.

3.11.4. Harvesting fractions

The sucrose gradient was harvested in 1.2 ml fractions from bottom to top, with fraction 0 representing the insoluble pellet at the base of the tube resuspended in MBS, fraction 1 representing the bottom of the tube and fraction 10 the top. A single light-scattering band centred on fractions 6-7 was observed within the 5-39% (w/v) sucrose region of the gradient corresponding to the DIG fraction. The bulk of the solubilized membrane protein was left in the 45% (w/v) sucrose region at the base of the gradient (Figure 3.1).
Isolation of DIGS by solubilization in nonionic detergent followed by buoyant density centrifugation. Cells/ membranes were solubilized in Triton X-100 sonicated on ice for 30 seconds and adjusted to 45% (w/v) sucrose by adding an equal volume of 90% sucrose. The samples were then layered under a 5-45% (w/v) discontinuous sucrose gradient and centrifuged for 18 hours at 140,000g. DIGs migrated up the sucrose gradient leaving the bulk of the solubilized membrane protein in the 45% (w/v) sucrose region at the base of the gradient.
3.12 METHODS TO EXAMINE THE PROTEINS IN THE RAFTS

Fractions 0-10 and the concentrated rafts (see below) were analysed by colorimetric assays, SDS-PAGE, Coomassie blue staining, silver staining, MALDI-TOF analysis and Western blotting.

3.12.1. Assays for known raft proteins

Proteins expected to be associated with the raft fraction were measured by Western blotting (flotillin) and colorimetric enzyme assay (alkaline phosphatase and AChE). Only fractions made from membrane samples were subjected to colorimetric assays since the haemoglobin would have interfered with fractions made from whole erythrocytes. Alkaline phosphatase activity was measured as described in section 3.5.9 and AChE activity was determined as described in section 3.5.8.

3.12.2. Assays to determine the presence of known red cell proteins in the rafts

Although the rafts are in fractions 5-8 of the sucrose gradient, all fractions were assayed to determine the proportion of proteins in the rafts compared with the Triton-soluble bottom of the gradient. Fractions 0-10 from sucrose gradients were loaded onto gels alongside high and low molecular weight markers. Proteins investigated for raft association were spectrin α, ankyrin (band 2.1), band 3, band
4.1, actin, stomatin, aquaporin-1, clathrin heavy chain, annexin V, annexin VI, and glycophorin A using Western blotting techniques.

3.12.3. Concentration of rafts

As the bulk of the red cell membrane proteins were at the bottom of the gradient (Triton-soluble) it was necessary to concentrate the rafts in order to visualise the proteins in them on a Coomassie blue stained gel. Fractions 5-8 (which contained rafts as determined by flotillin blots and alkaline phosphatase activity) were diluted 6-fold in MBS (to reduce the sucrose concentration) containing protease inhibitors and centrifuged at 100,000g (in the SW28 rotor in Beckman UltraClear 25mm x 89mm tubes) for 90 minutes at 0°C (Beckman Optima™ LE-80K) (Parkin et al., 1999b). These were electrophoresed on 9% gels for silver staining, in-gel digestion and MALDI-TOF analysis and 12% gels for Coomassie blue staining.

3.12.4. Coomassie blue and silver stained gels of patient and control rafts

In order to analyse raft proteins in patients and controls, fractions 0-4, the concentrated rafts (the pooled and concentrated fractions 5-8) and ghosts from the patient or control were loaded on to gels and SDS-PAGE (12% gels) was conducted. Gels were stained with Coomassie blue or silver stain.
3.12.5. In-gel digestion

A cube of approximately 1mm³ was cut from each band of interest. The resulting pieces of gel were swollen three times in 100µl of 50mM NH₄HCO₃ for 15 minutes until the pH ranged between 7 and 8 as measured with universal indicator paper. Gels were cut into smaller pieces and dehydrated for 10 minutes in acetonitrile. The liquid was removed. This step was repeated 3 times. Gels were then dried in the Speedvac (Savant) for 30 minutes. Gels were rehydrated in 50µl of 100mM NH₄HCO₃ containing 10mM DTT for 30 minutes at 56°C, then dehydrated in acetonitrile as above and dried in the Speedvac for 30 minutes.

Dried gel pieces were incubated in a mixture of 5µl of 75ng/µl trypsin (Promega) and 5µl of 70% dimethylformamide (DMF) at 30°C overnight.

3.12.6. MALDI-TOF mass spectrometry

The tryptic digest (0.5µl) was spotted on the target plate and allowed to dry before adding 0.5µl matrix solution (a saturated solution of α-cyano-4-hydroxysuccinamic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid) over the top. The spots were allowed to dry and MALDI-TOF spectra were taken of the samples (Bruker Biflex III) and annotated using Bruker DataAnalysis software. The peaks identified were processed using the programmes MS-Fit (www.falcon.ludwig.ucl.ac.uk/ucsfbin3.2/msfit) and Mascot and Matrix Science.
(www.matrix.science.com) and analysed by comparison with three separate databases (OWL, NCBI and Swiss Prot). Mass tolerance was limited to relative value of +/- 200ppm (parts per million), and proteins matched according to the probability scores that were calculated by each programme.
CHAPTER 4

ATP-DEPENDENT ENDOCYTIC VESICULATION IN HEREDITARY STOMATOCYTOSES PATIENTS AND THE EFFECT OF DMA ON THIS AND THE CATION LEAK

4.1 AIM

The aim of the work to be described in this chapter was to measure ATP-dependent endocytic vesiculation in the erythrocyte membrane of a spectrum of patients with different variants of the hereditary stomatocytosis syndromes and controls. The subsidiary aim was to examine the effect of the crosslinking agent DMA on both the process of ATP-dependent vesiculation and on the cation leak.

4.2 INTRODUCTION

The work in this chapter was prompted by the early work of Mentzer et al. (1976), who found that this vesiculation process was completely absent in one American OHSt patient ('WD'). Further, in this patient, both the defect in vesiculation and the cation leak could be corrected by the crosslinking agent DMA. We sought to extend these studies to the British pedigrees available to us. By using the AChE assay (Ellman et al., 1961) and Penniston and Greens’ method for making phosphate-free ghosts resealed with ATP (Penniston and Green, 1968) ATP-dependent endocytic vesiculation was measured (Chapter 3).
The work confirms Mentzer's 1978 observation that endocytic vesiculation of the erythrocyte membrane can be totally absent in OHSt and that the addition of DMA corrects this defect and the leak (Mentzer et al., 1978).

4.3 METHODS

Table 2.1 (Chapter 2) gives the patients studied in this chapter. Phosphate-free ghosts were prepared from patients and controls (as described in methods, section 3.6) and ATP-dependent endocytic vesiculation was initiated (methods, section 3.7) and measured by the AChE assay (methods, section 3.5.8). Where indicated, erythrocytes were pre-treated with DMA or DMP (see methods, section 3.9.1).

4.4 RESULTS

4.4.1. Preliminary experiments with normal controls

Validation of AChE assay technique in normal red cell ghosts.

To check that, in this laboratory, the AChE measurements truly reflected endocytic events, AChE measurements were compared with electron microscopical studies. The data are shown in figures 4.1 (transmission EM) and 4.2 (AChE assay). In all experiments, ITP was used as a negative control for ATP. Results were expressed as percentage reduction in AChE activity in presence of ATP compared to ITP. The AChE assay showed that in this particular experiment the AChE activity was reduced by 28.5% in the ATP-treated ghosts as
compared with ITP. To confirm that this reduction could be interpreted as endocytosis, electron microscopy was performed. The electron micrographs (Figure 4.1) confirm that in the ATP-treated ghosts, numerous intracellular vesicles were present, consistent with the 28.5% reduction in the surface marker enzyme, AChE.
Ghosts were incubated with 5mM ATP or ITP and the AChE activity was measured for each and the endocytic vesiculation was calculated. In this particular experiment the reduction of AChE in the presence of ATP was 28.5%. To confirm that this disappearance could be interpreted as endocytosis, the ghosts were fixed and transmission electron microscopy was performed. This shows the appearance of the ghosts under transmission electron microscopy with and without ATP. In the control (no ATP, upper panel) no vesicles can be seen inside the ghosts. In the presence of ATP (ATP, lower panel) there are seen to be about 10-20 intracellular vesicles within the outer membrane of the cell.
Ghosts were incubated with 5mM ATP or ITP and the AChE activity was measured for each and the endocytic vesiculation was calculated. In this particular experiment the reduction of AChE in the presence of ATP was 28.5%. To confirm that this disappearance could be interpreted as endocytic vesiculation, the ghosts were fixed and transmission electron microscopy was performed (Fig 4.1).
Variations in ATP-dependent endocytic vesiculation between different normal controls and in the same normal control from day to day.

The AChE assay was used to measure ATP-dependent endocytic vesiculation in ghosts from 19 normal control individuals recruited from the laboratory. Figure 4.3a shows the mean and standard deviation endocytic vesiculation of these 19 separate experiments on control ghosts, in which ghosts were resealed in the presence of different concentrations of ATP (or ITP) and constant Mg, Ca and Tris, after which the ghosts were incubated at 37°C to allow endocytosis to occur. There was a large standard deviation. Figure 4.3b shows a typical curve from one single experiment which suggests that the large standard deviation in figure 4.3a is not due to variations within the same experiment, but more likely could be due to variations from experiment to experiment. The data presented in table 4.1 illustrates that there was both considerable day-to-day and interindividual variation. This emphasises the importance of performing a control experiment at the same time as each patient. It is worth noting that the normal individuals A-P appear to be ranked in the same order of percentage endocytic vesiculation from day-to-day. In order to give some kind of day-to-day consistency in these studies, a single individual was used as the daily internal control in most studies (control E table 4.1). This 29% mean endocytic vesiculation of individual E was close to
the 27% mean endocytic vesiculation of individuals A-P (Table 4.1). This value is consistent with previous reports (Jarrett and Penniston, 1976; Birchmeier and Singer, 1977a; Birchmeier et al., 1979).
ATP dependent endocytic vesiculation was measured at 0-12.5 mM ATP in ghosts from 19 normal individuals recruited from the laboratory. Endocytic vesiculation was measured by reduction in AChE activity using the 96 well plate method. Ghosts were made from controls and were incubated with 0-12.5 mM ATP.

A. Results from 19 control experiments. The means and standard deviations from these experiments are displayed in the graph. There is a large standard deviation.

B. A typical curve from a single control experiment. This does not appear to be very different suggesting that the large standard deviation in A is due to day-to-day or interindividual variations.
Table 4.1 Day-to-day (rows) and interindividual (columns) variations in ATP-dependent endocytic vesiculation in normal controls.

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ATP-dependent endocytic vesiculation in normal control ghosts.

ATP-dependent endocytic vesiculation was measured at 5mM ATP in ghosts from normal individuals recruited from the laboratory. Endocytic vesiculation was measured by reduction in AChE activity using the 96 well plate method. Ghosts were made from controls and were incubated with 5mM ATP.

Variations between different controls performed at the same time are shown in the 5 columns, Jg37, 40, 41, 42 and 45, each representing a separate experiment (performed on separate days). Day-to-day variations from the same individual are shown in the rows A to P, each letter representing a different individual. Letter E represents the individual who was used as a control for the patient data most of the time. The mean value of control E (29%) is close to the mean value from all the individuals (27%) showing that this was a representative control.
4.4.2. Experiments with OHSt patients

The effect of DMA on normal cells was examined initially. Washed whole erythrocytes from 8 normal controls recruited from the laboratory were incubated with 2mM DMA at 37°C for 30 min before the preparation of phosphate-free ghosts and the measurement of ATP-dependent endocytic vesiculation (Figure 4.4). The data illustrate that incubation of whole erythrocytes with 2mM DMA had no effect on ATP-dependent endocytic vesiculation.

In the vast majority of patient studies, ATP-dependent endocytic vesiculation was measured over the concentration range of 0-12.5mM ATP. However, in the preliminary OHSt patient experiments, only 5mM ATP was used (since this was the value at which maximal endocytic vesiculation occurred in normal controls). Some initial experiments using DMA or DMP (dimethyl pimelimidate) were performed at the same time. Table 4.2 presents these data. In both the Manchester and Brighton pedigrees, which are typical OHSt pedigrees, it appears that the patients had a complete defect in endocytosis. This defect was corrected by DMA, but not by DMP. Endocytosis always occurred with control erythrocyte ghosts, and was unaffected by DMA or DMP. These findings are consistent with those of Mentzer et al. (1978). DMA and DMP differ solely in the length of the carbon cross bridge. DMA has 8 carbons and DMP has 9. They have
the same reactive groups at the end. Their structures are shown in Figure 1.7 (Chapter 1).

Since it was possible that these results might have occurred because the OHSSt cells had a different affinity for ATP, it was decided to look at a wide range of ATP concentrations. Figure 4.5 illustrates absence of ATP-dependent endocytic vesiculation in the Brighton pedigree using the range from 0-12.5mM or 15mM ATP. These results confirm that ATP-dependent endocytic vesiculation does not occur to any significant extent in the Brighton pedigree at any ATP concentration up to 15mM.

Figure 4.6 shows the effect of incubating whole erythrocytes with varying concentrations of DMA on (a) the ghost endocytosis and (b) the ouabain+bumetanide-resistant K⁺ influx in control cells and those from a patient (Manchester pedigree, patient B-III-2) with OHSt. The data show that DMA corrected both the defect in endocytic vesiculation and the 'leak' K⁺ influx almost to a normal level. In the control, DMA had no effect on endocytic vesiculation (also shown in Figure 4.4) and had no effect on the 'leak' K⁺ influx (also shown in table 4.3).

To determine whether DMA can act on the washed, broken membranes, phosphate-free ghosts were incubated in the cold with 0-1 mM DMA and ATP-
dependent endocytic vesiculation at 5mM ATP was measured. MOPS lysis buffer was used to substitute for Tris lysis buffer at all stages. This was because the Tris reacts with DMA and stops it from working. Figure 4.7 shows the effect of 0-1 mM DMA on the actual membranes in control ghosts and those from another patient (Brighton) with OHSt. The DMA had no effect on ATP-dependent endocytic vesiculation when incubated with the membranes in this experiment.

The Harrow case was haematologically similar to the two original OHSt pedigrees, but not identical. The haemoglobin measurement was not as low as the others were and the MCV was not so high. The deficiency of stomatin is less than that of the original cases (Chetty, Stewart, unpublished). In this atypical OHSt Harrow case, there was no obvious defect in ATP-dependent endocytic vesiculation (Figure 4.8) though it was slightly lower than the parallel control. DMA had no effect on endocytic vesiculation in the patient's cells (Figure 4.9) and had no effect on the 'leak' K⁺ influx (Table 4.3). This showed that endocytic vesiculation can be present in OHSt cases and that if it is present, then 2mM DMA does not affect endocytic vesiculation or the flux.
Figure 4.4 Effect of 2mM DMA on ATP-dependent endocytic vesiculation in control ghosts.

Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed three times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP-dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts by reduction in AChE activity using the 96 well plate method. The graph shows the mean and standard deviation of 8 separate experiments.
Table 4.2 Maximal ATP-dependent endocytic vesiculation in OHSt and control ghosts

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>OHSt pedigree</th>
<th>Condition</th>
<th>Patient endocytic vesiculation %</th>
<th>Control endocytic vesiculation %</th>
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<tr>
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<td>+2mM DMA</td>
<td>17</td>
<td>46</td>
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<td></td>
<td></td>
<td>+6.325mM DMA</td>
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<td>33</td>
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<tr>
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<td>36</td>
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<td>14</td>
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<td></td>
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ATP-dependent endocytic vesiculation in OHSt and control ghosts. Erythrocytes were washed three times in 4°C phosphate buffered saline before resuspending them at a haematocrit of 5% in 2mM DMA, DMP or sucrose (no DMA or DMP) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS, pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the spectrophotometer.
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-15mM (upper panel) and 0-12.5mM (lower panel) at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The graphs show that ATP-dependent endocytic vesiculation was defective in this patient (Brighton pedigree, family label A).
Figure 4.6 (Expt JB8) Effect of cross-linker DMA on cation leak and ATP-dependent endocytic vesiculation in normal and OHSt red cells (Manchester, family label B, Patient B-III-2). DMA corrects both processes in OHSt cells, and has no effect in normals.

a. Endocytic vesiculation

b. Flux

Erythrocyes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 0-6.3mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. An aliquot was taken from each conditions for preparation of ghosts for the endocytosis assay (A) and an aliquot was taken for the flux study (B). The data show:

A. Absence of endocytic vesiculation in OHSt cells (as reported by AChE assay), with restoration of endocytic vesiculation (upper panel) and

B. Correction of ion leak by treatment with increasing concentrations of the cross-linker DMA (lower panel).
Figure 4.7 Test to determine if DMA was effective on unresealed OHSt red cell membranes, rather than whole cells (Brighton pedigree, family label A).

One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of MOPS lysis buffer pH 7.4 (10mM MOPS-NMDG pH 7.4, 1mM CaCl$_2$ and 2.5mM MgCl$_2$) with a final concentration of DMA ranging from 0-1mM at 4°C for 1 hour. The final concentration of ATP was made up to 5mM and the membranes were incubated at 37°C for 35 minutes, after which AChE activity was measured using the 96 well plate method. The data show that when unresealed ghosts were incubated directly with the membranes, it had no effect on ATP-dependent endocytic vesiculation in either the OHSt patient (Brighton pedigree, family label A) or the control.
Figure 4.8 (Expt jg33) ATP-dependent endocytic vesiculation in an atypical OHSt case (Harrow pedigree, family label C).

One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation was present in this stomatin-deficient patient (Harrow pedigree, family label C).
Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mMDMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this stomatin-deficient patient (Harrow pedigree, family label C) or on the control. Neither was an effect seen on the cation leak (Table 4.3).
4.4.3 Other pedigrees

Figures 4.10-4.19 show measurements of endocytic vesiculation by AChE reduction in all the other available pedigrees (letters D-M). The data show that ATP-dependent endocytic vesiculation was present in all the patients and all of the controls. In one case of cryohydrocytosis, (Figure 4.10, experiment jf17, Watford), the percent AChE activity reduction in the patient was identical to that in the control. However, in all other cases in all types of HSt, the percentage reduction in AChE was less than that in the parallel control. In 17 tests of 14 individuals with 6 distinguishable conditions, the mean ATP-dependent endocytic vesiculation at 5mM [ATP] was 21.2 ± 2.5%, while in the controls it was 31 ± 2.1% (mean ± SEM, n=17). A two-tailed paired t-test on these paired data returned the probability of 0.99 that the difference was significantly different from zero. However, it could be argued that the data is not strong enough to make a definite conclusion from this. The day-to-day and interindividual variation in controls is such, and the patients are a heterogeneous group, that it would be difficult to argue that this reduction is significant.

Figures 4.20-4.26 show the effect of incubation of whole erythrocytes from the other patients with 2mM DMA, prior to making ghosts, on ATP-dependent endocytic vesiculation. The data show that in every case, 2mM DMA
had no effect on ATP-dependent endocytic vesiculation. Table 4.3 summarises all the patient data. The table shows the percent AChE activity at 5mM ATP, in the absence and presence of DMA, with measurements of ouabain+ bumetanide-resistant ('leak') K⁺ influx on the same samples, also without and with DMA. I am grateful to Miss. H Jarvis and Mrs. MC Chetty for the flux data. The table reiterates that ATP-dependent endocytic vesiculation was completely absent in the Manchester and Brighton OHSt pedigrees and that in all of the other forms, endocytic vesiculation was present, though perhaps lower than normal. It shows that in the two original pedigrees, where there was a defect in endocytic vesiculation, both this and the flux could be corrected by DMA. In all other cases, where there was no obvious defect in endocytic vesiculation, DMA had no effect on either endocytic vesiculation or on the flux.
Figure 4.10 ATP-dependent endocytic vesiculation in a cryohydrocytosis pedigree (Bushey pedigree family label H).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5 mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation was present in these cryohydrocytosis patients (Bushey pedigree, family label H).
Figure 4.11 ATP-dependent endocytic vesiculation in a cryohydrocytosis patient (Hurstpierpoint pedigree family label L).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation was present in this cryohydrocytosis patient (Hurstpierpoint pedigree, family label L).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation was present in this cryohydrocytosis patient (Hemel Hempstead pedigree, family label F).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation was present in this cryohydrocytosis patient (Watford pedigree, family label G).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation as present in this DHSt patient (Uxbridge, Omagh pedigree, family label D).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation as present in this DHSt patient (Stondon Massey, Essex pedigree, family label E).
Figure 4.16 ATP-dependent endocytic vesiculation in a FP2 pedigree (Chiswick pedigree, family label L).

One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation as present in this FP2 patient (Chiswick pedigree, family label L).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5 mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation is present in this FP1 patient (Edinburgh pedigree, family label N).
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation is present in this patient (Blackburn pedigree, family label J).
Figure 4.19 ATP-dependent endocytic vesiculation in a unique case (Woking pedigree, family label K).

**JG11**

![Graph showing % reduction in ACrE vs [ATP]: mM for JG11.](image)

**JG23**

![Graph showing % reduction in ACrE vs [ATP]: mM for JG23.](image)

**JG25**

![Graph showing % reduction in ACrE vs [ATP]: mM for JG25.](image)
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that ATP-dependent endocytic vesiculation is present in this patient (Woking pedigree, family label K).
Figure 4.20 Expt jf18. Effect of 2mM DMA on ATP-dependent endocytic vesiculation in ghosts from a patient with cryohydrocytosis (Watford pedigree, family label G).

Patient

![Graph showing ATP-induced endocytic vesiculation for Patient with and without DMA.](image)

Control

![Graph showing ATP-induced endocytic vesiculation for Control with and without DMA.](image)

Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a hematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% hematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this cryohydrocytosis patient (Watford pedigree, family label G). Neither was an effect seen on the cation leak (Table 4.3).
Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this DHSt patient (Uxbridge, Omagh pedigree, family label D). Neither was an effect seen on the cation leak (Table 4.3).
Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this DHSt patient (Stondon Massey, Essex pedigree, family label E). Neither was an effect seen on the cation leak (Table 4.3).
Figure 4.23 (Expt JG28.and 29) Effect of 2mM DMA on ATP-dependent endocytic vesiculation in ghosts from a patient with FP II (Patient 2, Chiswick pedigree, family label L).

Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5 mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this patient (Chiswick pedigree, family label L). Neither was an effect seen on the cation leak (Table 4.3).
Figure 4.24 (Expt JG21 and 22). Effect of 2mM DMA on ATP-dependent endocytic vesiculation in ghosts from a patient with FP I (Edinburgh pedigree, family label H).

Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this patient (Edinburgh pedigree, family label H). Neither was an effect seen on the cation leak (Table 4.3).
Figure 4.25 (Expt JG2). Effect of 2mM DMA on ATP-dependent endocytic vesiculation in ghosts from a patient with a unique form of HSt (Blackburn pedigree, family label J).

Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA had no effect on the ATP-dependent endocytic vesiculation in this patient (Blackburn pedigree, family label J). Neither was an effect seen on the cation leak (Table 4.3).
Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data show that DMA has no effect on ATP-dependent endocytic vesiculation in this patient (Woking pedigree, family label K). Neither was an effect seen on the cation leak (Table 4.3). No control was performed for this experiment.
Table 4.3. Endocytic vesiculation in different hereditary stomatocytosis kindreds, with the effect of DMA on endocytic vesiculation and ‘leak’ K influx. ATP-dependent endocytic vesiculation was measured by the AChE assay as described in Methods. K influx was measured using $^{86}$Rb as a tracer in the following medium (mM): Na, 145; K, 5; Cl, 150; MOPS-Na pH 7.4, 15; glucose, 5; ouabain, 0.1, bumetanide, 0.1. If required cells were pre-treated in DMA at 2mM as described in ‘Methods’. Key: OHSt, overhydrated HSt; DHSt, dehydrated HSt; CHC, cryohydrocytosis; FP, familial pseudohyperkalaemia; AChE, acetylcholinesterase activity. Stomatin: ‘↓’ implies reduced level in membrane; ‘+’, normal level in membrane.

Table 4.3.

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NORMAL 25-35% 25-35% 0.005-0.010 (37°C) no change
4.5 SUMMARY AND DISCUSSION

These results confirm and extend the observation by Mentzer et al. (1978) that there can be a defect in ATP-dependent endocytic vesiculation in OHSt patients. ATP-dependent endocytic vesiculation was absent only in the two original Manchester and Brighton OHSt pedigrees. It is absolutely clear that there was a defect in these cases. Both of these cases that show the obvious defect in endocytic vesiculation have stomatin deficiency. However, a third unrelated pedigree (Harrow) who is likewise stomatin-deficient, showed no defect in endocytic vesiculation. In all the other cases of HSt, endocytic vesiculation was present though it appeared to be reduced, in most cases anywhere between 5-50%. However, due to the nature of the variability of these experiments (day-to-day and interindividual) there was reluctance to conclude that this difference was real though there certainly was a trend to less vesiculation in the patients.

DMA corrected the endocytosis and the cation leak in both the Manchester and Brighton OHSt pedigrees, but had no effect on either the endocytosis or cation leak in other kindreds. Unless there was an obvious defect in endocytic vesiculation, DMA had no effect on the ion leak. If a defect in endocytic vesiculation was present, both it and the ion leak could be corrected by
DMA. These differences reflect the heterogeneity between the different patients and suggest that there is a link between the two processes.

The finding that DMP did not have any effect is consistent with previous findings that shorter or longer (DMP is longer) related reagents are either less effective than DMA, or have no effect at all (Mentzer et al., 1978). The mechanism of action of DMA is not understood and there are many possible explanations as to how it might work. It is a promiscuously reactive agent, reacting with many cellular components. It affects both the membranes and lipids and it has been suggested (Jozwiak et al., 1992) that the modified proteins may perturb the interactions of the components of the membrane, leading to alterations of the membrane organisation in the polar headgroup region (as opposed to the deeper regions of the membrane). The results here also show that it is necessary to incubate whole erythrocytes, rather than broken membranes, with DMA in order to elicit a corrective effect on OHSt erythrocytes (Figure 4.7). The reason for this is unknown, but it is possible that the action of DMA may require a cytoplasmic protein.

Before these data emerged it might have been concluded that stomatin was necessary for ATP-dependent endocytic vesiculation. However, the Harrow case, which is virtually stomatin deficient, had no obvious defect in endocytic
vesiculation. From this, it can be concluded that it is not the stomatin deficiency itself, or the abnormal intracellular Na\textsuperscript{+} and K\textsuperscript{+} concentrations, occurring in the stomatin-deficient cases which causes the defect in endocytic vesiculation.

Furthermore, the finding that endocytic vesiculation can be restored to stomatin-deficient red cell ghosts by treating whole red cells with DMA makes it very unlikely that stomatin is necessary since DMA would not be able to put stomatin back in the red cell which does not have protein-synthetic activity.

It is hypothesised that in the normal case a process of endocytic vesiculation may remove an unknown ion channel from the membrane. Thus in OHSt there is a defect in the vesiculation process and this could cause this ion channel to persist in the membrane. If it remains in the membrane, it could cause a non-selective 'passive leak' hence increasing the cation permeability.

A possible hypothesis for the action of DMA is that it recruits membrane proteins either across the surface or even from the cytosol. It could aggregate membrane proteins, hence causing endocytosis. This in turn might inactivate the ion channel by removing it from the membrane. These theories are consistent with other research into the mechanism of action of DMA. For example, DMA indirectly inhibits membrane transporters therefore reducing K\textsuperscript{+} loss from, and dehydration of, HbS cells (cells containing haemoglobin S) (Gibson et al., 2000).
CHAPTER 5

ATP-DEPENDENT ENDOCYTIC VESICULATION IN ERYTHROCYTE GHOSTS AND THE EFFECT OF DMA IN PATIENTS WITH SICKLE CELL DISEASE

5.1 AIM

The aim of the work to be described in this chapter was to measure ATP-dependent endocytic vesiculation, and any potential effect of DMA upon it, in the erythrocyte membrane of patients with sickle cell disease compared with that of controls.

5.2 INTRODUCTION

The main clinical features of sickle cell disease are a haemolytic anaemia and microvascular occlusion (Lew et al., 1985). Sickle cells contain haemoglobin S (HbS), which has a lower oxygen affinity than normal adult haemoglobin, HbA. HbS has a tendency to polymerise. After it polymerises the cell dehydrates by loss of K⁺ which can be lost from the cell by the Ca²⁺-activated K⁺ channel, the K⁺-Cl⁻ cotransporter or due to the combined effect of cellular deformation and oxidative damage to the membrane (Brugnara, 1993).

In sickle cell disease, membranes of circulating erythrocytes actively undergo both exocytosis and endocytosis (Westerman et al., 1979; Allan and
Raval, 1983). Lew et al. (1985) studied the mechanism of dehydration of sickle cells that are thought to be responsible for the haemolytic anaemia and microvascular occlusion. It was suggested that sickle cells (and to a lesser extent normal human red cells) possess intracellular vesicles with ATP-dependent Ca\(^{2+}\) accumulating capacity and suggested that virtually all measurable Ca\(^{2+}\) of fresh sickle cells was contained within these vesicles. This would help explain the mechanism of cell dehydration. Ca\(^{2+}\) would activate the red cell Ca\(^{2+}\)-sensitive K\(^{+}\) channels causing progressive loss of KCl and water. All sickle red cells and ghosts were found to contain enclosed vesicles (Lew et al., 1985). The sickle cell vesicles were more numerous and much larger than those found in normal cells. They also varied more in shape, size and numbers per cell, were often multi-lobed and contained electron-dense inclusions. This work showed that both normal and abnormal red cells contained intracellular vesicles, which could be the result of a physiological endocytic process.

These Ca\(^{2+}\) containing vesicles in sickle cells have been found by others (Williamson et al., 1990; Williamson et al., 1992) and the number of cells containing such vesicles has been found to increase upon deoxygenation (Williamson et al., 1992). Cytoplasmic vesicles present in sickle cells have been found to express on their surface plasma membrane Ca-ATPase (Lew et al., 1985;
Williamson et al., 1990; Williamson et al., 1992), suggesting that they arise by endocytic vesiculation from the plasma membrane (Williamson et al., 1992).

Evidence to suggest that an abnormal endocytic process occurs in sickle cells giving rise to these vesicles was shown by using the non-specific endocytic marker Lucifer Yellow. The results suggested that a slow, abnormal endocytic vesiculation takes place in these cells which may be the source of the Ca\textsuperscript{2+}-containing vesicles (Williamson et al., 1990). Further, the calcium channel antagonist bepridil inhibited the sickling of deoxygenated sickle cells and was shown to act by decreasing the surface area of sickle cell membranes with a concomitant lowering of cell deformity. This was shown by electron microscopy to be a result of endocytic vesiculation. Therefore it was suggested that bendripil was inhibiting sickling by induction of endocytic vesiculation and loss of cell deformity (Johnson et al., 1994).

DMA, which has such a striking corrective effect on OHSt cells (Chapter 4 and (Mentzer et al., 1976; Schroter and Ungefehr, 1976; Mentzer et al., 1978)), has been demonstrated to be an effective anti-sickling agent in completely deoxygenated sickle cells both in vitro (Lubin et al., 1975; Pennathur-Das et al., 1984; Gibson et al., 2000) and in vivo (Guis et al., 1984). Lubin et al. (1975) investigated DMA as an antisickling agent. Like cyanate, another inhibitor of
sickling (Gillette et al., 1973), DMA alters oxygen affinity of HbS. However, it was found that, unlike cyanate, it inhibited sickling in completely deoxygenated erythrocytes. This indicated that factors other than altered oxygen affinity must also be involved. It also blocked the usual K\(^+\) loss and increase in viscosity which accompany sickling. It was reported to have few deleterious effects \textit{in vitro}. It has been suggested that DMA might have a direct effect on the membrane or membrane–haemoglobin interaction which stabilises the cell in the discoid configuration and prevents cation alterations (Lubin et al., 1975).

DMA has been shown to reduce K\(^+\) loss from, and dehydration of, sickle cells. It has been found to inhibit all three pathways, which could be responsible for these effects: the deoxygenation-induced cation-selective channel, the Ca\(^{2+}\)-activated K\(^+\) channel and the K\(^+\)-Cl\(^-\) cotransporter. It was found that the DMA was acting by indirectly inhibiting these channels mainly due to the prevention of HbS polymerisation and sickling (Gibson et al., 2000).

The effect of the pretreatment of sickle cells with DMA \textit{in vitro} before returning them to the circulation has been studied (Guis et al., 1984). The treated cells lasted longer in the circulation than that usually observed in sickle cell disease. However, on the second infusion, the cells did not last as long as the initial study and an antibody was detected in the serum of the subjects against the
DMA-treated cells. This problem would have to be overcome for any DMA therapy to be effective.

Since sickle cells show abnormal cation transport, appear to feature an abnormal endocytic process, and DMA has antisickling effects and reduces the leak, a common pathological process between OHSt and sickle cell disease was considered. To investigate this hypothesis, ATP-dependent endocytic vesiculation measurements and the effect of DMA on this process in sickle cells was compared to that of controls to see if there was a defect, and whether, if a defect existed, it might be corrected by DMA.

5.3 METHODS

All patients had homozygous sickle cell disease, HbSS except for patient 3 who had HbSC and were under the care of the Department of Haematology, UCH. None had been transfused within the previous 6 months. All were on hydroxyurea except for patient 5. I would like to thank Prof. JB Porter and colleagues for provision of clinical samples. The methods used were the same as those used in the previous chapter.

5.4 RESULTS

Figure 5.1 shows the results of a comparison of ATP-dependent endocytic vesiculation in sickle cell ghosts compared to normal controls. Figure 5.2 shows
the effect of 2mM DMA on the patients and controls. The data show that, in the
patients tested, ATP-dependent endocytic vesiculation was present, though
reduced. The mean percentage ATP-dependent endocytic vesiculation at 5mM
ATP was 11% in sickle cell ghosts compared with 26.5% in control ghosts. There
was a single experiment where endocytic vesiculation was completely absent, but
in another sample from the same patient, endocytic vesiculation was present.
DMA was found to have no effect on ATP-dependent endocytic vesiculation in
any of the patients or controls.
Figure 5.1 ATP-dependent endocytic vesiculation in red cell ghosts from sickle cell patients and normal controls.

**Patient 1**

**JG44**

![Graph showing ATP-dependent endocytic vesiculation for Patient 1.]

**Patient 2**

**JG48**

![Graph showing ATP-dependent endocytic vesiculation for Patient 2.]

178
Figure 5.1 continued

Patient 2

JG43

\[
\text{% reduction in ACHE} \\
\text{[ATP]: mM}
\]

JG47

\[
\text{% reduction in ACHE} \\
\text{[ATP]: mM}
\]
Figure 5.1 continued

**patient 3**

**JH15**

![Graph showing the percentage reduction in AChE activity for patient 3 and control. The x-axis represents [ATP] in mM, ranging from 0 to 15, and the y-axis represents the percentage reduction in AChE activity, ranging from -10 to 40. The graph plots data points for both patient and control groups.]

**JH1**

![Graph showing the percentage reduction in AChE activity for patient 1 and control. The x-axis represents [ATP] in mM, ranging from 0 to 15, and the y-axis represents the percentage reduction in AChE activity, ranging from -5 to 35. The graph plots data points for both patient and control groups.]

180
Figure 5.1 continued

patient 4

JH22

JH3
One volume of packed phosphate-free ghosts from patients and controls were resuspended in 50 volumes of Tris lysis buffer with a final concentration of ATP or ITP ranging from 0-12.5mM at 4°C. Ghosts were incubated for 35 min at 37°C. AChE activity was measured using the 96 well plate method and endocytic vesiculation was taken as the % reduction in AChE. The data show that in all cases, there was less ATP-dependent endocytic vesiculation in the patient ghosts when compared to the control.
Erythrocytes were washed three times in 4°C PBS pH 7.4 before resuspending them at a haematocrit of 5% in 2mM DMA or sucrose (no DMA) made up in HBSS. This was incubated at 37°C for 30 minutes and then washed 3 times in 4°C TBS pH 7.4. The final pellet was diluted to 50% haematocrit and phosphate-free ghosts were prepared. ATP-dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts. Endocytic vesiculation was measured by AChE activity using the 96 well plate method. The data reiterates that ATP-dependent endocytic vesiculation is reduced in sickle cell ghosts (upper panel) when compared with controls (lower panel) and that DMA has no effect on endocytic vesiculation (filled squares compared with open circles) in either sickle cell or control ghosts.
5.5 SUMMARY AND DISCUSSION

In general, there is no obvious and consistent defect in ATP-dependent endocytic vesiculation in sickle cell ghosts though it is on average reduced to 42% of the control. Although endocytic vesiculation is reduced in these cases this could be because the membrane is in some way 'damaged' (oxidised) by the abnormal haemoglobin. Oxidant damage has been suggested as the underlying mechanism for phospholipid asymmetry in the erythrocyte membrane and has been shown to interfere with aminophospholipid flipase activity (de Jong et al., 1997).

On a single occasion ghosts prepared from sickle cell erythrocytes showed a total defect but an experimental problem cannot be excluded as a cause for this single abnormality. Nevertheless it is possible that this process could vary from day to day in sickle cell erythrocytes and could correlate with sickling episodes. Interestingly, this patient was the only patient with HbSC as opposed to HbSS.

The DMA had no effect on ATP-dependent endocytic vesiculation. Although this initially suggests that the main cause of sickling probably has nothing to do with endocytic vesiculation (since sickling can be corrected by DMA), the methods used to examine endocytic vesiculation here are not very physiological. The vesicles seen in sickle erythrocytes are not seen in normal red cells and occur under normal physiological conditions.
Further studies, including studies of ATP-dependent endocytic vesiculation in deoxygenated sickle cells and from patients in different clinical states, are required.
CHAPTER 6

INVESTIGATION INTO THE EXISTENCE OF RAFTS IN NORMAL AND OVERHYDRATED HEREDITARY STOMATOCYTOSIS (OHST) ERYTHROCYTES AND ASSOCIATION OF STOMATIN WITH THE RAFTS

6.1 AIM

The work to be described in this chapter was designed to determine whether rafts existed in the erythrocyte membrane. It was also designed to determine whether stomatin was associated with the rafts (and if so to what extent). Three stomatin-deficient OHSt pedigrees (Brighton, Manchester and Harrow) were examined in order to determine whether rafts existed in these patients.

6.2 INTRODUCTION

A number of preliminary observations led to the hypothesis that erythrocyte rafts might be defective in some way in stomatin-deficient OHSt patients. The role of stomatin as a lipid transfer protein (Ho and Stewart, unpublished) suggested a possible function for stomatin in lipid rafts. Stomatin is homologous to flotillin (Edgar and Polak, 2001; Bickel et al., 1997), known to be a raft protein in other cells (Bickel et al., 1997; Lee et al., 1998; Parkin et al., 1999a). In other cells,
rafts have been demonstrated to be a focus for endocytosis (Simons and Ikonen, 1997) which was shown in Chapter 4 to be defective in OHSt.

At the time the work described in this chapter was performed, the existence of rafts in erythrocytes had only briefly been investigated (Civenni et al., 1998) and had not previously been established. Therefore, the techniques used were adapted from work on rafts in other cells (Parkin et al., 1999b). It was also not known whether the raft marker flotillin is present in the erythrocyte membrane and this needed to be confirmed. The GPI-anchored protein alkaline phosphatase is known to be present in erythrocyte membranes (Kadlubowski and Agutter, 1977; Van Den Hoek and Zail, 1977; Goldemberg et al., 1988; Ruz et al., 1992; Goldemberg et al., 1996). In this chapter the GPI-anchored proteins alkaline phosphatase and AChE have been used as a raft marker since GPI-anchored proteins are generally associated with the rafts (Hooper, 1999).

6.3 METHODS

OHSt blood was sampled from the Brighton, Manchester and Harrow pedigrees (Chapter 2) and washed taking extra care to remove all white cells since these are rich in alkaline phosphatase (Elder et al., 1971; Aoyagi and Furusawa, 1995; Iqbal et al., 2000). In order to find out whether rafts exist in erythrocytes various
methods of the Hooper laboratory were employed (Parkin et al., 1999b), but this had to be adapted for erythrocytes.

Erythrocytes (at 10% haematocrit) or their membranes (at a concentration of 0.4mg/ml) were solubilized in Triton X-100 under the conditions described below. Rafts were prepared as described in the methods (section 3.11), but detergent concentration and the means by which the cells or membranes were disrupted was varied (see below) to find the optimal conditions for raft preparation. Fractions were assayed for flotillin, alkaline phosphatase, AChE and protein (see methods, sections 3.5.8-3.5.10).

Once the ideal conditions for preparing rafts had been established (methods section 3.11), rafts were then prepared from patient and control blood and the presence of rafts in patients was compared with that of controls (section 6.4.3) and the presence of stomatin in rafts was examined (section 6.4.4).

6.4 RESULTS

6.4.1. Finding optimum conditions for the preparation of the rafts from whole erythrocytes.

A preliminary experiment was performed (kind courtesy of Dr. ET Parkin [School of Biochemistry and Molecular Biology, Leeds University, Leeds, UK]). Fresh OHSt (Manchester) blood and control blood was solubilized in an equal
volume of cold 1% Triton X-100 before needle homogenisation and underlaying beneath a 45-5% sucrose gradient. They were centrifuged as triplicates for 18 hours at 140000g in an SW50 Beckman rotor and fractions were harvested from the bottom (0) to the top (10). Each fraction was assayed for the raft markers flotillin and caveolin. In this experiment flotillin was found in fractions 5-8 (not shown). This illustrated that flotillin was present in red cell membranes of both patients and controls and could be used as a raft marker and that rafts had been formed. Caveolin was found to be absent from red cells (not shown).

This technique was used to prepare rafts, but it was found that in order to get the rafts (as determined by a clearly visible, single, light scattering band), a sonication step (30 seconds in six 5 second bursts, 80% total time using a Branson Sonifer) was required rather than needle homogenisation. Figure 6.1a shows a flotillin blot after sonicating the cells and figure 6.1b shows a stomatin blot for all three conditions (no treatment, needle homogenisation and sonication). Sonicating the blood gave both flotillin and stomatin in the DIG region.

Stomatin or carbonic anhydrase was always left at the bottom of the gradient (Triton-soluble) and it was difficult to distinguish the two. Therefore, some experiments were performed using membranes as opposed to whole
erythrocytes. Gradient gels (7-17%) were also employed in an attempt to further resolve and distinguish the two proteins (Figure 6.7 b and d).
Figure 6.1 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal red cells broken by different means. (a) Anti-flotillin and (b) anti-stomatin antibodies.

Red cells were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

a. Anti-flotillin

Means of cell disruption

sonicated

--Rafts--

45----------------------5%
sucrose w/v

b. Anti-stomatin

no treatment

needle homogenized

sonicated

--Rafts--

45----------------------5%
sucrose w/v

Normal intact erythrocytes were solubilized in Triton X-100 and either untreated, needle homogenized 20 times or sonicated for 30 seconds.

A. Flotillin was located in the detergent-insoluble buoyant raft region (fractions 5-8) after rafts were prepared following a sonication step.

B. Stomatin was located in the detergent-insoluble buoyant raft region (fractions 5-8) after rafts were prepared following a sonication step.
6.4.2. Finding the ideal conditions for isolating rafts from erythrocyte membranes.

By using membranes as opposed to whole erythrocytes the majority of the haemoglobin and carbonic anhydrase are removed from the material used to make the rafts. This was felt to be a particularly important step, especially during experiments where the conditions were being optimised since it allows the colorimetric assays (alkaline phosphatase, protein and AChE) to be performed.

Preparation of membranes

The standard technique for making membranes in our lab is that of Dodge et al, 1963 with additional EDTA. However, this technique could not be employed since EDTA might disrupt the rafts (Parkin et al., 1996a). However, when EDTA was omitted from the hypotonic buffer it proved impossible to achieve white, haemoglobin-free ghosts at the usual 1:20 dilution used by the standard technique without multiple (7-8) washes. Therefore, membranes were prepared by lysing and washing in a larger volume of hypotonic buffer (1 volume of packed erythrocytes: 100 volume of hypotonic phosphate buffer). This technique was not as reliable as required. About 70% of the time, membranes were white after 4 washes, but sometimes more washes were required to achieve haemoglobin-free membranes. This was found to affect the banding patterns of the membranes.
(Figure 6.2) and led to concern that the cytoskeleton might be disrupted.

Therefore, membranes not turning white after 4 washes were discarded.

Membranes were resuspended in MBS at a protein concentration of 0.4mg/ml and rafts were prepared (methods section 3.11.3).
Normal red cell membranes were prepared by lysing cells in hypotonic buffer and washing until the membranes turned white. The number of washes taken for the membranes to turn white varied from sample to sample. Membranes were subjected to SDS-PAGE and stained with Coomassie blue to examine how this affected the banding patterns. Membranes shown were washed over 4 times (lanes 1-3) or 4 times (lane 4) to achieve white membranes. The data show that there is variation in the banding pattern from sample to sample. The membranes subjected to only 4 washes (lane 4) had a banding pattern more similar to that seen normally and the red cell membrane proteins appeared to be better preserved.
Detergent concentration

Membranes at 0.4mg/ml in MBS were solubilized in an equal volume of 1% Triton X-100 before sonication (at 80% total time 1.5 power for 30 sec) and subjected to raft separation (methods, section 3.11). Flotillin, protein and alkaline phosphatase assays were performed and all these were found to be in the bottom of the gradient (Triton-soluble), suggesting that the rafts were not being formed. It was therefore questioned whether there was too high a detergent: protein ratio since altering this ratio has previously been shown to affect the association of proteins with rafts in other cells (Parkin et al., 1999a).

An experiment was performed in which membranes at a concentration of 0.4mg/ml were solubilized in equal volumes of 1%, 0.5% and 0.25% Triton X-100 in MBS. Protein and alkaline phosphatase protein assays were performed for fractions 0-10 (Figure 6.3a and b). These data show that the bulk of the protein is at the bottom of the gradient (Triton-soluble) whereas most of the raft marker alkaline phosphatase is in fractions 5-8 (raft fraction). The proportion of alkaline phosphatase in the raft fraction was optimal at 0.5% Triton X-100 suggesting that solubilizing 0.4mg/ml membranes in an equal volume of 0.5% Triton X-100 is the best detergent concentration to prepare rafts from membranes. The same
samples were blotted for stomatin (Figure 6.3c) and this too gave the highest proportion of stomatin in the raft fraction at the 0.5% detergent concentration.
Figure 6.3 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal red cell membranes solubilized with different concentrations of Triton X-100.

1 vol red cell membranes prepared without EDTA were subject to solubilization in 1 vol ice-cold Triton X-100 at different concentrations (v/v), sonicated for 30 seconds and made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich Detergent-insoluble rafts at fractions 5-8.

A. Protein assay in fractions 0-10 from sucrose gradient separation of normal membranes using different concentrations of Triton to solubilize the cells.

B. Alkaline phosphatase activity in fractions 0-10 from sucrose gradient separation of normal membranes using different concentrations of Triton to solubilize the cells.
C. Distribution of stomatin in fractions 0-10 from sucrose gradient separation of normal membranes using different concentrations of Triton to solubilize the cells.

Detergent concentration:

1%

0.5%

0.25%

These results show that when 1 vol. 0.4mg/ml membranes were solubilised with 1 vol. of 0.25, 0.5 or 1% Triton X-100 before sonication, then:
(a) At all detergent concentrations, the bulk of the protein (over 90%) was at the bottom of the gradient (Triton-soluble);
(b) The greatest proportion of alkaline phosphatase was in the detergent-insoluble buoyant raft (fractions 5-8) when 0.5% Triton X-100 was used;
(c) The greatest proportion of stomatin was in the detergent-insoluble buoyant raft (fractions 5-8) when 0.5% Triton X-100 was used.
**Sonication**

After solubilization in an equal volume of 0.5% Triton X-100, membranes were subjected to different means of disruption (without treatment, needle homogenised or sonication (80% total time, 1.5 power level)) and rafts were prepared. Protein assays and alkaline phosphatase assays were performed (Figure 6.4). The data show that in all conditions, the bulk of the protein was at the bottom of the gradient (Triton-soluble). It also shows that sonication gave the highest proportion of alkaline phosphatase in the rafts compared with the other conditions. This suggested that sonication was the best way to prepare rafts from membranes, similar to intact erythrocytes.
Figure 6.4  Sucrose gradient separation of buoyant detergent-insoluble fractions from normal red cell membranes broken by different means. Red cell membranes prepared without EDTA were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

A. Protein concentration in fractions 0-10 from sucrose gradient separation of normal membranes using different conditions to break up the membranes before Triton solubilization.

B. Alkaline phosphatase activity in fractions 0-10 from sucrose gradient separation of normal membranes using different conditions to break up membranes before Triton solubilization.

In all conditions the bulk of the protein was at the bottom (Triton-soluble) of the gradient. Sonication gave the highest proportion of the alkaline phosphatase in the detergent-insoluble buoyant raft (fractions 5-8).
Due to a collaboration with Prof. NM Hooper [School of Biochemistry and Molecular Biology, Leeds University, Leeds, UK], the initial results described in this chapter were performed in his laboratory whilst the techniques were being established. However, the remainder of the results presented in this chapter and the following chapters took place at UCL. On return to UCL, it was possible to use the same equipment except that the sonicator was a different brand (Soniprep 150).

Due to the change in sonicator, an experiment was performed to find the ideal power level (microns) with which to sonicate the membranes (Figure 6.5). Membranes were sonicated for 30 seconds (six 5 second bursts) on ice at different power levels (1.5, 3, 4.5, 6, 9, 12 and 15 microns), rafts were prepared and fractions were assayed to determine the power level resulting in the optimal amount of raft markers in the DIG fraction (Figure 6.5a protein, b alkaline phosphatase, c AChE, d flotillin, e stomatin). These data show that all the raft markers (flotillin, alkaline phosphatase and AChE) were optimally isolated in the rafts using 9 microns to sonicate the membranes, so this was used for further experiments.
Figure 6.5 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal membranes after different sonication power levels (microns).

Red cell membranes prepared without EDTA were subject to solubilization in ice-cold Triton X-100, sonicated at different power levels (microns), made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

A. Protein assay

- Sonication power level (microns):
  - 1.5
  - 3
  - 4.5
  - 6
  - 9
  - 12

- Protein concentration (µg/10^4 µl)

- Fraction — Rafts —

45—5%
sucrose w/v

B. Alkaline phosphatase activity

- Sonication power level (microns):
  - 1.5
  - 4.5
  - 6
  - 9
  - 12

- Alkaline phosphatase activity (mU/mg phospholipid)

- Fraction — Rafts —

45—5%
sucrose w/v

A. Protein assay: The bulk of the protein in each condition was found at the bottom of the gradient (Triton-soluble).

B. Alkaline phosphatase activity: A power level of 9 microns gave the highest proportion of alkaline phosphatase in the detergent-insoluble buoyant raft (fractions 5-8).
C. Acetylcholinesterase activity: A power level of 9 microns gave the highest proportion of acetylcholinesterase in the detergent-insoluble buoyant raft (fractions 5-8).

D. Anti-flotillin antibody. All the flotillin was located at the bottom of the gradient (Triton-soluble) when the cells were sonicated at 1.5 microns. Flotillin is in the detergent-insoluble buoyant raft (fractions 5-8) at all other sonication levels shown, but a power level of 9 microns gives the highest proportion of flotillin in the rafts.

E. Anti-stomatin antibody. A power level of 9 microns resulted in stomatin being part in the detergent-insoluble buoyant raft (fractions 5-8) and part at the bottom of the gradient (Triton-soluble).
6.4.3. Do the OHSt patients have rafts?

The results so far have established that there are rafts in red cell membranes. To determine whether rafts are present in OHSt erythrocytes, rafts were prepared, by the methods established above, (also see methods, section 3.11) from both intact erythrocytes and erythrocyte membranes from OHSt patients and normal controls. Fractions were subjected to SDS-PAGE, blotted to PVDF membranes and probed with an anti-flotillin antibody. Where membranes were used as the 'start material', protein, alkaline phosphatase and AChE assays were also performed (Figure 6.6 Manchester membranes, Figure 6.7 flotillin a Brighton, b Manchester and c Harrow). These data show that the patients do have rafts. The AChE data show a possible shift in where the rafts are between patients and controls. However, this may well be insignificant and due inaccuracies in the harvesting technique.
Figure 6.6 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal and OHSt (Manchester) red cell membranes. Red cell membranes prepared without EDTA were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

A. Protein concentration in the various fractions. Rafts prepared from normal and OHSt red cell membranes.

B. Alkaline phosphatase and acetyl cholinesterase activities. Rafts prepared from normal and OHSt red cell membranes.
C. Rafts prepared from normal (left) and OHSt (right) red cell membranes in duplicate. Anti-flotillin antibody. The raft marker protein flotillin is present in the buoyant fraction, demonstrating that rafts exist in OHSt membranes, in spite of the absence of stomatin.

In both OHSt and normal red cells subjected to raft separation the following was observed:
A. The bulk of the protein was located at the bottom (Triton-soluble) of the gradient;
B. The alkaline phosphatase and AChE were located mainly in the raft region;
C. Flotillin was located exclusively in the raft fraction.
Figure 6.7 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal red cells or membranes, Brighton, Harrow and Manchester pedigrees. Anti-flotillin antibody.

Red cells (a and b) or membranes (c and d) were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

A. Rafts prepared from intact erythrocytes

i. Normal
   OHSt Brighton

ii. Normal
   OHSt Manchester

   --Rafts--
   45—---------------5%
sucrose w/v

iii. Normal
   OHSt Harrow

iv. Normal
   OHSt Harrow

   --Rafts--
   45—---------------5%
sucrose w/v

B. Rafts prepared from membranes

i. Normal
   OHSt Manchester

   --Rafts--
   45—---------------5%
sucrose w/v

In all cases the flotillin was located in the detergent-insoluble buoyant raft (fractions 5-8).
6.4.4. Is stomatin in the rafts?

Most of the samples already shown were probed with an anti-stomatin antibody and it was found to be raft associated (Figure 6.1c, 6.3c and 6.5e) in the conditions giving flotillin, alkaline phosphatase and AChE optimally in the raft region (fractions 5-8) of the gradient. However, some was at the bottom of the gradient (Triton-soluble).

Further rafts were prepared from control samples, subjected to SDS-PAGE, blotted to PVDF membranes and probed with an anti-flotillin antibody (to confirm that raft separation had occurred (not shown)) and an anti-stomatin antibody (Figure 6.8). Rafts prepared from erythrocytes were subjected to SDS-PAGE using 12% gels (Figure 6.8a) and using gradient gels to separate the stomatin from what is suspected to be carbonic anhydrase (Figure 6.8b). Rafts were also prepared from erythrocyte membranes to remove the carbonic anhydrase, subjected to SDS-PAGE using 12% gels (6.8c) and gradient gels (6.8d). These data suggest that stomatin is raft associated though not as strongly as flotillin.

6.4.5. Is the small amount of stomatin in patient blood in the rafts?

Rafts were prepared from patient and control samples, subjected to SDS-PAGE, blotted to PVDF membranes and probed with an anti-stomatin antibody. Films
were overexposed (by leaving overnight) to determine whether the small amount of stomatin in patients was raft associated (Figure 6.9a Brighton, b Manchester, c Harrow). These data show that it was.
Figure 6.8 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal red cells or membranes. Anti-stomatin antibody. Red cells (a and b) or membranes (c and d) were subjected to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

<table>
<thead>
<tr>
<th>Rafts prepared from:</th>
<th>Gel percentage:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact erythrocytes</td>
<td>45--------------------------5% mks</td>
</tr>
<tr>
<td>Membranes</td>
<td>45--------------------------5% mks</td>
</tr>
<tr>
<td></td>
<td>sucrose w/v</td>
</tr>
<tr>
<td></td>
<td>12%</td>
</tr>
</tbody>
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Intact erythrocytes (a and b top 2 panels) or membranes (c and d lower 2 panels) were subjected to raft separation. 12% (a and c) or 7-17% gradient gels (b and d) were made and fractions 0-10 were subjected to SDS-PAGE and Western blotting with an anti-stomatin antibody. What is presumed to be carbonic anhydrase (CA) can be seen in the gradient gels resolving at a slightly lower molecular weight to stomatin. The data show that stomatin was located partially in the detergent-insoluble buoyant raft (fractions 5-8) and partially at the bottom of the gradient (Triton-soluble).
Figure 6.9 Sucrose gradient separation of buoyant detergent-insoluble fractions from OHSt red cells. Anti-stomatin antibody. OHSt red cells from (a)Brighton, (b)Manchester, and (c)Harrow were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Buoyant, lipid-rich detergent-insoluble rafts at fractions 5-8.

Intact erythrocytes were subjected to raft separation and fractions 0-10 were subjected to SDS-PAGE and Western blotting with an anti-stomatin antibody. The films were left overnight with the blots to overexpose to enable the small amounts of stomatin to be visualised. The data show that in all pedigrees stomatin was located partially in the detergent-insoluble buoyant raft (fractions 5-8) and partially at the bottom of the gradient (Triton-soluble). In the Harrow pedigree (lower panel, C) it appears that most of the stomatin was located at the bottom of the gradient in this experiment, though this could be due to non-specific reaction with carbonic anhydrase.
6.5 SUMMARY AND DISCUSSION

The preparation of rafts from membranes had the advantages of allowing the colorimetric assays to be performed, by removing the haemoglobin, and also at the same time removed what is likely to be carbonic anhydrase (28kDa), which was found to cause cross-reactivity when Western blots were probed with antibodies to proteins resolving in the same region of the gel (see section 6.4.4 and 8.4.3). However, due to the extra steps in preparation and not being able to use EDTA, there was concern that this risked disruption of the cytoskeleton. Therefore, for the majority of experiments (Chapter 7 and 8), where possible, rafts were prepared from intact erythrocytes.

In this chapter stomatin was found to be partially raft associated. This finding could imply that both some of the stomatin is in the rafts and a separate portion is not. Alternatively it could suggest that stomatin spans a region of membrane bridging both the raft and the fluid part of the membrane. This result will be discussed in the context of other work later.

This chapter also found flotillin to be a red cell membrane protein. This has allowed its use as a raft marker. Flotillin was found to be most useful, since it was located almost exclusively in the raft region (see table 8.1 for densitometry...
results). Therefore, in future experiments, fractions were assayed for flotillin to ensure that rafts had been formed.

It was confirmed here that rafts exist in erythrocyte membranes and that patients have rafts. Although this work shows that patients have rafts, they must in some way be defective. The patients do not have stomatin in their red cells, which has here been shown to be a raft protein. In the following chapters a detailed analysis has been performed to find other possible defects in the rafts from these patients.

This chapter has also highlighted that the technique is very sensitive to slight changes in conditions. Conditions have to be exact and consideration of the correct detergent: protein ratio and means of disruption of the 'start material' are necessary for consistent results. The harvesting of fractions by syringe and needle is a crude technique. A shift in the distribution of AChE was apparent between OHSt and controls (Figure 6.6). This may well be insignificant and a sampling problem. It could be argued that it would have been better to find the exact density of each fraction being subjected to the various assays, though for most experiments this did not seem necessary.

The interpretation of these data alone is difficult, but recent works on stomatin have given clues to its function and provides an insight into the
relevance of these findings. Snyers et al. (1997) showed that stomatin concentrates preferentially in the plasma membrane folds and protrusions in UAC cells and that stomatin-containing vesicles, present in the cytoplasm of these cells, belong at least partly to the endocytic pathway (Snyers et al., 1999). In addition, stomatin appeared to colocalize to some extent with cortical actin microfilaments, even after disruption of actin filaments by cytochalasin D, suggesting that stomatin is linked to the cortical actin cytoskeleton (Snyers et al., 1997). These findings suggested a possible structural role of stomatin either in the formation of membrane folds and/or anchorage to the actin cytoskeleton (Snyers et al., 1998). These findings suggest that stomatin may be involved in endocytic vesiculation and it is feasible that it is involved in the ATP-dependent endocytic vesiculation shown to be defective in OHSt erythrocyte ghosts (Chapter 4). The relevance of the anchorage of stomatin to the cytoskeleton is unknown and investigation of the extent to which the cytoskeleton is associated with lipid rafts may provide insight into this (Chapter 7).

Snyers et al. (1998) demonstrated that the fundamental structure of stomatin is oligomeric (meaning that it associates with itself) and forms complexes consisting of 9-12 stomatin molecules. This was interpreted as suggesting a possible function as a scaffolding protein perhaps forming
specialised membrane structures (Snyers et al., 1999). C-terminal truncated stomatin was unable to incorporate into these oligomers suggesting that the C-terminus is involved in their formation (Snyers et al., 1998).

Since the work described in this chapter was performed, stomatin has been implicated by others as a lipid raft protein (Snyers et al., 1999; Salzer and Prohaska, 2001; Sedensky et al., 2001) and has been shown to be partially present in detergent-insoluble membrane domains in UAC cells (Snyers et al., 1999). This is consistent with the results presented in this chapter which showed some stomatin to be Triton-soluble.

Interestingly, Snyers et al. (1999) found that the C-terminally truncated stomatin (non-oligomeric) was excluded from the lipid rafts suggesting that the oligomeric state of stomatin is important for the association with the lipid rafts. This needs confirmation in erythrocytes. It would be interesting to determine whether in erythrocytes, the oligomeric stomatin is raft associated and that some stomatin is in a non-oligomeric form. This could explain why it is partially in the rafts and partially Triton-soluble. It would be interesting to examine factors influencing oligomerisation of stomatin in the intact erythrocyte as it is feasible that such factors could be involved in the pathogenesis of OHSt.
Stomatin was found to colocalize with the GPI-anchored proteins placental alkaline phosphatase (PLAP) and membrane folate receptor alpha (MFRα) which were present in the lipid rafts and internalised in vesicles containing stomatin. It was concluded that stomatin and GPI-anchored proteins are linked through lipid rafts and undergo the same sorting events (Snyers et al., 1999). These results indicate a link between endocytic vesiculation and rafts and suggest that stomatin may be involved in a raft-based vesiculation process. Since the OHSt erythrocytes have a defect in their rafts (since they are deficient the raft-associated protein stomatin) and also have a defect in an in vitro process if ATP-dependent endocytic vesiculation, it is conceivable that the pathophysiology of the disease may involve a defective raft-based endocytic vesiculation process, in some way causing the cation leak.

Similar topological and biochemical characteristics between stomatin and caveolin have been described (Parton, 1996; Snyers et al., 1998) including anchorage to the cytoplasmic leaflet of membranes, homo-oligomerisation and palmitoylation. However, the expression level of caveolin was much lower than stomatin and the cellular localisation of caveolin showed a very different pattern to stomatin in these cells. These data suggest that a functional link between stomatin and caveolin is unlikely (Snyers et al., 1999).
The finding that stomatin is raft associated in erythrocytes implies a defect in rafts in OHSt erythrocytes. However, the gene for stomatin is not mutated in these patients (Wang et al. 1992; GW Stewart, AC Argent, JF Ajetunmobi, MC Chetty, unpublished). This suggests that another protein must be involved. Work suggesting a defect in other proteins which are homologous to stomatin and interact with it has therefore raised interest and might be able to explain the importance of various characteristics of stomatin, such as oligomerisation and raft association and finally how stomatin might be lost from the cells:

Wang and Morrow (2000) reported the cloning and characterisation of the human stomatin homologue, stomatin-like protein 2 (SLP-2), which is found in many tissues including erythrocytes. In the erythrocyte it associates with the spectrin-actin cytoskeleton (and probably other integral membrane proteins) but is not itself integral to the membrane bilayer. It is at least partially an oligomeric protein complex in the erythrocyte membrane and it is homologous to the C-terminus of stomatin, lacking the hydrophobic N-terminus. Wang and Morrow (2000) put these findings together to suggest that SLP-2 might associate with stomatin and hypothesise that if this is the case, then SLP-2 could be the way in which stomatin-containing rafts might be linked to the cytoskeleton therefore playing a role in the regulation of ion channel conductances or the organisation of
rafts. They hypothesised that SLP-2 is required for the maintenance or attachment of stomatin to the cytoskeleton and defective SLP-2 function could result in the loss of stomatin from the erythrocyte (Wang and Morrow, 2000).

The STORP (stomatin related protein) gene, homologous to the gene encoding stomatin, has also raised interest (Gilles et al., 2000). It encodes a protein with a C-terminus homologous to the non specific lipid transfer protein found in UNC-24. Gilles et al. (2000) suggest that stomatin is part of a complex of proteins around the ion channel, possibly involved in its regulation or a cytoskeletal anchor. They suggest that STORP may form part of this complex (Gilles et al., 2000) though characterisation of this protein is required.

Sedensky et al. (2001) have found evidence to suggest that the stomatin homologues unc-1 (which has been shown to interact with an ion channel) and unc-24 (which codes for a protein with non specific lipid transfer activity) interact in C. elegans. They demonstrated that antibodies against UNC-1 resulted in a punctate pattern of staining consistent with lipid rafts which was abolished in the absence of UNC-24. Western blots indicated that UNC-24 probably affects the stability of the UNC-1 protein. They concluded that UNC-24 may therefore be necessary for the correct placement of UNC-1 in the cell membrane, its oligomerisation and organization of lipid rafts. This interaction may suggest how
the defect in OHSt erythrocyte rafts could be relevant to the disease, since to be relevant and/or causative (as opposed to coincidental) it must somehow cause an increase in the erythrocyte membrane permeability.

Only recently have rafts been investigated in erythrocytes (Civenni et al., 1998; Ilangumaran and Hoessli, 1998; Lauer et al., 2000; Nelson and Buckley, 2000; Salzer and Prohaska, 2001; Samuel et al., 2001) and stomatin has been confirmed to be raft associated in erythrocytes (Lauer et al., 2000; Salzer and Prohaska, 2001; Samuel et al., 2001). Since the work described in the following chapters was performed at the same time and not influenced by this work (Lauer et al., 2000; Salzer and Prohaska, 2001; Samuel et al., 2001), the findings from this literature will be discussed more fully in later chapters.
CHAPTER 7

INVESTIGATION OF RAFT COMPOSITION IN NORMAL AND OVERHYDRATED HEREDITARY STOMATOCYTOSIS (OHST) ERYTHROCYTES

7.1 AIM

The aim of the work to be described in this chapter was to investigate the extent to which a series of proteins, including known erythrocyte skeletal proteins, known integral membrane proteins and a number of raft proteins known from other cell types, were associated with rafts in normal and abnormal human red cells.

7.2 INTRODUCTION

The protein content of the human red cell membrane is as well understood as any other cell type in biology. However, when the work to be described in this chapter was performed, the extent to which these proteins are associated with the sphingomyelin+cholesterol-rich raft had not previously been investigated. Using buoyant sucrose density separation and Western blotting with specific antibodies, it was sought to determine the extent to which a series of well-known red cell proteins were associated with these rafts.
7.3 METHODS

The work sought to investigate the protein composition of rafts in normal and OHSt (Brighton and Manchester pedigrees) human red cell membranes. The most concentrated source of membranes would have been those prepared by ‘hypotonic lysis’ (based on the method of Dodge et al, 1963, with the addition of EDTA. Because this method involves the use of EDTA, it has the potential to disrupt the rafts (Parkin et al., 1996a). Since preparation of membranes without EDTA were problematic (see Chapter 6), unless it was necessary to use membranes (immunoblotting for proteins resolving in the same region of the gel as carbonic anhydrase i.e. stomatin and aquaporin-1 blots), whole cells were used as the ‘start’ material for these studies.

Rafts were prepared as described in the main methods section. Protease inhibitors were added throughout the experiments (as described in methods, section 3.11.3) since experiments were performed in conjunction with those in chapter 9 which required their use (see section 9.4.1). Briefly, erythrocytes or membranes were solubilized in 0.5% Triton X-100 and sonicated on ice before being made to 45% sucrose and 0.25% Triton X-100. This was underlayed beneath a 39-5% discontinuous (step) sucrose gradient and centrifuged for 18 hours at 140,000g at 0°C. Eleven fractions were harvested, the pellet being
denoted 'zero', 1 being the bottom of the tube and 10 being the top. The rafts were in fractions 5-8.

Fractions 0-10 were subjected to SDS-PAGE, blotted to PVDF membranes and probed with a variety of antibodies. Blots were analysed by densitometry to find the percentage of a particular protein in the rafts (fractions 5-8) compared with the total amount of that protein throughout the gradient (fractions 0-10) using NIH image software, version 1.62.

7.4 RESULTS

7.4.1. Distribution of actin in the rafts

Figure 7.1 shows Western blots of fractions 0-10 of a raft separation probed with an anti-actin antibody from duplicate experiments from 2 patient samples, one from the Manchester pedigree and one from the Brighton pedigree. In the control, on average 21% of actin was raft associated whereas in the patient only 2% was raft associated (Figure 7.1) as calculated by densitometry. These results demonstrate that in these two OHSt patients the proportion of actin that was associated with the raft fraction was reduced to about 10% when compared to the control.

Since DMA was shown to correct the ion leak and endocytic vesiculation (Figure 4.6 and Table 4.2, Chapter 4 and (Mentzer et al., 1978)) in these patients,
it was questioned whether prior treatment of whole cells with DMA might restore this deficiency of actin in the rafts. Figure 7.2 shows the result of a Western blot in which the quantity of raft-based actin in control and DMA-treated OHSt erythrocytes was compared. DMA had no effect on the distribution of actin in this experiment.

7.4.2. Distribution of other cytoskeletal membrane proteins in the rafts

Beta spectrin binds actin (Karinch et al., 1990) and has binding sites for ankyrin (Kennedy et al., 1991) and band 4.1 (Becker et al., 1990). It was therefore considered that spectrin α (and possibly ankyrin and band 4.1) might follow a similar pattern, the proportion of spectrin α in the rafts being reduced in the patients when compared with the control. A Western blot of fractions 0-10 after sucrose density fractionation from the Manchester pedigree and control was probed with an anti-spectrin α antibody (Figure 7.3). There might be a slight reduction in the proportion of spectrin α in the rafts in patients when compared to controls, but by eye it was much less convincing than the actin results (Figure 7.1). By densitometry, the proportion of spectrin α in the rafts in controls was 18.6%, which was 3-fold higher than that seen in the patients rafts (Table 1.1).

The other cytoskeletal membrane proteins band 2.1 (an ankyrin isoform), and band 4.1 are also shown (Figure 7.3). There is a small proportion of band 2.1
in the rafts in both patients and controls. Again, there might be a slight reduction
in the proportion of band 2.1 in the rafts in patients when compared to controls,
but by eye it was much less convincing than the actin results. By densitometry,
the proportion of band 2.1 in the rafts in controls was 6.62%, which was more
than 3-fold higher than that seen in the patient (1.95%).

The proportion of band 4.1 that was raft associated varied between two
separate experiments, though the patients and controls were still comparable.
There was no convincing difference in the distribution of band 4.1 between the
patients and controls (Figure 7.3 and table 7.1).

7.4.3. Distribution of other membrane proteins in the rafts.

Band 3 and glycophorin A are integral proteins that span the membrane
completely, while the cytoskeletal proteins are proteins that are linked together
underneath the lipid bilayer by a series of electrostatic interactions that can be
broken by manipulation of salt conditions. Fractions were subjected to SDS-
PAGE, blotted to PVDF membranes and probed with anti-band 3 and anti-
glycophorin A antibodies (Figure 7.4, first page). There is a small proportion of
band 3 and in the rafts in both patients and controls. There was no convincing
difference in the distribution of this protein between the patients and controls.
Glycophorin A (Figure 7.4) was completely absent from the rafts, all being present at the bottom of the gradient (Triton-soluble) in both patient and controls. Much more seemed to be present in the control when compared with the patient. To determine whether this was 'real' or an artefact (due to for example, faster degradation of one sample), membranes from 2 patients and 2 controls were probed with an anti-glycophorin A antibody and no difference was seen (not shown).

Since it is conceivable that clathrin could colocalize with the rafts (since clathrin coats certain vesicles it could be associated with rafts), fractions 0-10 from raft preparations from the Brighton and Manchester pedigrees were blotted with an anti-clathrin antibody (Figure 7.4, second page). These data show that clathrin was completely absent from the rafts in both the patients and control.

 Annexin V (35kDa) and annexin VI (70kDa) were totally absent from the rafts in both patients and controls (Figure 7.4, second page). There was a doublet in the patient at 70kDa in the annexin VI blot that was absent from the controls. This was reproducible on three occasions when raft separations were blotted, but no difference was seen between patients and controls on whole membranes.

Aquaporin-1 is a known red cell membrane protein (Agre et al., 1994) and is caveolae-associated in other cell types (Schnitzer and Oh, 1996; Page et al., 225...
1998). I sought to determine whether aquaporin-1 was raft associated in red cells.

A technical problem arose, in that aquaporin-1 had a similar molecular weight to
the abundant cytosolic protein carbonic anhydrase, which gave a false-positive
signal on the blots. Experiments using different blocking conditions including
addition of carbonic anhydrase to the block and antibody solutions were
unsuccesful.

Therefore, fractions from both red cell and red cell membrane raft
preparations were subjected to SDS-PAGE (using 12% gels for membranes and
gradient gels for whole red cell preparations), blotted to PVDF membranes
probed with an anti-aquaporin-1 antibody (Figure 7.5). Gradient gels (7-17%)
successfully separated the carbonic anhydrase from the aquaporin-1 when whole
cell raft preparations were used and aquaporin-1 was shown to be partially in the
rafts in controls (about 45%) and slightly less was in the rafts in the patients
(about 35%). When rafts prepared from membranes were blotted with the same
antibody the proportion of aquaporin-1 in the rafts in the control (about 40%) was
4-fold higher than when compared with the patient (about 10%). From these
results it was concluded that the OHSt cases studied there may be reduced
aquaporin-1 in the patient rafts when compared with a control.
Table 7.1 summarises the data from densitometry analysis of the Western blots shown in this chapter.
Table 7.1 Proportion (%) of various proteins found in the rafts in patients and controls

<table>
<thead>
<tr>
<th>Protein</th>
<th>Proportion (%) in rafts (fractions 5-8)</th>
<th>Manchester OHSt</th>
<th>Control</th>
<th>Brighton OHSt</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td></td>
<td>3.2</td>
<td>23.1</td>
<td>8.6</td>
<td>29</td>
</tr>
<tr>
<td>Actin, 2nd go</td>
<td></td>
<td>1.3</td>
<td>25.2</td>
<td>0.4</td>
<td>28.4</td>
</tr>
<tr>
<td>Actin, 3rd go</td>
<td></td>
<td>0</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spectrin α</td>
<td></td>
<td>6.25</td>
<td>18.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 2.1</td>
<td></td>
<td>1.95</td>
<td>6.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 3</td>
<td></td>
<td>7.7</td>
<td>11.1</td>
<td>0</td>
<td>3.9</td>
</tr>
<tr>
<td>Band 4.1</td>
<td></td>
<td>39.6</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 4.1</td>
<td></td>
<td>1.56</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td></td>
<td>1.2</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin VI</td>
<td></td>
<td>0</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flotilllin</td>
<td></td>
<td>81.5</td>
<td>88.3</td>
<td>97.7</td>
<td>90.3</td>
</tr>
<tr>
<td>Glycophorin A</td>
<td></td>
<td>0.26</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clathrin HC</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stomatin (membrane</td>
<td></td>
<td></td>
<td>54</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>preperation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aquaporin-1</td>
<td></td>
<td>35.5</td>
<td>43.7</td>
<td>31.5</td>
<td>42.3</td>
</tr>
<tr>
<td>Aquaporin-1 (membrane</td>
<td></td>
<td>10.7</td>
<td>43.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>preparation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of blots by densitometry using the programme NIH image. All studies were performed on intact erythrocytes unless stated otherwise. Numbers refer to the density of the raft fractions (5-8) compared with the density of the total fractions (0-10) and are expressed as percentages. Figures should be taken as an estimate only due to saturation of some of the darker bands.
Red cells were subject to solubilization in ice-cold Triton X-100, made 45% sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Detergent-insoluble rafts at fractions 5-8. Fractions were run on 12% SDS gels, blotted to PVDF membranes and probed with an anti-actin antibody. The proportion of actin in the rafts in controls was 27.1% and in the patients 2.7% (mean of 3 densitometry results).
Red cells were incubated at 37°C with or without 2mM DMA, subject to solubilization in ice-cold Triton X-100, made 45% sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Detergent-insoluble rafts at fractions 5-8. Fractions were run on 12% SDS gels, blotted to PVDF membranes and probed with an anti-actin antibody. The data show that pretreatment of red cells with 2mM DMA had no effect on the proportion of actin in the rafts in OHSt cells.
Red cells were subject to solubilization in ice-cold Triton X-100, made 45% sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Detergent-insoluble rafts at fractions 5-8. Fractions were run on 9% SDS gels, blotted and probed with antibodies as labelled. The data show that a small proportion of each of the cytoskeletal proteins was present in the rafts in OHSt and control cells. It appears that there may be a small reduction in the proportion of spectrin and band 2.1 which was raft associated in the patient cells when compared with controls.
Figure 7.4 Sucrose gradient separation of buoyant detergent-insoluble fractions from normal and OHSt red cells. Antibodies to other membrane proteins: band 3, glycophorin A, clathrin, annexin V and annexin VI.

Band 3 protein, family B, jj2

Control

45--Rafts--5%
sucrose w/v

90kDa

Band 3 protein, family A, jj6

Control

45--Rafts--5%
sucrose w/v

90kDa

Glycophorin A, family B, jj6

Control

45--Rafts--0%
sucrose w/v

80kDa
Red cells were subject to solubilization in ice-cold Triton X-100, made 45% sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Detergent-insoluble rafts at fractions 5-8.Fractions were subjected to SDS-PAGE, blotted to PVDF membranes and probed with the antibodies indicated. Acrylamide percentages were as follows: 9% for band 3, glycophorin A, annexin V and annexinVI and 7% for clathrin HC. The data show that a small proportion of band 3 appears to be raft associated whilst the other proteins (glycophorin A, annexin V, annexin VI and clathrin HC) were completely absent from the rafts in these blots. Annexin VI appeared as a doublet at 70kDa in separations from patient cells, but not controls.
Red cells or membranes (prepared without EDTA) were subject to solubilization in ice-cold Triton X-100, made 45% in sucrose, then centrifuged under a 5-45% sucrose gradient. Fraction 1, bottom of gradient (45% sucrose); fraction 10, top (5% sucrose). Detergent-insoluble rafts at fractions 5-8. Fractions were subjected to SDS-PAGE on a 7-17% polyacrylamide gel, blotted to PVDF membranes and probed with an anti-aquaporin-1 antibody. These data show that aquaporin-1 was raft associated and a reduced proportion of aquaporin-1 was raft associated in patients when compared with controls. The gradient gels separated the 28kDa aquaporin-1 from non-specific reaction (likely to be carbonic anhydrase). The preparation of rafts from membranes (carbonic anhydrase-free) removed this non-specific reaction.
7.5 SUMMARY AND DISCUSSION

The results here show that the rafts in OHSt and control erythrocytes contain spectrin α, band 2.1, band 4.1, and aquaporin-1 and do not contain clathrin, glycophorin A, annexin V or annexin VI. The results also suggest that there was a reduction in the proportion of certain proteins in the rafts in OHSt patients when compared with controls (actin, spectrin, band 2.1 and aquaporin-1).

The most striking difference between OHSt and control cells is that rafts in OHSt are deficient in actin. This finding is consistent with the results from previous chapters. In chapter 4 it was shown that in OHSt, there is a defect in ATP-dependent endocytic vesiculation that can be corrected by the crosslinker DMA, which also corrects the ion leak. The actin-cytoskeleton has been shown to be involved in endocytic vesiculation in many cell types (Birchmeier and Singer, 1977b; Knowles et al., 1997; Lamaze et al., 1997; Bobrowska-Hagerstrand et al., 1998; Li et al., 1998; Ayscough, 2000; Jeng and Welch, 2001) and previous research has suggested rafts as the focus for endocytic vesiculation and degradation of proteins (Kurzchalia and Parton, 1999; Rozelle et al., 2000). It is possible that the stomatin and actin are associated with each other since they are both in the rafts though this association still needs to be confirmed with immunoprecipitation studies. This would not be inconsistent with previous
research since stomatin has been shown to be colocalised with actin filaments in cultured amniotic cells (Snyers et al., 1997).

Perhaps absence of stomatin, in OHSt, results in less actin in the rafts. If the rafts are involved in endocytic vesiculation and the actin is reduced from the raft fraction in these patients this could be the cause of the endocytic vesiculation defect seen in these patients. The DMA might somehow bridge structures together in the membrane, its actions reflecting that of stomatin. However, it was shown here that addition of DMA does not bring actin back into the rafts in OHSt cells. The fact that adding DMA to OHSt cells before making rafts has no influence on the distribution of actin excludes the possibility that the action of DMA is to move actin into the raft fraction.

Spectrin and band 2.1 may have a similar pattern to actin due to their arrangement with each other and to actin in the cytoskeleton. However, band 4.1 which is bound to beta spectrin (Becker et al., 1990) does not follow this pattern, no convincing difference being seen between patients and controls.

Another difference between the patients and controls studied here was that the patients had a lower proportion of aquaporin-1 in their rafts. This could potentially contribute to their leakiness (though it is a water channel not an ion channel so is unlikely to be the cause of it) or it may have no effect or any effect...
may be masked by the cation leak and osmosis. Aquaporin-1 typically runs as a
doublet, due to the presence of a proportion of glycosylated subunits with slower
electrophoretic mobility (Maunsbach et al., 1997). It is possible that the upper
band labelled 'non-specific' in Fig. 7.5 was the glycosylated aquaporin-1, present
only in the lower (Triton-soluble) part of the gradient and not in the buoyant
fraction. However, this second, upper, band was not present when washed
membranes were used as the start material for the experiment (Fig 7.5, lowermost
panel), suggesting that the uppermost band is more likely to be due to carbonic
anhydrase.

Glycophorin A, clathrin and annexin V and VI were both found to be
completely absent from the rafts here, in both patients and controls. In other cells
various isoforms of annexin have been located in the rafts though this has been
found to be highly dependent on calcium concentration (Parkin et al., 1996a;
Babiychuk and Draeger, 2000) which was absent from the buffers here.

The Harrow OHSt case and other types of HSt were not studied here since
it was decided it would be preferable to look at one patient type in more detail.
However, this would be very interesting to look at in the future.

While the work described in this thesis was in progress, the Prohaska
group published similar findings to those here (Salzer and Prohaska, 2001). Lipid
rafts were isolated from human erythrocytes and their major protein components
were identified. Apart from GPI anchored proteins, the most abundant integral
raft proteins were found to be stomatin, flotillin-1 and flotillin-2.

Acetylcholinesterase was also shown to be raft related. The major cytoskeletal
proteins, spectrin α, actin, band 4.1 and 4.2 were shown to be partly raft
associated. Flotillin-1 and -2 were found in the rafts of OHSt patients that were
deficient in stomatin. From this it was concluded that it is conceivable that OHSt
erthrocytes have a defect in either the assembly or maintenance of lipid-rafts
causing loss of stomatin and possibly other raft proteins (Salzer and Prohaska,
2001). These findings are consistent with the work found here though they did
not report any comparison of the proportion of the proteins in the rafts in patients
compared with controls.

Another group examining a raft related mode of entry of the malarial
parasite into erythrocytes also published findings on rafts prepared from human
erthrocytes (Samuel et al., 2001). They showed that GPI-anchored proteins, the
transmembrane Duffy antigen and the cytoplasmically associated signalling
molecule Gs, could be isolated in detergent-resistant membranes that float with
densities expected for rafts on sucrose gradients (Lauer et al., 2000). It was
further shown (Samuel et al., 2001) that these detergent-resistant domains
constitute 4% of the plasma membrane. Similarly to the results found here, they found that the total protein content of the DIG fraction was reduced compared with the bottom of the gradient (Triton-soluble) but contained multiple protein bands on an SDS gel (consistent with the work found in the following chapter).

The raft fractions were enriched in flotillin, stomatin, and aquaporin-1.

However, unlike the work performed here or that by Salzer and Prohaska (2001) they were unable to detect any of the cytoskeletal proteins: spectrin, actin, ankyrin or band 4.1 in the rafts (Samuel et al., 2001). It must be pointed out that they used EDTA in preparing their membranes. This might explain the conflicting results since EDTA is thought to disrupt the rafts (Parkin et al., 1996a).

Furthermore, glycophorin A (Lauer et al., 2000) and band 3 (Lauer et al., 2000; Samuel et al., 2001) were shown to be absent from the rafts. This is mostly consistent with the work here, though a small proportion of band 3 was raft associated in the work described in this thesis. In my experiments contamination of the raft fraction is a possibility or it could be due to their use of EDTA. Measurement of the exact density of the fractions alongside harvesting of the fractions is required to determine if any contamination is occurring.
On comparison of my work with the more recent literature (Lauer et al., 2000; Salzer and Prohaska, 2001; Samuel et al., 2001), although the results here have on the whole been consistent with other work, it appears that proteins found in the raft fraction vary depending on the technique used. Isolation of rafts with or without EDTA would be a very useful control experiment to determine whether this is the cause of the differences found between groups. Progress in this field requires a single method of isolating rafts to be established so that work between groups can be more easily compared.

The work described in this chapter has confirmed that there is a defect in the rafts in typical OHSt patients, but has only covered a few of the hundreds of red cell membrane proteins. It is only possible to speculate about ones that might be in the rafts. Therefore, in the next chapter, the protein content of the rafts is explored using tools of one-dimensional gels and mass spectrometry. A relationship between this defect in rafts and the defect in endocytic vesiculation and this is examined in Chapter 9, but without these data it is hard to speculate about what the results here could mean and their potential relevance to the clinical picture.
CHAPTER 8

FURTHER INVESTIGATION INTO THE PROTEIN CONTENT OF THE RAFTS BY PROTEOMICS

8.1 AIM

The work to be described in this chapter was aimed at further identification of the protein content of the rafts in the erythrocyte membrane in OHSt patients and controls.

8.2 INTRODUCTION

In chapter 6, it was demonstrated that rafts existed in human red cells. Some known red cell membrane proteins were investigated in Chapter 7, to see if they were raft associated and if so to what degree in patients and controls. The rafts make up a small amount of the bulk of the membrane protein (under 10%) and the techniques used in Chapters 6 and 7 only looked at proteins that were already suspected to be in the rafts. In the experiments here, it was sought to determine further the protein content of these rafts by proteomics, in order to build up a more complete picture of the components of patient and control erythrocyte rafts.

8.3 METHODS

Rafts were prepared from OHSt and control erythrocytes as described in section 3.11. Fractions 0-10 were subjected to SDS-PAGE, blotted to PVDF membranes
and probed with an anti-flotillin antibody to ensure that raft separation had occurred. Rafts (fractions 5-8) were pooled and concentrated (see methods, section 3.12.3) such that protein analysis could be performed, using one-dimensional gels stained with Coomassie blue and mass spectrometry.

A cocktail of protease inhibitors was included throughout the experiments (see methods, section 3.11.3 for details) to avoid excessive protein degradation (below).

Further protein analysis is still in progress by other laboratory members. Rafts have also been prepared for lipid analysis, which is currently being performed by Anna Nicolaou [Department of Pharmacology, University of Bradford].

8.4 RESULTS

8.4.1. Preliminary experiments to optimise the method for concentration of rafts while avoiding excessive protein degradation.

Coomassie blue stained gels of concentrated rafts without protease inhibitors did not show distinct bands suggesting that either protein degradation might be occurring or the high lipid content was causing this effect. Therefore, raft separations were performed with or without inclusion of a cocktail of protease inhibitors throughout the preparation (see methods, section 3.11.3). Fractions
were harvested and rafts were concentrated (section 3.12.3). SDS-PAGE was performed and gels were stained with Coomassie blue (Figure 8.1) or Western blotted and probed with an anti-flotillin antibody (Figure 8.2), in order to determine the effect of the protease inhibitors on protein degradation. The results show that a significant amount of protein degradation occurred during raft concentration and that this was lessened by the addition of the protease inhibitors.

To further ensure that the protease inhibitors used did not alter raft separation either in patient or control samples, rafts were prepared with protease inhibitors in a number of both patient and control blood samples. Fractions 0-10 were subjected to SDS-PAGE, blotted to PVDF membranes and probed with the anti-flotillin antibody. From this it was seen that the protease inhibitors had no effect on raft formation. Therefore, this cocktail of inhibitors was used in all experiments to stop degradation in all future preparations.
Whole normal red cells were subjected to raft separation with (+inh) or without (-inh) protease inhibitors. Carbonic anhydrase (28kD; CA) and Hb (15kD) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, discrete bands are only clearly visible where protease inhibitors were included (right panel). Degradation occurred when inhibitors were not included (left panel). Some spectrin (Sp) and band 3 (B3) are visible in the raft fraction, but the distribution is not identical to a standard membrane preparation (right lane). Flotillin (Fl) is visible at 48kDa and stomatin (St) is present at 32kDa in raft fraction.
Whole normal red cells were subjected to raft separation with (+ inh) or without (- inh) protease inhibitors. Rafts were concentrated, subjected to SDS-PAGE, blotted to PVDF membranes and probed with an anti-flotillin antibody. When protease inhibitors were included (right panel), there was more immunoreactivity with flotillin (fl) at 48kDa compared with the no protease inhibitor condition (left panel) which shows less reactivity at 48kDa and shows what is presumed to be degradation products at lower molecular weights. These blots suggest that there is more degradation of flotillin in the no protease inhibitor condition. The positive controls used are the pooled, but not concentrated rafts (fractions 5-8).
8.4.2. Patient and control rafts analysed by SDS-PAGE

Figures 8.3-8.7 show Coomassie blue stained SDS-PAGE of fractions 0-4 (pellet plus Triton-soluble material) and the concentrated rafts (fractions 5-8) prepared from the sucrose gradient separation of red cells. A sample containing simple purified membranes was also electrophoresed to aid in the identification of proteins (Figure 8.3 Brighton experiment jj1, Figure 8.4 Brighton experiment jj4, Figure 8.5 Manchester experiment jj2, Figure 8.6 Manchester experiment jj5, Figure 8.7 Harrow).

In all three patients, a band at 32kDa is missing which is present in the control. This is presumed to be stomatin. In the Manchester and Brighton pedigrees there is an apparent difference at 70kDa (Figures 8.3-8.6) where a raft protein 'X' is absent (Figure 8.5 and 8.6 Manchester), hardly detectable (Figure 8.4 Brighton), or hardly detectable though perhaps appearing as a faint doublet (Figure 8.3 Brighton) in OHSt, but present in control rafts. This difference was not seen in the Harrow pedigree (Figure 8.7), but the staining intensity was darker and the experiment was not repeated. In the Harrow pedigree there appeared to be a raft protein 'Y' at 28kDa but virtually nothing in the ghosts, but in the control this protein 'Y' was only detectable in the ghosts (Figure 8.7). There is no difference between the banding pattern in the DMA or control condition in
Manchester rafts (Figure 8.8). Table 8.1 summarises the presence of red cell proteins in rafts and ghosts from patients and controls as determined by Coomassie blue staining and by Western blotting (Chapter 7).
Whole red cells were subjected to raft separation. Carbonic anhydrase (28kD: CA) and Hb (15kD) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, some spectrin (Sp) and band 3 (B3) are visible, but the distribution is not identical to a standard membrane preparation (right lane). Flotillin (Fl) is visible at 48kD in both normal and abnormal raft fractions. Stomatin is present in normal red cells but invisible in OHSt, as expected. A protein 'X' at 70kDa is also apparently absent or as a faint doublet in OHSt cells on this Coomassie stain.
Whole red cells were subjected to raft separation. Carbonic anhydrase (29kDa; CA) and Hb (15kDa) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, some spectrin (Sp) and band 3 (B3) are visible, but the distribution is not identical to a standard membrane preparation (right lane). Flotillin (Fl) is visible at 48kDa in both normal and abnormal raft fractions. Stomatin is present in normal red cells but invisible in OHSt, as expected. A protein 'X' at 70kDa is also only just detectable in OHSt cells on this Coomassie stain.
Whole red cells were subjected to raft separation. Carbonic anhydrase (28kD; CA) and Hb (15kD) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, some spectrin (Sp) and band 3 (B3) are visible, but the distribution is not identical to a standard membrane preparation (right lane). Flotillin (Fl) is visible at 48kD in both normal and abnormal raft fractions. Stomatin is present in normal red cells but invisible in OHSt, as expected. A protein 'X' at 70kDa is also apparently absent in OHSt cells on this Coomassie stain.
Whole red cells were subjected to raft separation. Carbonic anhydrase (28kD; CA) and Hb (15kD) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, some spectrin (Sp) and band 3 (B3) are visible, but the distribution is not identical to a standard 'membrane preparation' (right lane). Flotillin (Fl) is visible at 48kD in both normal and abnormal raft fractions. Stomatin is present in normal red cells but invisible in OHSt, as expected. A protein 'X' at 70kDa is also apparently absent in from OHSt cells on this Coomassie stain.
Whole red cells were subjected to raft separation. Carbonic anhydrase (28kD: CA) and Hb (15kD) predominate in the soluble fraction (lanes 1-4) but membrane proteins can also be seen. In the raft fraction, concentrated by dilution of sucrose followed by centrifugation, some spectrin (Sp) and band 3 (B3) are visible, but the distribution is not identical to a standard membrane preparation (right lane). Flotillin (Fl) is visible at 48kD in both normal and abnormal raft fractions. Stomatin is present in normal red cells but invisible in OHSt, as expected. A protein 'Y' at 28kDa is also apparently in the rafts, but not the ghosts in OHSt cells unlike the control where it is only visible in the ghosts on this Coomassie stain.
Whole OHSt red cells were incubated with or without 2mM DMA, washed in TBS and subjected to raft separation. No difference in the banding pattern of rafts was seen in this Coomassie stained gel after pretreatment of erythrocytes with DMA.
Table 8.1 Table describing bands visible by Coomassie blue (CB) and Western blotting (WB) in Manchester/ Brighton

OHSt and control ghosts and raft preparations.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW kDa (gel)</th>
<th>Control ghosts on CB gel</th>
<th>OHSt ghosts on CB gel</th>
<th>Rafts in control blood</th>
<th>Rafts in OHSt blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-spectrin</td>
<td>240</td>
<td>visible</td>
<td>visible</td>
<td>visible by WB and on CB</td>
<td>visible by WB and on CB</td>
</tr>
<tr>
<td>β-spectrin</td>
<td>220</td>
<td>visible</td>
<td>visible</td>
<td>visible on CB</td>
<td>visible on CB</td>
</tr>
<tr>
<td>ankyrin</td>
<td>210</td>
<td>indistinguishable from spectrin</td>
<td>indistinguishable from spectrin</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>adducin</td>
<td>97+103</td>
<td>not visible</td>
<td>not visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>band 3</td>
<td>90-100</td>
<td>visible</td>
<td>visible</td>
<td>visible on CB and WB</td>
<td>visible on CB and WB</td>
</tr>
<tr>
<td>4.1</td>
<td>80+78</td>
<td>visible</td>
<td>visible</td>
<td>visible on CB and WB</td>
<td>visible on CB and WB</td>
</tr>
<tr>
<td>4.2</td>
<td>72</td>
<td>indistinguishable from 4.1</td>
<td>indistinguishable from 4.1</td>
<td>WB not done, indistinguishable from 4.1 on CB, ? protein 'X'</td>
<td>WB not done, indistinguishable from 4.1 on CB, ? protein 'X'</td>
</tr>
<tr>
<td>dematin (4.9)</td>
<td>48+52</td>
<td>visible</td>
<td>visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>p55</td>
<td>55</td>
<td>not visible</td>
<td>not visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>actin (5)</td>
<td>43</td>
<td>visible</td>
<td>visible</td>
<td>visible by WB and on CB</td>
<td>reduced on WB</td>
</tr>
<tr>
<td>G3PD (6)</td>
<td>35</td>
<td>visible</td>
<td>visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>Band 7 stomatin</td>
<td>31</td>
<td>visible</td>
<td>not visible</td>
<td>visible by WB and on CB</td>
<td>tiny amounts by WB, absent from CB</td>
</tr>
<tr>
<td>tropomyosin</td>
<td>27+29</td>
<td>not visible</td>
<td>not visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
<tr>
<td>Aquaporin-1</td>
<td>28</td>
<td>not visible</td>
<td>not visible</td>
<td>visible by WB</td>
<td>visible by WB at lower amounts</td>
</tr>
<tr>
<td>Protein 8</td>
<td>23</td>
<td>visible</td>
<td>visible</td>
<td>WB not done, not visible on CB</td>
<td>WB not done, not visible on CB</td>
</tr>
</tbody>
</table>

where WB = Western blot and CB refers to 'Coomassie blue'
The Coomassie blue stain has advantages of being quantitative, unlike silver staining, but even after the concentration of the rafts there was little protein. This meant that the bands were difficult to discern. Therefore, concentrated rafts and ghosts from patients and controls were subjected to SDS-PAGE and silver stained (Figure 8.9). The only clear and consistent difference was that a 32kDa protein (which is presumed to be stomatin) was absent from OHSt rafts and ghosts. The multitude of bands apparent due to the sensitivity of silver staining also made it difficult to decide if the patients and control rafts had different banding patterns. Part of the problem could be due to the fact that the samples were degraded and although every step was taken to ensure that the cells were treated the same way, it is possible that they degraded at different rates, thereby producing artefacts.

However, silver stains, stained with less intensity were useful for matrix assisted laser desorption ionisation-time of flight (MALDI-TOF) analysis (below) and with less intensive staining less bands were visible and differences between patient (Manchester) and control rafts became apparent. Bands were undetectable in patient, but not control, rafts at 150kDa, 70kDa (presumably protein X) and 32kDa (Figure 8.10). These areas of the gel were labelled D, H and L respectively.
Whole red cells from patients and controls were subjected to raft separation, concentrated, subjected to SDS-PAGE (using 9% acrylamide gels) alongside membranes from the same patients and controls, and silver stained. There are a vast number of bands using this technique, most unidentifiable. Spectrin (Sp), band 3 (B3), flotillin (Fl), carbonic anhydrase (CA) and haemoglobin (Hb) can all be seen in both patient and control rafts. Stomatin (St) is visible in controls, but only appears as a faint band in patients.
Whole red cells were subjected to raft separation, concentrated, subjected to SDS-PAGE and silver staining. Stomatin (labelled I here) is present in normal red cells but invisible in OHSt (labelled L), as expected. The bands labelled as d and h in controls and are also apparently absent from the OHSt cells. The missing bands in OHSt have been labelled D and H respectively. The bands a to m in controls and OHSt were excised from the gel. Areas D, H and L were also since these areas were where the bands were missing in OHSt. The gels samples were digested and subjected to MALDI-TOF analysis.
8.4.3. MALDI-TOF analysis

Raft samples prepared from the Manchester pedigree and control erythrocytes were electrophoresed on a large one dimensional 9% polyacrylamide gel, silver stained and the bands, labelled a-m, excised (Figure 8.10). It can be seen that the bands which were labelled d, h, and l, at 150, 70 and 32kDa respectively appeared in the control, but not the patient (labelled D, H and L). All visible bands in patient and control, and areas of the patient gel where these bands had appeared in the control, were excised and digested for MALDI-TOF analysis (methods section 3.12).

MALDI-TOF analysis was performed and data analysed as described in methods (section 3.12). A very large amount of data was obtained, which will not be described in detail here. Table 8.2 shows the two most statistically significant results as determined by MOWSE scores (based on the probability that a match is significant) for each excised band. Using the Matrix Science program (Table 8.2a), a score of 61 or more implies that the probability of that result being insignificant is less than 0.05 (www.matrixscience.com) so scores below 61 were rejected. With the MSFIT programme (Table 8.2b), scores of over $10^3$ were considered significant (personal communication with D Bradley [Prof. J Godavac-Zimmerman laboratory, Rayne institute, UCL]). MSFIT gave many
more significant matches for each band, but for simplicity the table only gives the two highest-scoring proteins for each band. On occasion, the browsers identified the same protein in two bands (this was most common for myosin and titin). It was presumed that at least one of these represented a degradation product. Such proteins were only given once in the table at the highest molecular weight.

Keratin was not included in the results since this was probably due to contamination.

Most bands on this one-dimensional analysis had numerous significant proteins. Of particular interest were the high number of high scores for titin and myosin in rafts of patients and controls.

8.4.4. Lipid analysis

Rafts prepared from OHSt (Brighton pedigree) and control erythrocytes are currently being examined by Anna Nicolaou [Department of Pharmacology, University of Bradford].
Table 8.2a Table showing 2 highest significant matches for each using the matrix science programmes for control and OHSt:

<table>
<thead>
<tr>
<th>Band and MW (kDa)</th>
<th>Matrix science</th>
<th>Control</th>
<th>MOWSE score if &gt;61 (p&lt;0.05)</th>
<th>OHSt</th>
<th>MOWSE score if &gt;61 (p&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. 270</td>
<td>spectrin alpha erythrocyte</td>
<td>151</td>
<td>spectrin alpha erythrocyte</td>
<td>112</td>
<td></td>
</tr>
<tr>
<td>b. 260</td>
<td>no match</td>
<td></td>
<td>no match</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. 250</td>
<td>no match</td>
<td></td>
<td>no match</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. 150</td>
<td>no match</td>
<td></td>
<td>myosin heavy polypeptide, skeletal muscle</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>bands e-m</td>
<td>no match</td>
<td></td>
<td>no match</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8.2b Table showing 2 highest significant matches for each band using the MSFIT database for control and OHSt:

<table>
<thead>
<tr>
<th>MSFIT</th>
<th>Control</th>
<th>MOWSE score if &gt; 1e+03</th>
<th>OHSt</th>
<th>MOWSE score if &gt; 1e+03</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. 270</td>
<td>spectrin alpha chain</td>
<td>7.56e+05</td>
<td>spectrin, alpha elliptocytosis 2</td>
<td>6.75e+16</td>
</tr>
<tr>
<td></td>
<td>spectrin alpha chain</td>
<td>6.75e+16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. 260</td>
<td>trabeculin-alpha</td>
<td>2.65e+04</td>
<td>spectrin beta erythrocytic spherocytosis</td>
<td>1.25e+07</td>
</tr>
<tr>
<td></td>
<td>spectrin beta erythrocytic</td>
<td>9.67e+04</td>
<td>spectrin rouen mutant coding sequence</td>
<td>9.31e+05</td>
</tr>
<tr>
<td>c. 250</td>
<td>titin</td>
<td>2.21e+15, but a score of 6.21e+84 at 73kDa</td>
<td>titin</td>
<td>4.93e+07</td>
</tr>
<tr>
<td></td>
<td>actin binding protein</td>
<td>5.15e+10</td>
<td>trabeculin alpha (actin binding protein)</td>
<td>7.31e+04</td>
</tr>
<tr>
<td>d. 150</td>
<td>fibrillin-1 precursor</td>
<td>8.73e+16</td>
<td>KIAA1262 protein</td>
<td>1.16e+04</td>
</tr>
<tr>
<td></td>
<td>ryanodine receptor</td>
<td>2.73e+15</td>
<td>microtubule-actin crosslinking factor</td>
<td>7.69e+03</td>
</tr>
<tr>
<td>e. 110</td>
<td>myosin, heavy polypeptide</td>
<td>5.84e+05</td>
<td>unnamed protein product</td>
<td>2.61e+07</td>
</tr>
<tr>
<td></td>
<td>protocadherin</td>
<td>1.5e+05</td>
<td>myosin, heavy polypeptide</td>
<td>2.2e+04</td>
</tr>
<tr>
<td>f. 100</td>
<td>collagen alpha-1</td>
<td>2.01e+06</td>
<td>HSTITIN</td>
<td>1.66e+07</td>
</tr>
<tr>
<td></td>
<td>fibrillin-1 precursor</td>
<td>1.3e+06</td>
<td>fibrillin-2 precursor</td>
<td>1.02e+04</td>
</tr>
<tr>
<td>Band and MW (kDa)</td>
<td>Control</td>
<td>MOWSE score if &gt; 1e+03</td>
<td>OHSt</td>
<td>MOWSE score if &gt; 1e+03</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>g. 80</td>
<td>no match</td>
<td></td>
<td>neurofibromin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>collagen alpha-1 precursor</td>
<td>1.23e+06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.52e+05</td>
</tr>
<tr>
<td>h. 73</td>
<td>nebulin</td>
<td>6.65e+47</td>
<td>fibrilllin-1 precursor</td>
<td>6.27e+06</td>
</tr>
<tr>
<td></td>
<td>elastic titin</td>
<td>5.45e+42</td>
<td>ryanodine receptor</td>
<td>1.77e+06</td>
</tr>
<tr>
<td>i. 55</td>
<td>HSTTITIN</td>
<td>2.37e+06</td>
<td>collagen type XII</td>
<td>3.26e+05</td>
</tr>
<tr>
<td></td>
<td>cardiac muscle titin</td>
<td>2.37e+06</td>
<td>laminin beta precursor</td>
<td>3.18e+05</td>
</tr>
<tr>
<td>j. 50</td>
<td>no match</td>
<td></td>
<td>zinc finger protein</td>
<td>3.28e+03</td>
</tr>
<tr>
<td>k. 48</td>
<td>unnamed protein product</td>
<td>1.23e+05</td>
<td>titin, cardiac muscle</td>
<td>2.03e+07</td>
</tr>
<tr>
<td></td>
<td>VLDL</td>
<td>1.2e+05</td>
<td>heparan sulfate proteoglycan precursor</td>
<td>2.41e+03</td>
</tr>
<tr>
<td>l. 32</td>
<td>ankyrin B</td>
<td>3.85e+05</td>
<td>kendrin</td>
<td>1.76e+03</td>
</tr>
<tr>
<td></td>
<td>ankyrin 2 isoform</td>
<td>3.82e+07</td>
<td>ankyrin 2</td>
<td>1.29e+03</td>
</tr>
<tr>
<td>m. 14</td>
<td>HSA6788 NID</td>
<td>4.04e+03</td>
<td>no match</td>
<td></td>
</tr>
</tbody>
</table>
8.5 SUMMARY AND DISCUSSION

This chapter showed that a raft protein at about 70kDa might have been deficient from the patient rafts in the Manchester and Brighton pedigrees (Figures 8.3-8.6).

This was confirmed by silver staining (Manchester pedigree) which also suggested that a 150kDa protein could be missing. The mass spectrometry provided preliminary data as to what some of these proteins might be. These included alpha and beta spectrin (Goodman et al., 1988), actin binding proteins (dos Remedios and Thomas, 2001), titin (Trinick, 1994; Maruyama, 1997)(with very high scores and in most of the bands), myosin (Fowler et al., 1985)(in most of the bands), ryanodine receptor (Takeshima, 1998), nebulin (Trinick, 1994), fibrillin (Ramirez and Pereira, 1999), collagen (Bergeon, 1967), fibrillin and collagen precursors, ankyrin (Bennett, 1992) and kendrin (Li et al., 2001).

Ideally the mass spectrometry experiments should have been repeated on at least 3 patient raft samples, but time did not permit. These data also need confirmation by means such as Western blotting (which in turn would demonstrate any patient and control differences since mass spectrometry cannot give quantitative results). This was not possible during the work described in this thesis due to time restraints, but will be continued in both the Stewart and Hooper laboratories shortly. Ideally, two-dimensional analysis should have been used.
because the bands contained multiple proteins and this would have further separated them.

Although these data appear to suggest a mutant beta spectrin (Table 8.2) this need not really mean that the mutant spectrin is actually present, since it could be the result of a contaminant (from the gel or other proteins on the gel) being present as well as spectrin. A contaminant from the gel, with some similar tryptic fragments to mutant beta spectrin could give an artefactual result. This result would need to be repeated on a very pure sample preferably by two-dimensional analysis in order to draw such a conclusion or to be sequenced.

A potential candidate for the 70kDa protein X absent from OHSt cells is band 4.2 pallidin and protein Y in the rafts in Harrow could be tropomyosin though this is purely speculative, and based on the molecular weights of these known red cell membrane proteins.

The mass spectrometry data suggest that myosin and titin are in the rafts of patients and controls. A form of myosin is known to be present in the erythrocyte (Fowler et al., 1985; Matovcik et al., 1986; Higashihara et al., 1989; Pasternack and Racusen, 1989; Racusen and Pasternack, 1990), although the gene has never been cloned and the exact sequence is not known.
A 200kDa polypeptide which cross reacted with affinity-purified antibodies to platelet myosin has previously been found in the erythrocyte. After haemolysis and preparation of ghosts, 20-40% of the myosin remained associated with the membranes, suggesting that it is both bound to the membrane and present in the cytosol. Immunofluorescence staining demonstrated that myosin was present in all cells and is localised in a punctate pattern throughout the cell (Fowler et al., 1985). Since the findings in this chapter suggest that myosin could be an erythrocyte raft protein, it could be argued that this punctate pattern is due to it being localised in the rafts.

It has been suggested that erythrocyte myosin could function together with tropomyosin (by interacting with the membrane-associated actin filaments) in an actomyosin contractile apparatus to influence ATP-dependent erythrocyte changes in erythrocyte shape (Fowler et al., 1985) and also membrane properties (Fowler, 1986). Erythrocyte myosin exhibited a characteristic pattern of myosin ATPase activities. It was present in a ratio of 80 actin monomers for every myosin molecule (Fowler et al., 1985). This is comparable to actin-myosin ratios in other non-muscle cells, and there is enough tropomyosin to almost completely coat all the short actin filaments in the membrane cytoskeleton (Fowler, 1986).
Regulatory myosin light chain (but not myosin heavy polypeptide as suggested here) has been found previously to be present in rafts. It was identified by mass spectrometry in the Triton-insoluble floating fraction along with actin, porin, comitin, a member of the CD36 family and the phospholipid anchored cell adhesion molecule gp80 in Dictyostelium (amoebae) cells (Harris et al., 2001).

Titin (also known as connectin) is an elastic protein present in skeletal and cardiac muscle in vertebrates (Maruyama et al., 1977). Titin interacts with myosin and actin filaments by aggregating them (Kimura et al., 1984) and in cardiac muscle the actin-titin association contributes to the stiffness (Linke et al., 1997). It has also been found in non muscle cells, for example human platelets, and has also been found in many invertebrates (Locker and Wild, 1986). Connectin-like protein was detected in erythrocyte membranes and fluorescent anti-connectin staining suggested it is located on the cytoplasmic surface of the membrane (Maruyama et al., 1977).
CHAPTER 9

INVESTIGATION OF THE MECHANISM OF ATP-DEPENDENT ENDOCYTIC VESICULATION IN NORMAL ERYTHROCYTE GHOSTS.

9.1 AIM

The aim of the work to be described here was to explore the molecular nature of the ATP-dependent endocytic vesiculation process in normal red cells, using pharmacological inhibitors of protein kinase activity, an inhibitor of ATPase activity, lipid raft disrupters, and some antibodies to candidate proteins which might potentially be involved in the process.

9.2 INTRODUCTION

The molecular nature of ATP-dependent endocytic vesiculation in erythrocyte ghosts is not fully understood. The work described in chapter 4 confirmed that there could be a complete defect in this process in ghosts prepared from OHSt cells. The work described in this chapter was designed to investigate this mechanism in normal red cell ghosts. It is hypothesised here that both stomatin (and possibly other red cell membrane proteins) and lipid rafts are involved in this process. The involvement of protein kinases and/ or an ATPase in the process, was also investigated. The work described in chapter 4 also suggested a
strong relationship between ATP-dependent endocytic vesiculation and the ion leak in the defective cells. Here, it is hypothesised that agents affecting one of these processes in normal cells will also affect the other process.

The effect of various antibodies (anti-stomatin, flotillin, spectrin α, actin, aquaporin-1 and annexin V), known raft disrupters (MBCD, nystatin, filipin, digitonin and saponin) and pharmacological inhibitors of phosphorylation processes including protein kinase inhibitors (staurosporine, wortmannin, A3 hydrochloride, H9 and genestein) was investigated. Other agents were employed: a general ATPase inhibitor (vanadate) and the alkylating agent NEM, known to be an inhibitor of the aminophospholipid flipase (de Jong et al., 1997; Hagerstrand et al., 1998) and a general inhibitor of vesiculation processes (Dachary-Prigent et al., 1993; Skiba et al., 1996; Simon et al., 1998). The substitution of ATP with ATPγS aimed to investigate whether cleavage of the phosphate bond was necessary for ATP-dependent endocytic vesiculation to occur.

9.3 METHODS

The methods involved in induction of (Chapter 3, section 3.7) and measurement (Chapter 3, section 3.5.8) of ATP-dependent endocytic vesiculation have previously been described. To investigate the action of specific antibodies or
chemicals on this process, the influence of various reagents on ATP-dependent endocytic vesiculation was measured. The experiments were easy to perform due to the ability to reseal reagents inside ghosts before inducing ATP-dependent endocytic vesiculation. Phosphate-free ghosts were prepared as described in the methods chapter (Chapter 3 section 3.6). They were kept at 0°C and molecules were added and the resulting suspension mixed. After an appropriate incubation time on ice (30 minutes before ATP addition for raft disrupters or antibodies, added at the same time as ATP for others) and addition of ATP or ITP, ghosts were warmed to 37°C. This both resealed the ghosts, now with the added molecules inside and initiated ATP-dependent endocytic vesiculation. Returning the ghosts to ice stopped endocytic vesiculation or at least slowed it to an insignificant level while the AChE assay was performed.

Lipid raft disrupters were employed to determine whether this vesiculation process is raft-based. Table 1.1 (Chapter 1) gives the mechanism of the raft disrupters employed. In certain experiments (nystatin Figure 9.3, filipin Figure 9.4A, MBCD Figure 9.6A) whole erythrocytes were incubated with raft disrupters before washing three times in TBS, pH 7.4, prior to the preparation of ghosts or measurement of fluxes. The washing step was important due to the known reversible effect of nystatin (which disappears after washing cells in
nystatin-free media) on increasing the permeability of the erythrocyte membrane
to Na⁺, K⁺ and Cl⁻ (Dalmark, 1975; Joiner and Lauf, 1978; Freedman and
Hoffman, 1979). ATP-dependent endocytic vesiculation in erythrocyte ghosts
and/or the ouabain+bumetanide-resistant K⁺ influx in whole erythrocytes was
measured.

In order for the AChE assay to truly reflect endocytic events, the ghosts
had to be intact and not lysed since this would expose any internalised AChE.
Incubation of intact erythrocytes with chemicals provided a simple way of
assessing whether lysis had occurred. When the cells were centrifuged during the
washing step, if lysis has occurred, haemoglobin was released and this was easily
detected in the supernatant. Any conditions causing visible lysis of cells were
discarded, before the experiment was performed, since this suggested damage to
the membrane and could potentially result in the ghosts not resealing.

Chemicals were dissolved in suitable solvents (ethanol, methanol, DMSO,
water) as described in methods (Chapter 3, Table 3.2). At no point was the
concentration of these more than 1% (v/v) and controls contained the appropriate
solvent.
The anti-stomatin antibody used in these experiments has already been described (see methods, section 3.4.1 and (Coles et al., 1999b)). Other antibodies used are presented in Table 3.3 (methods, section 3.4).

9.4 RESULTS

9.4.1. Stomatin antibody experiments

Erythrocyte ghosts were incubated with an antibody to stomatin at 4°C for 30 minutes prior to adding ATP to see if it inhibited the process of ATP-dependent endocytic vesiculation. Figure 9.1 A shows the effect of the antibody on this process and it was found to be inhibitory at extremely low dilutions with an IC₅₀ of 31ng/ml. At the same time, ghosts were incubated with an anti-GST antibody at a concentration of 1.5µg/ml (an ‘isotype control’). Normal endocytic vesiculation was seen in both this condition and the ‘no antibody’ condition.

The action of the anti-stomatin antibody could have been due to non-specific binding. To eliminate the possibility that the inhibitory effect was artefactual, ghosts were incubated on ice with various concentrations (0-2.5µg/ml) of the soluble stomatin fusion protein D13-7 for 10 minutes prior to the addition of a fixed concentration of anti-stomatin antibody (63ng/ml), which had previously been shown to inhibit endocytic vesiculation (by about 90%), but not
completely (Figure 9.1B). This figure shows that the inhibitory activity was "competed off" with D13-7 (stomatin).

Another potential explanation of the action of the anti-stomatin antibody could have been a mechanism based on 'rigidification' of the membrane, i.e. a stiffening action by virtue of the coating of the cytoplasmic aspect of the membrane with an antibody to a common protein. To try to control for such an effect, Fab fragments of the antibody were prepared (see methods, section 3.4.2) and employed in the same assay in three separate experiments (Figure 9.1C). These data show that inhibition of endocytic vesiculation occurred with anti-stomatin Fab fragments. The concentration of the Fab fragments was unknown since the amount made was insufficient to perform a protein assay, though they were detected on a Western blot at a dilution of 1:100 (see methods, section 3.3).
Figure. 9.1 Inhibition of ATP-dependent endocytic vesiculation with an anti-stomatin antibody, Fabs and competition of this antibody by the stomatin fusion protein D13-7.

A: Inhibition of endocytosis by anti-stomatin antibody

B: Competition by stomatin fusion protein

C: Inhibition by Fab fragments of anti-stomatin antibody

Erythrocyte ghosts were prepared and were incubated on ice for 30 minutes with an anti-stomatin antibody (A) or Fab fragments from an anti-stomatin antibody (C) before addition of ATP to a final concentration of 5mM and measurement of ATP-dependent endocytic vesiculation. The effect of the anti-stomatin antibody was competed out by addition of D13-7 to the ghosts 10 minutes prior to addition of the antibody (B). It can be seen that the anti-stomatin antibody and Fab fragments inhibit the process and the effect of the antibody can be competed out by the stomatin fusion protein D13-7.
9.4.2. Experiments using other antibodies

The effect of the anti-flotillin antibody on this process and other antibodies to red cell membrane or raft proteins was assessed by incubation of erythrocyte ghosts at 4°C for 30 minutes with these antibodies prior to addition of ATP. Figure 9.2 shows the data for anti-flotillin, anti-actin, anti-spectrin α, anti-aquaporin-1 and anti-annexin V. From this, it can be seen that the antibodies tested had no apparent effect. The highest concentration used for each antibody (1: 200 dilution was used for all antibodies though expressed as concentrations in Figure 9.2) was higher than that used for Western blotting (where a 1:500 dilution was used for all antibodies except for anti-spectrin α which used a 1:750 dilution (Figure 7.7 and Figure 8.1-8.5)).
Figure 9.2 Effect of various antibodies on ATP-dependent endocytic vesiculation in erythrocyte ghosts.

jj12 and jj16 anti-flotilin  jj13 anti-actin

Erythrocyte ghosts were prepared and were incubated for 30 minutes on ice with the above antibodies at the concentrations indicated (where known). The highest concentration of anti-spectrin was a 1:200 dilution. The highest concentrations used were higher than those suitable for probing of Western blots. The data show that none of the antibodies appear to have any effect on ATP-dependent endocytic vesiculation.
9.4.3. The effect of compounds known to disrupt lipid rafts on the ATP-dependent endocytic vesiculation and 'leak' K\(^+\) influx

To investigate the possibility that the cholesterol-dependent raft might be implicated in this process, the effect of various compounds known to disrupt lipid rafts (Hooper, 1999) was investigated. Whole erythrocytes from normal individuals were resuspended at a haematocrit of 2% and incubated with 0-5\(\mu\)g/ml nystatin for 30 minutes in the dark at room temperature, followed by three washes in TBS, pH 7.4, prior to making ghosts and the performance of the endocytosis experiment (Figure 9.3A) or the measurement of ouabain+bumetanide-resistant K\(^+\) influx on the whole erythrocytes (Figure 9.3B). Nystatin was shown to inhibit the endocytic process (Figure 9.3A) (IC\(_{50}\)=15ng/ml) and increase the 'leak' K\(^+\) influx (Figure 9.3B) (EC\(_{50}\)=0.035ng/ml). Figure 9.4A shows the effect of incubation of whole erythrocytes with 7.5\(\mu\)g/ml filipin (30 min. 37\(^\circ\)C), followed by three washes in TBS, pH 7.4, prior to preparation of ghosts on ATP-dependent endocytic vesiculation at 0-12.5mM ATP. Figure 9.5 shows the effect of incubating whole erythrocytes with 0-100\(\mu\)g/ml filipin, followed by three washes in TBS, pH 7.4, on the ouabain+bumetanide-resistant K\(^+\) influx. Filipin had no effect on either endocytic vesiculation or the ouabain+bumetanide-resistant K\(^+\) influx. Incubation of whole
erythrocytes with 10mM MBCD (Figure 9.6A), and washing three times in TBS, pH 7.4 prior to making ghosts caused complete inhibition of endocytic vesiculation at all concentrations of ATP tested.

In other experiments (filipin Figure 9.4B, MBCD Figure 9.6B, saponin Figure 9.7 and digitonin Figure 9.8), raft disrupters at various concentrations were added to the actual ghosts and ATP-dependent endocytic vesiculation was measured. In all cases (including filipin, which had not had an effect when incubated with the whole erythrocytes on either endocytic vesiculation or the flux), there was complete inhibition of endocytic vesiculation. The IC₅₀'s were calculated as follows: MBCD=2mM, digitonin=5.4μM, saponin=4.73μg/ml, filipin=5μg/ml which were comparable to the published data (Table 9.1).
A. Effect of nystatin on ATP-dependent endocytic vesiculation in normal ghosts (expt jb17).

Intact erythrocytes were incubated with 0-5μg/ml nystatin (30 min, room temperature, in the dark). Nystatin was removed by three washes in TBS, pH 7.4.

A. ATP-dependent endocytic vesiculation was measured at 5mM ATP
B. Ouabain + bumetanide-resistant K influxes were measured (courtesy of MC Chetty).

The data show that incubation of erythrocytes with nystatin irreversibly caused inhibition of ATP-dependent endocytic vesiculation (IC50=15ng/ml) and increased the ouabain+bumetanide-resistant K influx (EC50=0.035ng/ml).
Figure 9.4 (Expt JJ10 and 11) Effect of filipin on ATP-dependent endocytic vesiculation in normal red cells and ghosts.

A. INCUBATION OF INTACT RED CELLS WITH FILIPIN

B. INCUBATION OF ERYTHROCYTE GHOSTS WITH FILIPIN

A. ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP in ghosts made from erythrocytes exposed to 7.5μg/ml filipin at 37°C for 30 min. before washing three times in TBS, pH 7.4.

B. ATP dependent endocytic vesiculation was measured at 5mM ATP in ghosts exposed to 0-125μg/ml filipin for 30 min on ice.

The data show that when intact erythrocytes were incubated with filipin and washed, before preparation of ghosts, it had no effect on ATP-dependent endocytic vesiculation, but when the ghosts themselves were exposed to filipin, ATP-dependent endocytic vesiculation was inhibited with an IC50 of 5μg/ml.
Intact erythrocytes were exposed to 0-100μg/ml filipin at 37°C for 30 min. Erythrocytes were washed three times in TBS pH 7.4 and ouabain + bumetanide resistant K influxes were measured (courtesy of MC Chetty). The data show that incubation of intact erythrocytes with filipin, followed by washing had no effect on the ouabain+bumetanide-resistant K influx.
Figure 9.6 Effect of MBCD added to erythrocytes (A) and ghosts (B) on ATP-dependent endocytic vesiculation

A. EFFECT ON INCUBATION OF INTACT RED CELLS WITH MBCD ON ATP-DEPENDENT ENDOCYTIC VESICULATION IN ERYTHROCYTE GHOSTS

B. EFFECT OF DIRECT INCUBATION OF GHOSTS WITH MBCD ON ATP-DEPENDENT ENDOCYTIC VESICULATION IN ERYTHROCYTE GHOSTS

A. Intact erythrocytes were exposed to 10mM MBCD at 37°C for 30 min. MBCD was removed by washing three times in TBS, pH 7.4. Ghosts were prepared and ATP dependent endocytic vesiculation was measured at 0-12.5mM ATP

B. ATP dependent endocytic vesiculation was measured at 5mM ATP in ghosts exposed to 0-12.5mM MBCD.

The data show that incubation of intact erythrocytes with MBCD irreversibly inhibited ATP-dependent endocytic vesiculation and that incubation of the ghosts with MBCD inhibited the processes (IC50=2mM).

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Figures 9.7 and 9.8. Effect of saponin (9.7) or digitonin (9.8) on ATP-dependent endocytic vesiculation in normal ghosts.

Normal erythrocyte ghosts were incubated on ice for 30 min. with saponin (upper panel, Fig. 9.7) or digitonin (lower panel, Fig. 9.8) at the concentrations indicated. ATP-dependent endocytic vesiculation was measured at 5mM ATP. The data show that both saponin (IC50=4.73µg/ml) and digitonin (IC50=5.4µM) inhibited ATP-dependent endocytic vesiculation when incubated directly with the ghosts.
Table 9.1. IC₅₀ measurements for effect of different raft disrupters on ATP-dependent endocytic vesiculation in normal human red cell membranes. Comparison with published data on effect of same substances on other biological systems.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conditions used</th>
<th>IC₅₀ (ATP-dependent endocytic vesiculation); EC₅₀ (flux)</th>
<th>Typical concentrations used in other systems or IC₅₀ or inhibition constant (Ki)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digiton</td>
<td>0-80μM incubated directly with ghosts on ice for 30 min.</td>
<td>5.4μM</td>
<td>Incubation of human epithelial A-498 cell membranes for 20 minutes with 40.5μM digiton prior to Triton extraction greatly perturbed the MAL/myc microenvironment suggesting disruption of detergent-insoluble microdomains (Millan et al., 1997).</td>
</tr>
<tr>
<td>Saponin</td>
<td>0-625μg/ml incubated directly with ghosts on ice for 30 min.</td>
<td>4.73μg/ml</td>
<td>1% (10mg/ml) saponin disrupted prion protein-containing rafts (Naslavsky et al., 1997).</td>
</tr>
<tr>
<td>MBCD</td>
<td>0-12.5mM incubated directly with ghosts on ice for 30 min; 10mM incubated with intact erythrocytes (30 min, 37°C at 2% haematocrit) followed by 3 washes.</td>
<td>2mM when incubated directly with ghosts. Complete inhibition when incubated with intact erythrocytes at all ATP concentrations.</td>
<td>10mM MBCD for 30 min at 30°C disrupted rafts (Yashiro-Ohtani et al., 2000); 10mM MBCD for 15 min 30°C strongly inhibited phagocytosis (Peyron et al., 2000).</td>
</tr>
<tr>
<td>Reagent</td>
<td>Conditions used</td>
<td>IC$<em>{50}$ (ATP-dependent endocytic vesiculation); EC$</em>{50}$ (flux)</td>
<td>Typical concentrations used in other systems or IC$_{50}$ or inhibition constant (k$_i$)</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Filipin</td>
<td>0-250µg/ml (382.5µM) incubated with ghosts on ice for 30 min; 7.5µg/ml (endocytic vesiculation assay) or 0-100µg/ml (flux assay) incubated with intact erythrocytes (30 min, 37°C at 2% haematocrit) followed by 3 washes.</td>
<td>5µg/ml when incubated with ghosts. No effect when incubated with intact erythrocytes on endocytic vesiculation or flux.</td>
<td>Incubation of MA104 cells with 10µg/ml filipin for 15 min shifted the folate receptor from the caveolae membrane fraction to the non caveolae membrane fraction (Smart et al., 1996); Inhibition of cholera toxin internalisation (IC$_{50}$=0.5nM=0.327ng/ml) (Orlandi and Fishman, 1998); 2µg/ml filipin for 15 min 30°C strongly inhibited phagocytosis (Peyron et al., 2000)</td>
</tr>
<tr>
<td>Nystatin</td>
<td>0-5µg/ml (for endocytic vesiculation assay) or 0-500ng/ml (flux assay) incubated with intact erythrocytes (30 min, room temperature, in dark at 2% haematocrit) followed by 3 washes.</td>
<td>Inhibited endocytic vesiculation with IC$_{50}$ 15ng/ml ; Increased the flux, half maximal effect at 0.035ng/ml</td>
<td>25µg/ml nystatin impaired VacA -induced cell vacuolation in HEp-2 cells (Ricci et al., 2000); 25µg/ml nystatin for 15 min 30°C strongly inhibited phagocytosis (Peyron et al., 2000)</td>
</tr>
</tbody>
</table>
Many of these raft disrupters have a detergent action. A potential artefact could have arisen in the AChE assay technique, since a detergent could have caused lysis of the ghosts, masking any reduction of AChE activity by the ATP-dependent process. However, the concentrations used in whole cell experiments had no visible lytic effect.

To further eliminate the possibility that the results with the raft disrupters might be artefactual and more a detergent action hence exposing all AChE, erythrocytes were incubated with 7.5µg/ml filipin and ghosts were made, fixed and transmission electron microscopy performed (Figure 9.9). The data show that the ghosts are intact. However, ATP-dependent endocytic vesiculation occurred in the presence of filipin unless it was directly incubated with the ghosts. It is a very weak control, but microscopy was limited.
Figure 9.9 Electron micrographs showing the ghosts made from erythrocytes incubated with 7.5μg/ml filipin for 30 mins at 37°C. (Pictures by GV Landon and PS Rowley [Dept of Histopathology, UCL])

Ghosts were prepared from red blood cells pretreated with 7.5μg/ml filipin at 37°C for 30 minutes. To confirm that the ghosts were intact, they were fixed and transmission electron microscopy was performed. The electron micrographs illustrate the ghost morphology after pretreatment of erythrocytes with the filipin. This confirms that the ghosts are intact and not lysed.
9.4.4. Experiments with other compounds

A variety of pharmaceutical compounds were tested for their effects on ATP-dependent endocytic vesiculation in normal ghosts. Compounds were mixed with the ghosts immediately before addition of ATP or ITP. Table 9.2 and figure 9.10 summarises the compounds tested and their effect on endocytic vesiculation.

These experiments were repeated up to 3 times at concentrations where they ought to have completely inhibited endocytic vesiculation if they were affecting the systems under study (see table 9.3). After this, more detailed experiments were performed using the compounds which exhibited a greater than 80% inhibitory effect in order to determine what concentrations were effective.

Table 9.2 Table showing the effect of various compounds on ATP dependent endocytic vesiculation in normal red cell ghosts.

<table>
<thead>
<tr>
<th>Compound and concentration</th>
<th>No of experiments (n)</th>
<th>Endocytosis % with compound +/- SD (n=3) or spread (n=2)</th>
<th>Endocytosis % without compound +/- SD (n=3) or spread (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanadate* 1mM</td>
<td>3</td>
<td>0 +/- 1</td>
<td>24 +/- 1.7</td>
</tr>
<tr>
<td>Staurosporine* 1.07mM</td>
<td>2</td>
<td>4 +/- 1</td>
<td>21 +/- 6.7</td>
</tr>
<tr>
<td>A3 hydrochloride 10μM</td>
<td>3</td>
<td>15.3 +/- 4.2</td>
<td>21 +/- 6.7</td>
</tr>
<tr>
<td>H9 10μM</td>
<td>3</td>
<td>21.7 +/- 5.9</td>
<td>21 +/- 6.7</td>
</tr>
<tr>
<td>Genestein 10μM</td>
<td>2</td>
<td>13.5 +/- 0.5</td>
<td>21 +/- 6.7</td>
</tr>
<tr>
<td>Wortmannin 10μM</td>
<td>3</td>
<td>21 +/- 4.5</td>
<td>21 +/- 6.7</td>
</tr>
<tr>
<td>NEM* 1mM</td>
<td>3</td>
<td>-0.3 +/- 1.41</td>
<td>22.5 +/- 1.2</td>
</tr>
</tbody>
</table>

* = Compounds exhibiting an inhibitory effect > 80%
Ghosts were prepared from normal erythrocytes and were either untreated (and exposed to vehicle only (methanol, ethanol, DMSO, water)) or treated and exposed to 1mM staurosporine, 10μM A3 hydrochloride, 10μM H9, 10μM genestein, 10μM wortmannin, 1mM N-ethyl-maleimide (NEM) or 1mM vanadate. ATP-dependent endocytic vesiculation was measured by reduction in AChE. The chart shows the mean and standard deviation (where n=3) or spread (where n=2). The data suggest that NEM, vanadate and staurosporine are inhibitors of ATP-dependent endocytic vesiculation at the concentrations tested.
Experiments to determine whether an ATPase is involved in ATP-dependent endocytic vesiculation

To investigate whether ATP-dependent endocytic vesiculation in ghosts involved an ATPase in the process, the effect of incubating ghosts with the ATPase inhibitor, vanadate, was explored. Figure 9.11 shows that vanadate inhibited ATP-dependent endocytic vesiculation (IC$_{50}$=1.3μM), as previously reported by Schrier et al. (1986b), who found that 30μM vanadate resulted in 97% inhibition of ATP-dependent endocytic vesiculation in erythrocyte ghosts. Primaquine-induced endocytosis was also blocked with an IC$_{50}$ of 7.5μM vanadate.

To test whether ATP hydrolysis is necessary for ATP-dependent endocytic vesiculation to occur, ATP$_\gamma$S (similar to ATP, but containing an uncleavable thio-ester bond between the β and γ phosphate groups) was used to substitute for ATP. ATP caused 33% disappearance of AChE when compared with ITP, as expected. ATP$_\gamma$S caused no disappearance of AChE activity on comparison with ITP. This shows that it is not just ATP as a molecule that is taking part: it really is getting cleaved.
Ghosts were prepared from normal erythrocytes and were exposed to 0-1 mM vanadate at the same time as addition of ATP/ITP. ATP-dependent endocytic vesiculation was measured at 5 mM ATP. The data show that vanadate inhibited ATP-dependent endocytic vesiculation (IC50=1.3 µM).
Protein kinase inhibitors

Protein kinase inhibitors such as staurosporine (Tamaoki et al., 1986; Takahashi et al., 1990; Yanagihara et al., 1991), wortmannin (Yano et al., 1995), A3 hydrochloride (Inagaki et al., 1986), H9 (Inagaki et al., 1985; Hirayama et al., 1990) and genestein (Migita et al., 1994) were tested for their effect on ATP-dependent endocytic vesiculation (Table 9.2, Figure 9.10 and Figure 9.12) at concentrations higher or equivalent to those typically used (Table 9.3). Apart from staurosporine, none had a significant inhibitory effect. Staurosporine had an inhibitory effect as shown in Figure 9.12 though at high concentrations (IC$_{50}$=1μM) which is much higher than typical IC$_{50}$s which tend to be in the low nanomolar concentrations (Schachttele et al., 1988; Tischler et al., 1990; Yanagihara et al., 1991).

N-ethyl maleimide (NEM)

The alkylating agent NEM (Roed, 1989; Nakajima et al., 1990; Luo et al., 1999) was found to be inhibitory as shown in Figure 9.13 which shows the average of 4 separate experiments (IC$_{50}$=0.28mM).
Figure 9.12 (Expt JE2) Effect of staurosporine on ATP-dependent endocytic vesiculation in normal ghosts

Ghosts were prepared from normal erythrocytes and were exposed to 0-10μM staurosporine at the same time as addition of ATP/ITP. ATP-dependent endocytic vesiculation was measured at 5mM ATP. The data show that staurosporine inhibited ATP-dependent endocytic vesiculation, but at high concentrations (IC50=1μM).
Figure 9.13 (ExptsJG10-13). Effect of NEM on ATP-dependent endocytic vesiculation in normal ghosts.

Ghosts were prepared from normal erythrocytes and were exposed to 0-1 mM NEM at the same time as addition of ATP/ITP. ATP-dependent endocytic vesiculation was measured at 5 mM ATP. The graph shows the mean of 4 experiments performed on different samples on different days and shows that NEM inhibited ATP-dependent endocytic vesiculation (IC50=0.3 mM).
Table. 9.3. IC$_{50}$ measurements for effect of chemical modifiers on ATP-dependent endocytic vesiculation in normal human red cell membranes. Comparison with published data on effect of same substances on other biological systems.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Highest concentration used</th>
<th>Inhibition of ATP-dependent endocytic vesiculation (IC$_{50}$)</th>
<th>Typical concentrations used in other systems or IC$_{50}$ (or inhibition constant (k$_i$) where stated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein kinase inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staurosporine</td>
<td>1.07mM</td>
<td>1µM</td>
<td>Protein kinase C with an IC$<em>{50}$ of 2.7nM (Tamaoki et al., 1986), myosin light chain kinase with an IC$</em>{50}$ of 1.3nM, protein kinase A with an IC$<em>{50}$ of 2.7nM , protein kinase G with an IC$</em>{50}$ of 8.5nM (Schachttele et al., 1988; Tischler et al., 1990; Yanagihara et al., 1991)</td>
</tr>
<tr>
<td>A3 hydrochloride</td>
<td>10µM</td>
<td>no inhibition at 10µM</td>
<td>Casein kinase I k$_i$=80µM, casein kinase II k$_i$= 5.1µM, myosin light chain kinase k$_i$=7.4µM, protein kinase A k$_i$=4.3µM, protein kinase C k$_i$=47µM, protein kinase G k$_i$= 3.8µM (Inagaki et al., 1986)</td>
</tr>
<tr>
<td>H9</td>
<td>10µM</td>
<td>no inhibition at 10µM</td>
<td>Protein kinase C k$_i$=18µM; cGMP k$_i$=0.87µM; cAMP-dependent protein kinase k$_i$=1.9µM (Inagaki et al., 1985)</td>
</tr>
<tr>
<td>Genestein</td>
<td>10µM</td>
<td>no inhibition at 10µM</td>
<td>5-10µg/ml cell cycle arrest (Volonte et al., 1999); 1-5µM increased tumour latency and decreased tumour multiplicity in skin cancer cells (Wei et al., 1995); inhibited dopamine uptake with a k$<em>i$ of 68nM (Doolen and Zahniser, 2001); inhibited epidermal growth factor receptor kinase (IC$</em>{50}$=2.6µM)(Akiyama et al., 1987),</td>
</tr>
<tr>
<td>Reagent</td>
<td>Highest concentration used</td>
<td>Inhibition of ATP-dependent endocytic vesiculation (IC$_{50}$)</td>
<td>Typical concentrations used in other systems or IC50 (or inhibition constant (k_i) where stated)</td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------</td>
<td>---------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>10µM</td>
<td>no inhibition at 10µM</td>
<td>Phosphatidylinositol-3 kinase (IC$_{50}$= 3nM)(Yano et al., 1995)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>1mM</td>
<td>0.3mM</td>
<td>0.5mM NEM blocked acetylcholine-activated K^+ current in bullfrog atrium (Nakajima et al., 1990)</td>
</tr>
<tr>
<td>Vanadate</td>
<td>1mM</td>
<td>1.3µM</td>
<td>30µM vanadate resulted in 97% inhibition of ATP-dependent endocytic vesiculation in erythrocyte ghosts and primaquine endocytosis is also blocked with an IC$_{50}$ of 7.5µM vanadate (Schrier et al., 1986b);</td>
</tr>
</tbody>
</table>
9.5 SUMMARY AND DISCUSSION

The anti-stomatin antibody and Fab fragments were found to inhibit ATP-dependent endocytic vesiculation. It was found that the stomatin fusion protein, D13-7, competed out this effect. Antibodies to GST, spectrin α, actin, annexin V, flotillin and aquaporin-1 had no effect. These results suggest that stomatin is involved in the vesiculation process. The finding that the anti-stomatin antibody was the only antibody to have an effect makes this experiment even stronger.

Although stomatin is involved in the endocytic vesiculation process, experiments in chapter 4 showed that it was not strictly necessary for the process to occur: first, it was shown that in the Harrow case a deficiency in stomatin had no defect on endocytic vesiculation. Second, in the Manchester and Brighton pedigrees that were stomatin-deficient but did have a defect in endocytic vesiculation, DMA corrected the endocytic vesiculation and leak. Therefore, it appears that when stomatin is present, the endocytic vesiculation process does involve stomatin, but can occur without it, in the presence of DMA. It is therefore suggested that stomatin may act as some kind of bridging structure and that its action can be mimicked by that of DMA.

The anti-spectrin α data was contradictory to previous work in which incorporation of anti-spectrin antibodies (but not Fab fragments of this) into
phosphate-free ghosts along with the MgATP in amounts less than equivalent to the spectrin significantly increased the shape changes caused by MgATP alone (Sheetz and Singer, 1977). The concentration of anti-spectrin used in my experiment was unknown, but the highest concentration used was 3.75 fold higher than that used for Western blotting.

The inhibition of ATP-dependent endocytic vesiculation by inhibitors which are known to disrupt rafts, suggest that a key role for cholesterol, which as it is a component of rafts implicates the involvement of a raft function in this process. These results are also consistent with the idea that stomatin, which was found to be a raft protein (see chapter 6), is involved in the endocytic vesiculation process.

Although it could be argued that these data are somewhat weakened by the finding that the anti-flotillin antibody had no effect on the endocytic vesiculation process, it is possible that it is in different rafts to stomatin. Alternatively, the concentration of antibody may have been insufficient (though the highest concentration used was 2.5-fold higher than that used for Western blotting), or the antibody may not have reacted with the 'native' protein.

Recent work by another group in which MBCD was used to disrupt raft-cholesterol in erythrocytes (Samuel et al., 2001) has added validity to my
experiments by providing an important control: MBCD was shown to deplete raft-cholesterol and disrupt association of all raft proteins (Samuel et al., 2001).

Although in theory MBCD ought to disrupt all raft proteins in erythrocytes as it has in other cells (Klein et al., 1995; Ilangumaran and Hoessli, 1998), this had not previously been demonstrated in my experiments or those of others. Interestingly, MBCD was found to block erythrocytic infection by the malaria parasite which occurs by an endocytic process (though very different to the in vitro methods used on a broken cell population here) suggesting that this type of vesiculation in erythrocytes is raft-associated (Samuel et al., 2001).

It could be argued that a potential problem with the work on the raft disrupters here, is that some of them could have worked by a detergent action, hence releasing AChE and giving an artefactual inhibition. This was addressed by performing experiments with a variety of raft disrupters. The concentrations used in whole cell experiments were low enough not to cause any lysis when added to whole erythrocytes before making ghosts. Therefore, it is probably safe to conclude that disrupting the rafts causes an inhibition of ATP-dependent endocytic vesiculation and that this process does indeed operate via lipid rafts.

The only EM data here is limited to a single experiment in which filipin was incubated with erythrocytes and ghosts were made and fixed for electron
microscopy. Although discussions with the electron microscopists established that the ghosts were intact, this is only with one compound and one not found to inhibit endocytic vesiculation unless incubated with the ghosts themselves. In hindsight, another method of measuring endocytic vesiculation would have been preferable to only using the AChE assay for these experiments, but unfortunately, this was the technique most readily available.

The finding that nystatin increased the flux as well as inhibited ATP-dependent endocytic vesiculation, suggests a relationship between endocytic vesiculation and an ion channel as already suggested by the DMA data (Chapter 4). Nystatin has been used experimentally to alter ion concentrations of erythrocytes by increasing the ion permeability of the membrane (Finklestein and Cass, 1968; Cass and Dalmark, 1973; Dalmark, 1975; Joiner and Lauf, 1978; Freedman and Hoffman, 1979). Cells are treated with 20-30μg/ml nystatin and the ion content of the suspending medium is progressively changed to alter the ion content of the cells, but its permeability effects are reversible and on returning cells to nystatin-free media the membrane becomes impermeable to ions (Cass and Dalmark, 1973). Here, the cells were thoroughly washed after treatment and produced an increase in the flux at incredibly low concentrations (EC50=0.035ng/ml). However, filipin, which does not have this effect (Finklestein
and Cass, 1968; Gent and Prestegard, 1976) had no effect on intact erythrocytes which were subsequently washed on either the ouabain+bumetanide-resistant K⁺ influx or on ATP-dependent endocytic vesiculation.

The action of nystatin is interesting. It is clear that nystatin is an effective raft disrupter in other systems (Rothberg et al., 1990). It has been used as a tool to increase the monovalent cation permeability of red cells and of sterol containing membranes whether artificial or natural (Finklestein and Cass, 1968; Cass and Dalmark, 1973).

It might be tempting to suggest that it increases monovalent permeability of red cells by inhibition of the vesiculation/channel silencing mechanism suggested here: if in normal cells, vesiculation silences an ion channel, then inhibition of vesiculation by nystatin could leave an ion channel in the membrane exposed to leak, resulting in the increase in the flux seen here. However, it must be pointed out that the action of nystatin is complex. It is capable of inducing high cation permeability in artificial membranes (which will not contain any protein) and therefore may be acting on red cells via other mechanisms.

There are two interesting parallels between the action of nystatin on artificial membranes and the stomatocytoses. First, its action requires the presence in the membrane of a beta-hydroxy sterol (Cass and Dalmark, 1973), an
essential biochemical element of a raft (Rothberg et al., 1990). Second, it has a paradoxical temperature dependence such that the leak induced by nystatin increases with decreasing temperature (Finklestein and Cass, 1968). This happens in normal, untreated, red cells below 8°C (Stewart et al., 1980) and in some (but by no means all) stomatocytosis cells below 23°C (Stewart and Ellory, 1985; Coles and Stewart, 1999; Coles et al., 1999a). So it might be suggested that the leak lesion in stomatocytosis could be due to a 'nystatin-like' abnormality, due perhaps to some abnormal membrane constituent that modifies cholesterol-containing structures, possibly rafts, and increases membrane permeability.

The non-hydrolyzable nucleotide analogue ATPγS did not support endocytic vesiculation when substituted for ATP, showing that cleavage of the phosphate bond is necessary for endocytic vesiculation to occur. This suggests that an ATPase might be implicated in the process, although it could also be a phosphorylation reaction in which the $P_i$ is transferred to a protein as a kind of controlling process. Vanadate may work by acting on an ATPase though vanadate does affect many biological reactions involving phosphate interchanges (Stankiewicz et al., 1995).

One possible target for the actions of vanadate include an ATP-dependent aminophospholipid flipase which has been suggested to be a 'prime mover' for
endocytic events (Devaux, 2000). The finding here, that NEM inhibited ATP-dependent endocytic vesiculation provides more evidence for this, since this ATP-dependent aminophospholipid flipase is known to be inhibited by NEM (Martin and Pagano, 1987). Although the results here are consistent with the suggestion that the ATP is being cleaved by the aminophospholipid flipase, there is no direct proof that it is this molecule that is crucial: to our knowledge, no specific inhibitor of this system exists. Another possible target for the action of vanadate, is myosin, another ATPase (Fowler et al., 1985), whose true function in the red cell remains obscure. Other ATPases exist, for example, the NaK ATPase and Ca ATPase.

Although, an inhibitory effect was observed with the protein kinase inhibitor staurosporine, this effect was only seen at high concentrations, \( IC_50 = 1 \mu M \) and is unlikely to have been due to inhibition of protein kinases. The absence of any inhibitory effect by the inhibitors of phosphorylation, staurosporine, wortmannin, A3 hydrochloride, genestein and H9, suggest that this mechanism is probably not involved or at least not essential for the endocytic vesiculation process to occur. However, other kinase inhibitors could have been tried. Since protein kinases phosphorylate almost all the membrane cytoskeletal proteins (Lux and Palek, 1995), perhaps controlling the rigidity of the
cytoskeleton, these results are perhaps surprising. They suggest that ATP-dependent endocytic vesiculation is not controlled by this mechanism.
CHAPTER 10:

GENERAL DISCUSSION

The work in this thesis has sought to shed light on the pathophysiological defect underlying the 'hereditary stomatocytosis' class of haemolytic anaemias. The work has focused on the most severe form of these conditions, overhydrated HSt, and has concentrated on, first, a defective process of vesiculation; and second, an abnormality in lipid rafts. As will be discussed, it seems possible that these findings are related, although there is as yet no real proof that these findings are first, relevant, and second, causative.

10.1 ATP-DEPENDENT ENDOCYTIC VESICULATION

As previously shown by Mentzer et al. (1978), ATP-dependent endocytic vesiculation of the erythrocyte membrane was absent in two stomatin-deficient OHSt patients and this defect in endocytic vesiculation and the cation leak were both corrected by pretreatment of erythrocytes with DMA. Endocytic vesiculation was present, though perhaps reduced, in erythrocyte membranes from all the other HSt pedigrees available to us in the UK (n=11) and in HbS erythrocyte membranes (n=5) and in all cases where it was present DMA had no effect on the endocytic vesiculation or the cation leak.
These data add another dimension to the marked heterogeneity of these conditions. Previous work in the Stewart laboratory focused on variations in temperature dependence of the 'leak' K⁺ influxes (Coles and Stewart, 1999; Coles et al., 1999a; Coles et al., 1999b) (Figure 2.2 Chapter 2) and the variants also display locus heterogeneity (Carella et al., 1999). The work here illustrates a cell biological dimension: it allows stomatin-deficient variants to be distinguished on the presence or absence of ATP-dependent endocytic vesiculation.

The relevance of the process of ATP-dependent endocytic vesiculation to the pathophysiology of the whole red cell can be questioned. Nevertheless, normal red cells do contain intracellular vesicles derived from the plasma membrane and although it has never been visualised (Lew et al., 1985), these must presumably be formed from the plasma membrane by an inwardly directed budding process. Perhaps the most telling point is that, on examination of different pedigrees, it was very clear that there was a very tight correlation between the defect in endocytic vesiculation and the effects of the crosslinker DMA. If endocytic vesiculation was absent, it could be corrected by DMA and DMA also corrected the cation leak. If endocytic vesiculation was present, the leak could not be corrected by DMA and DMA had no effect on endocytic vesiculation. This correspondence was striking and suggested an important
relationship between the leak and the defect in endocytic vesiculation, and the possible significance of this will be discussed below.

The inhibition of ATP-dependent endocytic vesiculation by the anti-stomatin antibody suggested that stomatin may be necessary for endocytic vesiculation to occur, but this cannot be the case. First, DMA can restore the process in a stomatin-deficient cell that lacks protein synthetic capacity; and second, the process was present even without DMA in the stomatin-deficient Harrow case. Thus we may say that the antibody result implies that stomatin is likely to be involved in the process in normal cells but is not strictly necessary for it. The Harrow case also indicates that the defect in endocytic vesiculation is not a result of the leak, since it was present in these equally leaky stomatin-deficient cells.

The studies using inhibitors of the ATP-dependent process suggest that it is dependent on an ATPase (inhibitory effect of vanadate, NEM) and on rafts (inhibitory effect of MBCD, nystatin, filipin, digitonin and saponin), but does not involve phosphorylation (no effect of protein kinase inhibitors). ATPγS could not substitute for ATP, implying that ATP hydrolysis is required. The identity of the presumed 'target' ATPase is not known, but it is known that the red cell membrane contains the aminophospholipid flipase and myosin, both of which are
ATPases and which could, in different ways, conceivably supply the mechanical forces for inward membrane bending. It was of interest that myosin was identified in the raft proteins (see below). The raft theme will be explored below.

Since DMA has a beneficial effect on sickle cells (Lubin et al., 1975; Waterman et al., 1975; Guis et al., 1984; Pennathur-Das et al., 1984; Gibson et al., 2000) the idea that sickle cells might share pathophysiology with OHSt was explored. However, although endocytic vesiculation was perhaps reduced in oxygenated sickle cells when compared with the control in all cases studied, it was present and DMA had no effect on this process. Although it now seems unlikely that the pathophysiology of these diseases are related, in hindsight, examination of the cells in a deoxygenated state (in which the cation leak is more exaggerated (Gibson et al., 2000)) may have been more informative.

10.2 CHOLESTEROL+SPHINGOMYELIN-RICH RAFTS

As described in the Introduction, investigation of the idea of laterally defined lipid rafts in red cells was prompted by an observation stemming from the cloning of the C. elegans gene, unc-24, which has a chimaeric structure, in which a membrane-bound stomatin-like N-terminus is associated with a C-terminus with predicted lipid transfer activity (Barnes et al., 1996).
Some preliminary work suggested that some kind of laterally defined domains might exist in red cell membranes. After preliminary experiments aimed at optimising methods, the following main points were established:

i. Rafts exist in normal human red cells;

ii. Stomatin is partly present in rafts in normal human red cells;

iii. GPI-anchored proteins such as acetylcholinesterase (AChE) are present in these rafts;

iv. Flotillin is present in both normal and OHSt human red cells and is almost entirely present in the rafts;

v. Caveolin is absent from the normal (and OHSt) red cell;

vi. Rafts exist in stomatin-deficient OHSt red cells;

vii. Rafts in OHSt red cells show a relative deficiency in actin;

viii. There are differences in Coomassie blue staining of raft proteins between OHSt and normal cells;

ix. Many other proteins are present in the raft fraction (to a variable degree) including spectrin α, band 2.1 (ankyrin), band 3, band 4.1, actin and aquaporin-1 and probably titin, myosin, spectrin β, and many others;

x. Glycophorin A, clathrin, annexin V and annexin VI are completely absent from rafts.
The fact that rafts can be found in stomatin-deficient red cells demonstrates that, although stomatin is found in rafts, it is not necessary for raft existence in red cells. Nevertheless a difference in the quantity of actin associated with the rafts emerged in these OHSt cells, possibly suggesting that stomatin may be a direct or indirect binding partner for actin in the red cell.

Although this difference in the proportion of actin in the rafts and deficiency in stomatin in patient cells were the only striking differences found between patient and control rafts, it can already be concluded that although the patients do have rafts they are defective in at least two ways. Furthermore, certain other proteins (spectrin α, band 2.1 and aquaporin-1) appeared to follow a similar pattern to actin, though less convincingly. It is conceivable that more differences will emerge with future work comparing patient and control rafts in red cell membranes.

It is worth noting that in many systems the actin-based cytoskeleton regulates the activity of a variety of ion channels, transport proteins and water channels and that interactions between the actin cytoskeleton play a key role in a multitude of cellular activities including maintenance of cell polarity (Mills et al., 1994). It is possible that it is the deficiency of actin in the rafts in these cells which results in the cation leak.
Recent work on erythrocyte rafts (Lauer et al., 2000; Salzer and Prohaska, 2001; Samuel et al., 2001) has also resulted in findings similar to those here though there certainly are discrepancies which may well be due to the different techniques used in isolating rafts.

10.3 A POSSIBLE PATHOLOGICAL MECHANISM FOR THE STOMATOCYTOSES.

These findings may be taken, along with evidence from other biological systems, to suggest a possible pathogenic scheme for OHSt, which can tie together these findings. There is as yet no positive proof that this mechanism is in fact the truth, but it represents the best mechanism yet for these conditions.

One function of lipid rafts is thought to lie in endocytic vésiculation (Simons and Toomre, 2000) and the findings here suggest that a raft-based endocytic vésiculation process exists in normal erythrocytes: the raft associated protein stomatin is involved in an in vitro process of ATP-dependent endocytic vésiculation and raft disrupters inhibit this process. The work by Snyers et al. (1999) implicating stomatins’ involvement in endocytic vésiculation and association with rafts in cultured amniotic cells is supportive of the idea of a raft-based vésiculation process in erythrocytes. This hypothesis demands that a raft-based vésiculation process occurs in normal unbroken red cell membranes.
The work here suggests that this raft-based endocytic vesiculation process is defective in OHSt erythrocytes: ATP-dependent endocytic vesiculation is defective in OHSt erythrocytes; erythrocytes from these patients have a defect in the composition of their rafts; the deficiency of stomatin from their erythrocytes which is involved in endocytic vesiculation and rafts both here and in other cells (Snyers et al., 1999) is supportive of this.

Further findings from the literature can be mentioned. Sequence homology comparisons suggest that stomatin is part of a group of proteins concerned with regulation of expression of membrane proteins (Tavernarakis et al., 1999). A stomatin homologue (MEC-2) interacts with subunits of an amiloride-sensitive ENaC channel (Huang et al., 1995). In the human hypertensive condition Liddle's syndrome, which has a dominant mutation of the ENaC channel, the channel leaks because it is exposed in the membrane too long, emphasising the importance of this form of channel regulation for this channel protein (Abriel et al., 1999). These three points can be put together to suggest that stomatin may somehow be involved in the regulation of the surface expression (and therefore the activity) of this ion channel. Although many other possibilities exist, a precedent for the OHSt mutation may be that of the action of dominant negative mutants of dynamin, which block clathrin-mediated endocytosis and
have been used to enhance surface exposure, and ion channel transport activity of ENaC channels (Shimkets et al., 1997).

This mechanism could explain the cation leak and the deficiency in stomatin whilst still explaining the small amounts of stomatin seen in red cells.

The normal process of endocytosis may 'snip off' a section of membrane containing an unknown ion channel, which if allowed to remain in the cell membrane mediates the increase in cation permeability by acting as a non-selective 'passive leak' channel i.e. a channel which persists in the membrane. In OHSt cells, a mutant (defective) collar protein could destabilise the stomatin binding site and prevent pinching off of the vesicle so that stomatin is degraded and the channel is left exposed to leak.

This mechanism could also explain the stomatocytic shape. A blockage to the inwardly directed 'pressure' of endocytosis could conceivably result in excessive packing of the inner leaflet of the bilayer, probably by the aminophospholipid flipase resulting in stomatocytosis. Figure 10.1 suggests this hypothetical model.
Figure 10.1 Speculative model of OHSt based on these data

**Figure 10.1.** Cartoon illustrating putative model which can explain main features of stomatocytosis
The DMA could promote clustering of the surface of the membrane, a common preliminary for endocytosis. DMA probably does something to the membrane replacing the action of stomatin in the OHSt (Manchester and Brighton) cells. DMA could aggregate membrane proteins hence causing endocytosis (this role is possibly normally by stomatin) and therefore inactivate the channel by recovering it from the membrane.

Stomatin’s involvement in ATP-dependent endocytic vesiculation (Figure 9.1, Chapter 9) can also be explained by this model: in normal cells it would bind to the protein (which is normal in these cells, but mutant in OHSt) and an antibody to stomatin could make this binding partner defective.

The definite proof of this mechanism requires first the isolation of the mutant gene; and second, direct demonstration of mis-trafficking of a channel protein. The number of channel molecules could be very small, as few as single figures per cell (Lew et al., 1982), so the technical difficulties are considerable.

Full scale endocytosis is not a characteristic feature of red cells and this explanation assumes that the ATP-dependent endocytic vesiculation studied here has some kind of physiological equivalent, perhaps analogous to the caveolus in vascular tissue. No such structure has been identified in red cells, although intracellular vesicles derived from the plasma membrane can be seen in normal
red cells. The endocytic vesiculation could be very small scale in erythrocytes, but still silence an ion channel. Further studies using electron microscopy to visualise the process in the whole erythrocyte including the effect of lipid raft disrupters and the effect of DMA treatment of whole erythrocytes on the membrane are required.

There are many alternatives to dynamin for possible mutant proteins. It could be argued that a mutant form of actin in OHSt could result both in less actin in the rafts and failure to bind stomatin allowing it to be degraded. Alternatively, it could be argued that the stomatin which is raft associated binds actin in normal cells, bringing actin into the rafts. In OHSt cells, deficient in stomatin the association of actin with the rafts could be lost. However, it is not known whether actin and stomatin are bound together and this highlights the importance of finding stomatins' binding partners.

The model is consistent with the fact that the stomatin knock-out mouse does not have stomatocytosis (Zhu et al., 1999). It was argued that the knock out mice had no HSt phenotype due to hereditary stomatocytoses being caused by a mutation in a protein other than stomatin. This protein would have been normal in the knock out mouse and therefore not resulted in the HSt phenotype. The results were interpreted as indicating that stomatin plays no direct role in this disorder.
and it was suggested that stomatin has no role in cation transport in red cells (Zhu et al., 1999).

Zhu et al. (1999) suggest various possible 'partner binding proteins' for stomatin: β-adducin was suggested as a potential candidate since a potential interaction between the proteins has been suggested (Sinard et al., 1994) though the association requires confirmation. Calpromotin was also suggested (Zhu et al., 1999) since stomatin has been found to bind to a calpromotin affinity column and calpromotin is involved in activation of the charybdotoxin-sensitive calcium-dependent potassium channel (Moore and Shriver, 1997).

Identification of recent stomatin homologues are also consistent with the idea of a protein closely associated with stomatin being defective (Gilles et al., 2000; Wang and Morrow, 2000; Sedensky et al., 2001).

Other explanations can be offered. It could be argued that the stomatin is lost as a reaction to the cation leak, either because of the high intracellular [Na], or less directly via an action mediated by the increased glycolytic activity in these cells (Wiley et al., 1979). In this context it is relevant to point out that stomatin co-immunoprecipitates with the glucose transporter (Zhang et al., 1999) and may be a regulator of it (Zhang et al., 2001). It should be pointed out however, that the cation leak cannot explain all the features of the disease, for example it cannot be
the sole cause for the defect in endocytic vesiculation: the Harrow, Woking and Blackburn cases all displayed ATP-dependent endocytic vesiculation and have considerable leaks not corrected by DMA.

Another possibility for the mechanism causing the features of HSt that would have been feasible before this work is now unlikely and can be excluded. It could have been argued that HSt was a haemoglobinopathy similar to sickle cell disease, but the results in this thesis suggest that they do not share a similar pathology as hypothesised at the start of this work.

10.4 PROBLEMS WITH THE WORK AND FUTURE DIRECTIONS

Further work investigating the binding associations of stomatin with its neighbours is clearly required and will be worth the technical difficulties likely to be encountered. There are difficulties in identifying such proteins due to the technical difficulties involved in solubilizing stomatin. It is hypothesised now that stomatin binds to actin, either directly or indirectly.

Stomatin's involvement in the red cell and the consequences of its loss in OHSt is not fully understood. Further, the studies with the knock out mice were interpreted as indicating that stomatin plays no direct role in this disorder and suggested that stomatin has no role in cation transport of red cells (Zhu et al., 1999). A clue to the role of stomatin in the normal red cell could come from
investigation of its role in non-erythroid tissues. There has not to date been a
completely stomatin-deficient case reported (i.e. with stomatin deficient from all
tissues). This suggests that a complete mutation of stomatin in humans would be
fatal unlike the knock out mouse, which lived to adulthood and was normal (Zhu
et al., 1999). Stomatin's wide distribution throughout the human and animal
kingdom suggests an important role in biology.

Other work in progress in the lab (B Fricke, M Chetty, G Stewart,
unpublished) suggests that stomatin is made in the immature developing
erythrocyte but is lost as the cell matures. It would be interesting to
know if the leak changed during this period of development. The simplest
way to separate young and old cells is by density, using centrifugation,
but these overhydrated cells do not separate into 'young' and 'old'
populations by any means of centrifugation and this experiment has not, so
far, been possible.

The Coomassie gels and mass spectrometry suggested an overwhelming
amount of data, impossible to fully analyse here due to time restraints. Numerous
proteins were suggested to be present in the rafts of erythrocyte membranes and
suggested possible differences between patients and controls. Here, MALDI-TOF
analysis was performed on a one-dimensional gel. A two-dimensional gel would separate the proteins further so they are easier to analyse.

One problem with this work is lack of OHSt patients and size of pedigrees. Not only are the pedigrees are too small for mapping studies; it also makes the work here less conclusive than if we had, say, 10 patients with the same condition.

They are also a very heterogeneous group of patients and there is the need to be careful to study them individually rather than as a group. The Harrow case was considered to be identical to the Brighton and Manchester cases, but is clearly different. It will be interesting to look at rafts in other variants.

Although the ATP-dependent endocytic vesiculation was present in most cases studied, the overt abnormality in endocytic vesiculation that occurred in the most severe cases could be a clue to the pathophysiology of all these conditions. The difficulty of whether the ATP-dependent endocytic vesiculation is a physiological process has already been discussed and the importance of visualising the process has been highlighted. More EM studies both of the HSt and control red cells during the various stages in this process and with DMA or raft disrupters could be very helpful.
Further work on the mechanism of the endocytic vesiculation process could identify which ATPase is responsible, its location in the membrane and raft association. Confirmation of various proteins' (myosin, titin, dynamin and many more) existence in erythrocyte membrane rafts could proceed investigation into how this raft-based endocytic machinery works together as a unit and where this fails in HSt.

We have not determined the exact density of the rafts in OHSt red cells. Aspiration by syringe and needle from sucrose gradients remains a crude (but for most purposes adequate) means of gathering fractions. It was planned to attempt to make a precise comparison of raft density in normal and abnormal cells using Pharmacia density marker beads as an internal standard, but these beads are not useful in sucrose. Dr L Forbes is currently attempting to make this analysis using OptiPrep gradients, but work has been delayed by transfusion in the patients.
CHAPTER 11

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