

Vesiculation Events and Bilayer-Cytoskeleton Interactions in the Human Erythrocyte

by

Carla Hagelberg

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University of London

Department of Physiology
University College and Middlesex
School of Medicine
University Street
London WC1E 6 JJ

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ABSTRACT

Changes in human erythrocyte morphology leading to echinocytosis and microvesicle release were studied. The results were generally consistent with the Bilayer Couple Hypothesis and it was calculated that expansion of the outer leaflet of the lipid bilayer by as little as 3% relative to the inner leaflet was sufficient to cause microvesicle release.

The protein and phospholipid composition of microvesicles released as a result of ATP-depletion, during storage, or after treatment with merocyanine 540, dimyristoylphosphatidylcholine or Ca^{2+} /A23187 was compared with the composition of the original cell membrane. These microvesicles were depleted in band 3, glycophorin and phosphatidylinositol 4,5-bisphosphate relative to phospholipid by 40% or more. This data was interpreted to mean that less than half of these membrane components are free to diffuse laterally in the lipid bilayer. Acetylcholinesterase was found to be enriched 2-3 fold in microvesicles, possibly because the removal of non-diffusing proteins from the vesiculating region of the lipid bilayer allows more space for freely-diffusing proteins like acetylcholinesterase to enter the microvesicle membrane.

The phosphorylation of human erythrocyte membrane proteins was investigated in order to determine if phosphorylation affected cytoskeletal interactions which could alter shape or vesicle release. Protein kinase C was activated by phorbol myristate acetate and protein kinase A by the incubation of erythrocytes with cAMP. The extent of the polyphosphoinositide breakdown after ATP-depletion in control and cAMP-treated cells was compared. In the treated cells the phosphatidylinositol 4,5-phosphate breakdown was greatly increased. The phosphorylation of membrane proteins could cause a reduced interaction between this lipid and the cytoskeleton, leading to a greater proportion of the lipid being broken down. The microvesicles produced from cAMP-treated erythrocytes were analysed to ascertain if any phosphoproteins partitioned into the microvesicles. Band 7 was the only phosphoprotein, besides Band 3, found to be present in the microvesicles.

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To John

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1. GENERAL INTRODUCTION

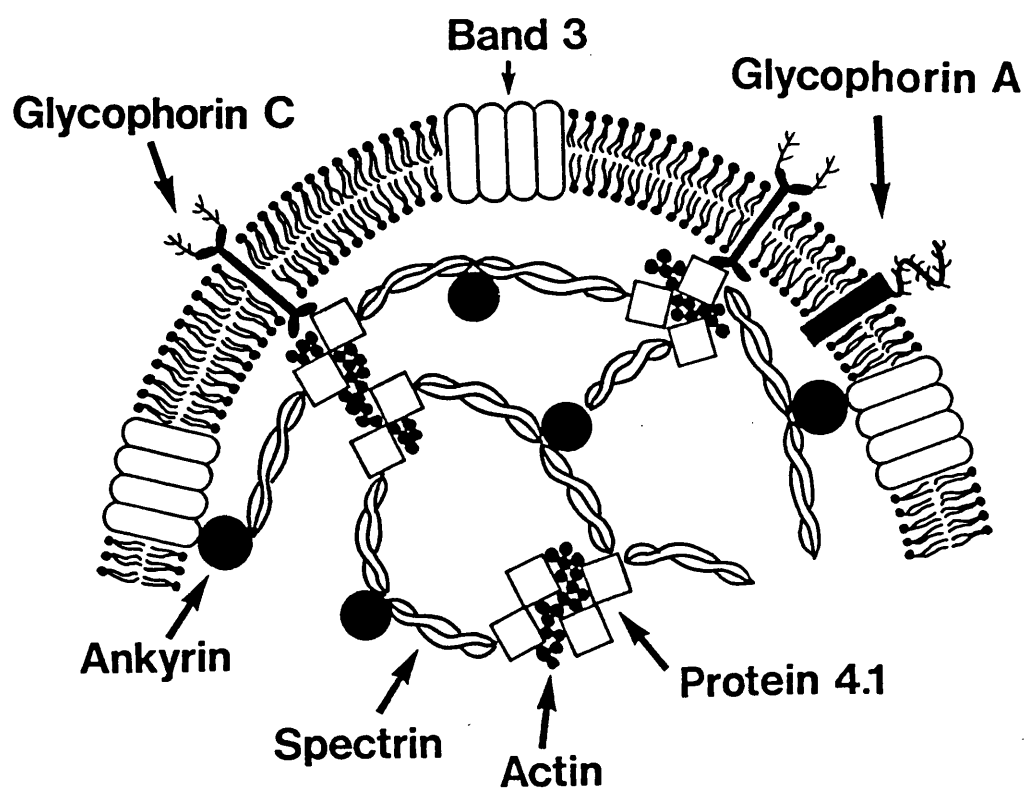
1.1. The Function of Erythrocytes

Red blood cells or erythrocytes are the most abundant of the blood cells, constituting 40-45% of blood volume. Their main function is to transport oxygen from the lungs to the tissues and carbon dioxide back to the lungs. Central to this process is haemoglobin, which binds oxygen and carbon dioxide reversibly. It is the major constituent of the erythrocyte, making up more than 90% of the cell's dry weight.

Mammalian erythrocytes have a relatively simple structure (see Figure 1.1). They have no nucleus and their only membrane is the plasma membrane. The structural simplicity of erythrocytes, their relative abundance (5×10^9 cells per millilitre of blood), and the fact that the plasma membrane is very easily isolated, has made them widely studied. Many of the studies on erythrocytes have therefore provided important information on membrane structure and function. Our understanding of regulatory processes, both within the erythrocyte as well as more complex, nucleated cells, has also increased through the study of this non-nucleated model system.

Figure 1.1 The Human Erythrocyte Membrane

Schematic model of the human erythrocyte membrane, from Bennett 1989.



1.2. The Structure of the Membrane

The erythrocyte membrane is made up of lipids and proteins, with approximately half its mass accounted for by lipids and the other half by proteins. The core of the membrane is a semi-permeable lipid bilayer, with the lipid molecules oriented in such a way that the polar groups are directed towards the aqueous environments and the non-polar groups are directed towards one another forming hydrophobic regions. Within this lipid membrane some proteins, containing non-polar domains, penetrate the bilayer (integral proteins) whilst others are associated with the membrane surface (peripheral proteins). Some of the peripheral proteins are associated with the membrane by non-covalent interactions with lipids or other proteins. Others, such as acetylcholinesterase (AChE), are covalently linked to the membrane. In this case the attachment is via phosphatidylinositol glycan on the outer surface of the plasma membrane. A proportion of the membrane proteins are free to diffuse laterally, whereas others are restricted by their interactions within the cytoskeletal structure.

The main integral proteins are band 3 (the anion channel) and glycophorin.

The peripheral proteins include those which make up the bulk of the membrane skeleton: spectrin, actin, band 4.1 and band 4.9.

Figure 1.2 shows the pattern obtained when human erythrocyte membrane proteins are separated by SDS- polyacrylamide gel electrophoresis (SDS- PAGE) and visualised using Coomassie Blue staining. Table 1.1 gives their molecular weights, assembly states and approximate numbers of copies per cell.

1.2.1. The Integral Membrane Proteins

Band 3, a glycoprotein of molecular weight 88-105 kDa, accounts for 25% of the erythrocyte membrane protein, making it the major protein constituent of the membrane bilayer (see reviews by Jennings 1989 and Reithmeier 1993). It consists of dimers of long folded polypeptide chains extending across the bilayer up to fourteen times. This protein consists of two domains: the amino-terminal 43kD cytosolic segment that provides a major site of association between the membrane and the cytoskeleton (by binding to ankyrin or band 4.1), and a carboxyl-terminal domain embedded in the membrane, which is responsible for ion transport.

Band 3 is asymmetrically situated within the membrane, with covalently attached carbohydrate groups restricted to the extracellular face. The transmembrane disposition of this protein was shown by freeze-fracture techniques, by which the membrane bilayer can be split into the two monolayers before examination by electron microscopy.

Figure 1.2 SDS-PAGE Pattern of Some Polypeptides of the Human Erythrocyte Membrane

Polypeptides were detected using SDS-PAGE and Coomassie staining according to Fairbanks *et al* 1971. Diagrammatic presentation is simplified from Marchesi *et al* 1976.

Molecular weights are shown on the right-hand side.

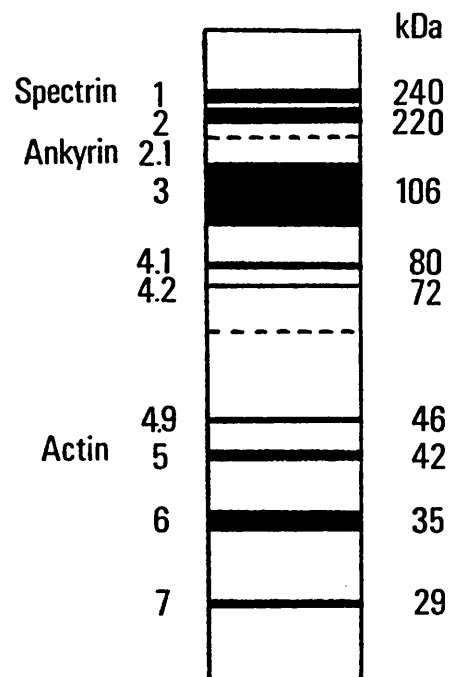


Table 1.1 Major Human Erythrocyte Membrane Proteins

From Bennett (1985)

Protein	Subunit (kDa)	Probable Assembly State	Approximate Copies per Cell
Peripheral proteins:			
Spectrin	260	tetramer	1×10^5
	225		
Ankyrin	215	monomer	1×10^5
Band 4.1	78	--	2×10^5
Band 4.2	72	--	2×10^5
Band 4.9	45	--	5×10^4
Actin	43	--	5×10^5
Glyceraldehyde- 3-phosphate dehydrogenase	35	tetramer	5×10^5
Band 7	29	--	5×10^5
Band 8	23	--	1×10^5
Tropomyosin	29	dimer	7×10^4
Integral Proteins:			
Band 3	89	dimer/tetramer	1×10^5
Glycophorin A	31	dimer	4×10^5
Glycophorin B	23	--	1×10^5
Glycophorin C	29	--	1×10^5

The major role of band 3 in the erythrocyte is as the anion transporter, exchanging HCO_3^- for Cl^- [Steck 1978], a process which is essential for transmembrane equilibration of CO_2 .

The **glycophorins**, a group of glycoproteins containing sialic acid, comprise three polypeptides, the most abundant of which is glycophorin A. This protein is a transmembrane protein with 80% of its total length located at the external surface of the cell. The external portion possesses about 16 oligosaccharide chains containing over 100 sugar residues, and these chains account for most of the erythrocyte's surface carbohydrate and negative charge. Although the function of the glycophorins in the erythrocyte is not wholly understood, it has been suggested that they act as surface receptors. They are also carriers of M and N blood group specificity [Marchesi 1976].

1.2.2. The Cytoskeleton

The normal shape of the erythrocyte is a discocyte, a flexible biconcave disk about $7\mu\text{m}$ in diameter. In this conformation the ratio of surface area to volume is not at a minimum, and the cell is able to go through extreme shape changes without undue stress on the membrane. During most of the erythrocytes' life-span of approximately 120 days in the human circulation, the normal discoid shape is thus able to undergo considerable distortion in response to shear forces and can negotiate small blood vessels that are less

than 3µm in diameter. Aged or pathological cells lose their flexibility and this results in splenic and hepatic trapping and ingestion by macrophages [Grimes 1980].

The bilayer portion of the plasma membrane is underpinned by a network of proteins on the cytosolic face. This network, known as the membrane skeleton, is essential for the maintenance of the cell's stability and shape.

This has been demonstrated by several findings [Cohen C M 1983]:

a) Hereditary abnormalities of the skeletal proteins are linked with membrane instability, leading to shortened erythrocyte survival and possibly anaemia; b) extraction of cytoskeletal proteins from erythrocyte ghosts by low ionic strength solution results in membrane vesiculation; c) when membrane lipids and integral proteins are extracted with the non-ionic detergent Triton-X100, a protein skeleton (Triton shell) remains, retaining the original shape of the erythrocyte [Yu *et al* 1973]. In later studies however, it was shown that Triton shells do not always have the same shape as the parent cells, implying that the bilayer also plays a significant role in the control of erythrocyte shape [Lange *et al* 1982].

1.2.3. Protein Components of the Cytoskeleton

The main component of the cytoskeleton is **spectrin** (review by Goodman *et al* 1988). It makes up 25% of the total membrane protein and 75% of the

cytoskeleton [Steck 1974]. Spectrin consists of two chains, alpha and beta, of approximate molecular weights of 260kDa and 225kDa respectively. The two chains form dimers, associated head to tail forming 200nm long flexible tetramers. These rod-shaped tetramers, helical in structure, can act as springs, extending when the erythrocyte is subject to mechanical forces and recovering when this stress is relaxed [McGough & Josephs 1990]. This characteristic contributes to the cell's elasticity and durability. The tails of these tetramers are associated with actin filaments and this interaction is promoted by protein 4.1.

In other animal cells, molecules with a 240kDa sub-unit and a similar structure to spectrin have been found [Carraway & Carraway 1989]. The role of these analogues in non-erythroid cells is not fully understood, although there is speculation that these spectrin-like molecules may be involved in the regulation of receptor mobility and function, by providing a link between the sub-membrane microfilaments and the plasma membrane.

Actin (or band 5) is believed to form filaments 5nm in diameter containing between 10 and 60 monomers [Brenner & Korn 1980; Atkinson *et al* 1982]. Associated with actin are other minor cytoskeletal proteins such as protein 4.2 and protein 4.9, adducin and tropomyosin and these may contribute to the assembly and stabilisation of the filaments.

Protein 4.1, which is important in providing a key linkage site between the membrane and the cytoskeleton, comprises about 5% of the cytoskeletal weight. It is made up of two similar peptides 4.1a and 4.1b of molecular weight 78 and 82 kDa [Cohen C M 1983].

Several possible binding sites for this protein have been proposed in the last few years, including interaction with band 3 [Pasternack *et al* 1985], a direct interaction with the lipid bilayer via phosphatidylserine [Shiffer *et al* 1988; Rybicki *et al* 1988] or a glycophorin and polyphosphoinositide complex. Both glycophorin A [Anderson and Marchesi 1985] or glycophorin C [Bennett 1989] have been suggested as interaction sites with the polyphosphoinositides, but glycophorin C is now thought more likely especially since glycophorin C-deficient cells exhibit a reduction in stability, whereas glycophorin A-deficient cells are normal [Reid *et al* 1987].

Ankyrin (band 2.1) is a 200kDa globular protein, important in maintaining the main association between the cytoskeleton and the lipid bilayer [Bennett 1982]. It is sensitive to attack by proteases, giving rise to lower molecular products associated with the membrane.

The spectrin-actin network is linked to the plasma membrane by an association of the beta-chain of the spectrin subunit with ankyrin, which is in turn bound to the cytoplasmic domain of the trans-membrane protein band 3. These associations restrict the lateral mobility of the integral proteins. This

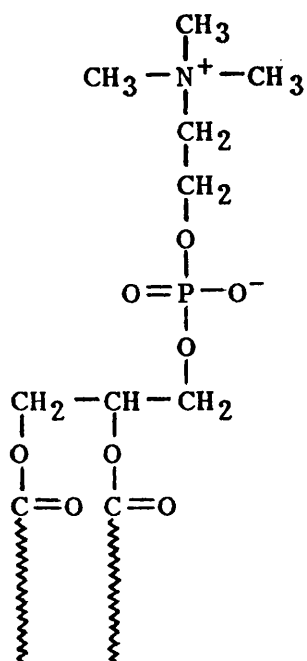
was shown in studies where an increase in the mobility of fluorescence-labelled band 3 and glycophorin was observed after the proteolytic fragmentation of spectrin [Bennett 1978].

1.2.4. The Lipid Bilayer

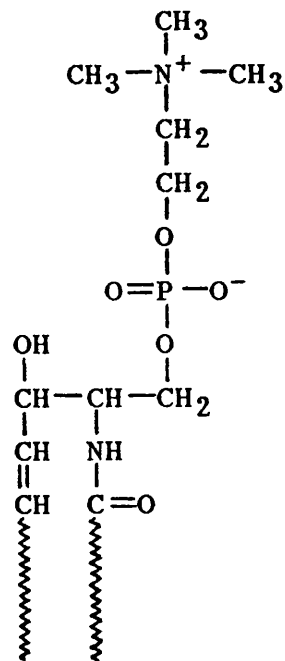
The erythrocyte plasma membrane is based on a lipid bilayer, made up of cholesterol, glycosphingolipids and phospholipids (including the inositol lipids). The major erythrocyte phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin. The first three consist of a glycerol backbone esterified with two hydrophobic fatty acid chains, each containing 14-24 carbon atoms with varying numbers of unsaturated bonds, and a hydrophilic phosphate-containing head group. Sphingomyelin has a sphingosine backbone with a fatty acid residue attached through an amide linkage (Figure 1.3).

The role of cholesterol is to modulate the fluidity of the lipid bilayer by inhibiting possible phospholipid phase transitions [Grimes 1980]. Because it can easily flip-flop between the lipid layers, cholesterol may give the erythrocyte flexibility by allowing the cell to change its membrane contour by moving between the monolayers in response to differential pressures in the two leaflets. A reduction in cholesterol leads to a decrease in the surface area, producing sphered, osmotically fragile cells.

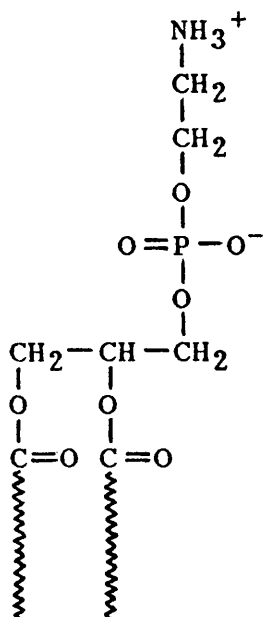
Figure 1.3 The structure of the major erythrocyte lipids



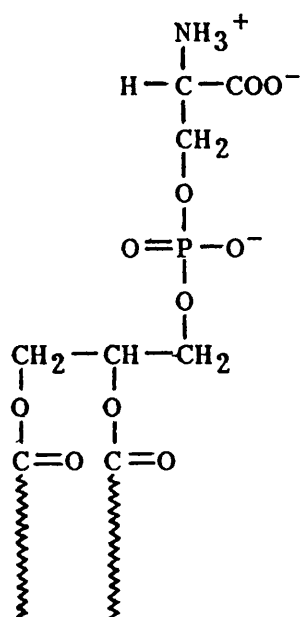
phosphatidylcholine



sphingomyelin



phosphatidylethanolamine



phosphatidylserine

Table 1.2 Lipid composition and asymmetrical distribution of lipids in human erythrocytes

Nelson 1972, Mitchell *et al* 1986; Zwall *et al* 1975;
Op den Kamp 1979.

Lipid	% of total lipid by weight	molar % of total phospholipid	% of lipid in outer leaflet	% of lipid in inner leaflet
Cholesterol	29		50	50
Glycolipid	3		100	0
Phosphatidylethanolamine	18	27.0 ± 2.7	20	80
Phosphatidylserine	8	15.8 ± 1.7	0	100
Phosphatidylcholine	17	25.4 ± 1.1	76	24
Sphingomyelin	18	24.5 ± 2.0	85	15
Phosphatidylinositol 4,5-bisphosphate	6	1.4 ± 0.3		
Phosphatidylinositol 4-phosphate		0.8 ± 0.1		
Phosphatidylinositol		1.2 ± 0.1		
Phosphatidic acid		2.2 ± 0.2		
lyso-Phosphatidylcholine		1.0 ± 0.3		
lyso-Phosphatidyl- ethanolamine		1.3 ± 0.3		

Increased cholesterol above normal levels also has a detrimental effect on membrane fluidity. Patients with liver disease have osmotically fragile erythrocytes and this has been linked to an increase in the cholesterol to phospholipid ratio [Grimes 1980].

Like most biological membranes, erythrocytes have an asymmetric distribution of phospholipids in the bilayer [Bretscher 1972, Zackowski & Devaux 1990]. In human erythrocytes, the outer lipid layer consists mainly of glycolipids and lipids containing a choline headgroup (phosphatidylcholine and sphingomyelin). The inner layer contains mainly lipids that possess a primary amino group (phosphatidylethanolamine and phosphatidylserine), as well as the majority of the inositol lipids [Ling *et al* 1989; Bütikofer *et al* 1990].

A difference also exists between the lipid fatty acid chains in the two leaflets of the bilayer. The fatty acids of the outer leaflet lipids are more saturated than those of the inner lipids. The unsaturation in the inner layer lipids could result in the inner layer having a more fluid structure. A double bond produces a kink in the hydrocarbon chain and this disrupts the ordered packing of the chains, thus increasing fluidity. The negative charge on phosphatidylserine and phosphatidylethanolamine also causes a difference in charge between the inner and outer half of the bilayer.

The distribution of lipids between the two membrane layers has been measured using mainly chemical and enzymatic methods. Incubation of cells with phospholipase A₂ or phospholipase C results in the hydrolysis of the external-facing lipids only. When lysed cells or isolated membranes are treated with a phospholipase, the lipids in both leaflets are accessible, and are therefore hydrolysed. Labelling the amino groups of phosphatidylethanolamine and phosphatidylserine by chemical agents, such as trinitrobenzene sulphonic acid, was another way used to demonstrate lipid asymmetry [Op den Kamp 1979].

1.3. Control of Phospholipid Asymmetry

Interactions between membrane phospholipids and the cytoskeleton may play an important role in the regulation of phospholipid asymmetry [Franck *et al* 1985; Mohandas *et al* 1985; Haest *et al* 1978], and could be significant in the regulation of shape changes.

An association of inner leaflet lipids with cytoskeletal proteins such as spectrin [Haest 1982] or protein 4.1 [Sato & Ohnishi 1983] has been suggested to be responsible for maintaining this asymmetry. Other studies dispute this since they showed that lipid asymmetry also exists in spectrin-poor microvesicles obtained by calcium/ionophore treatment [Raval & Allan 1984], or by shearing ghosts under pressure [Scott *et al* 1984]. It is worth

noting that microvesicles eventually lose their phospholipid asymmetry, although this is a relatively slow process.

These results imply that spectrin/lipid interaction is not vital in maintaining asymmetry and that other factors are important in the maintenance of lipid asymmetry. Further studies also support this suggestion. In heat-treated erythrocytes phospholipid asymmetry is maintained, even though spectrin has undergone structural changes [Gudi *et al* 1990]. Similarly, in diamide-treated cells in which cross-linking of the skeletal proteins was achieved, the association between the cytoskeleton and the bilayer is disrupted, but the phospholipid asymmetry also remains intact [Middlekoop *et al* 1989].

Asymmetry is probably also maintained by ATP-dependent translocation of phosphatidylserine and phosphatidylethanolamine towards the inner monolayer [Seigneuret & Devaux 1984; Zachowski & Devaux 1990], since outside to inside translocation of phosphatidylserine occurs when this lipid is added to normal erythrocytes but not when added to ATP-depleted cells.

The maintenance of phosphatidylserine asymmetry is also believed to be dependent on the oxidative status of the cytoskeleton as well as that of a 32kDa polypeptide [Connor & Schroit 1990]. This polypeptide may be the Rhesus blood group protein [Schroit *et al* 1990]. An argument against this is that cells that are devoid of the Rhesus protein (Rh_{null} cells) translocate lipids normally, so this protein cannot be the translocase [Smith & Daleke

1990]. However, Schroit *et al* (1990) explained this result by suggesting that the Rhesus protein is present in Rh_{null} cells in a new antigenically silent phenotype.

The predominance of phosphatidylcholine and sphingomyelin in the outer lipid layer has only been explained in terms of passive redistribution in response to the active movement of the aminophospholipids into the inner leaflet [Van Meer & Op den Kamp 1982]. It is worth noting however, that sphingomyelin has been found to be originally synthesised on luminal surfaces and so will be exported to the external surface [Allan & Kallen 1993].

1.3.1. The Role of Lipid Asymmetry

Changes in phospholipid asymmetry have been proposed to be related to membrane fusion. In studies where erythrocytes were exposed to Ca²⁺ and ionophore, microvesicles were shed from the cells as a consequence of a membrane fusion event [Allan & Michell 1976]. Allan & Raval (1984) did not detect an immediate exposure of phosphatidylserine to the outside following microvesiculation. However Comfurius *et al* (1990) showed that this microvesiculation did correlate with the exposure of phosphatidylserine to the outside, as demonstrated by an increase in procoagulant activity. In other studies where erythrocytes were induced to swell osmotically, it was also

shown that prior incubation with Ca^{2+} and ionophore leads to increased cell fusion and this is associated with the exposure of phosphatidylserine to the outside together with an increase in procoagulant activity [Baldwin *et al* 1990].

Loss of lipid asymmetry in erythrocytes may also serve as a mechanism for the destruction of pathological or aged cells. The exposure of phosphatidylserine on the outside may be a gradual process resulting from a loss of metabolic competence that signals the recognition and destruction of defective cells by phagocytes [Tanaka & Schroit 1983].

1.4. Shape Changes and Vesicle Formation in Human Erythrocytes

1.4.1. Factors Affecting Morphology

The discoid shape of normal erythrocytes can be altered *in vitro* by a variety of treatments or agents in order to study the relationship between membrane morphology and deformability. Shape transformations can be induced by a decrease in extracellular pH [LaCelle 1969], ATP-depletion [Nakao 1960; Weed *et al* 1969], increased intracellular calcium concentration [Allan *et al* 1976], modifications to the cytoskeletal proteins [Goodman & Shiffer 1983; Wagner 1987], or through unilateral expansion of one of the lipid leaflets by

drugs [Sheetz & Singer 1974]. Under these treatments either echinocytes (sphered cells covered with an irregular arrangement of spicules) or stomatocytes (cup-shaped cells) may be formed.

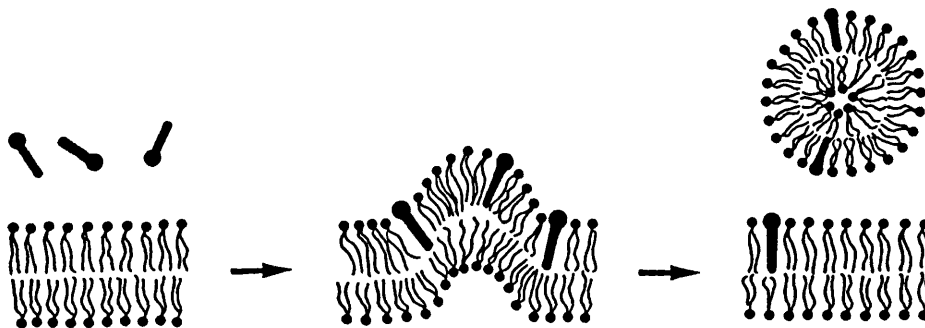
These changes in cell morphology are potentially reversible, i.e. the discocytic shape can be regained if the causative agent is removed or the cell is allowed to resynthesise ATP [Anderson & Lovrien 1981]. However, the changes become irreversible at the point where part of the surface of the cell is lost by budding (Figure 1.4), leading eventually to the formation of spherocytes.

Membrane material is either endocytosed in the form of internal vesicles (from stomatocytes) [Allan & Walklin 1988], or external vesicles are released (from echinocytes) [Rumbsby *et al* 1977; Ott *et al* 1981; Wagner *et al* 1984; Allan *et al* 1989].

The mechanism for the transformation of cells from discocyte to echinocyte or stomatocyte is as yet not fully understood, although several theories have been proposed. Consensus however, has not been reached on whether changes in morphology are wholly dependent on the disruption of the membrane or the cytoskeleton alone, or a combination of the two. The following paragraphs will give details of the different instances of shape change and vesicle release and will describe the possible mechanisms for these events.

Figure 1.4 **Morphological changes in the erythrocyte leading to echinocytosis and microvesiculation**

Picture showing membrane bending and vesiculation in response to insertion of an exogenous molecule.



1.4.2. Intercalation of Amphipathic Molecules into the Bilayer

Changes in morphology from discocyte to echinocyte or discocyte to stomatocyte induced by the intercalation of amphipathic molecules into the lipid bilayer have been widely studied [Deuticke 1968; Murphy 1973; Brecher & Bessis 1972; Evans 1974; Ott *et al* 1981; Allan *et al* 1989]. Anionic amphipaths, which are repelled by the negative charge on the inner leaflet, or cationic amphipaths, which are charged at neutral pH and not able to cross the membrane, expand the outer leaflet to form echinocytes. The phenomenon of echinocyte formation was explained by the bilayer couple hypothesis of Sheetz & Singer (1974) which postulated that perturbations to the membrane caused by various amphipaths results in the two halves of the lipid bilayer undergoing a differential change in area, whilst remaining coupled to one another. In other words, a differential expansion of one leaflet relative to the other is accommodated by membrane bending and a change in shape (Figure 1.4).

Shape changes are also observed when certain cationic amphipaths such as chlorpromazine, which are partly uncharged at neutral pH, diffuse across the bilayer and are retained in the inner leaflet due to the attraction of the negatively charged phospholipids on the inner leaflet of the membrane. This causes an expansion of the inner leaflet relative to the outer leaflet, thus

forcing the membrane to curve inwards, leading to the production of stomatocytes.

Although the Sheetz and Singer bilayer hypothesis was later put into question by Conrad & Singer (1981), their theory is supported by many other studies and still provides the most comprehensive explanation for the shape changes observed, either through the differential expansion or contraction of one of the monolayers.

In the Conrad & Singer study the partitioning of amphipaths into synthetic vesicles and biological membranes was measured. They found that although these molecules bound to the synthetic membranes they did not intercalate into the biological membranes. They concluded that biological membranes must have a high "internal pressure" that prevents the insertion of these exogenous molecules and that the apparent binding of the amphipaths to the biological membranes is due to their tendency to form micelles in aqueous solutions.

It is difficult to accept the conclusions made by Conrad and Singer in light of other numerous studies, particularly those using spin-labelled lipids or fluorescent amphipaths, which demonstrated (by measuring environment-dependent changes in the molecule's attributes), that these compounds are not just adsorbed to the cell surface, but are intercalated into the erythrocyte membrane [Ferrell *et al* 1981; Matayoshi 1980; Allan *et al* 1989]. The

distribution of exogenous phospholipids incorporated into erythrocytes can also be clearly monitored by looking at the changes in morphology. Adding dilauroyl-phosphatidylcholine results in a discocyte to echinocyte shape change whereas addition of dilauroyl-phosphatidylserine result in the production of stomatocytes [Daleke & Huestis 1989]. In all these studies a clear correlation was shown between the amount of amphipath transferred to the cells and the resulting cell shape.

As described in the following sections, many instances of shape change that occur under very different conditions, such as metabolic depletion or increased intracellular calcium concentration, can be explained in terms of the Sheetz and Singer bilayer hypothesis. This theory has also been extended further by others to cover conditions where the area of one of the bilayer leaflets is reduced by the degradation or removal of lipid [Allan *et al* 1975, 1979; Haest *et al* 1981; Daleke & Huestis 1989; Ferrell & Huestis 1984]. It appears that changes in the bilayer are the most significant events involved in the alteration of erythrocyte shape, but the concept that these changes may depend in part on rearrangements of the cytoskeleton cannot be dismissed.

1.4.3. Influence of Metabolic Depletion on Erythrocyte Shape

ATP depletion of normal erythrocytes occurs after the cold storage of blood for several weeks, but also when erythrocytes are incubated under sterile

conditions at 37°C for over 12 hours without added glucose. Parallel with the decline of ATP, the cells lose their flexibility, change from discocytes through echinocytes to spheres [Nakao 1960], and lose membrane lipid and protein in the form of microvesicles [Lutz *et al* 1977]. A decrease in cellular ATP also leads to a decrease in the phosphorylation of the major cytoskeletal protein, spectrin [Birchmeier & Singer 1977; Sheetz & Singer 1977].

It was postulated by Mohandas *et al* (1978) that the phosphorylation of spectrin plays a role in cell deformability and morphology. This was explained in terms of the dephosphorylation of this protein leading to a rearrangement of the cytoskeleton resulting in the contraction of the inner monolayer. Dephosphorylation of spectrin may also lead to decreased protection against oxidative damage that promotes cross-linking, resulting in morphological changes.

However, the correlation between spectrin dephosphorylation and morphology was put into question by later studies which showed that shape changes induced by ATP-depletion preceded the decrease in phosphorylation [Anderson & Tyler 1980]. Since spectrin dephosphorylation is now not considered to be the determining factor, another explanation is that the shape change is promoted by the conversion of PIP₂ to PI and phosphatidic acid to diacylglycerol [Müller *et al* 1981; Ferrell & Huestis 1984]. This breakdown produces lipids with a smaller, less charged headgroup, leading to

a difference in the inner leaflet area relative to the outer leaflet. In addition, the production of diacylglycerol may assist membrane fusion and thus promote vesiculation. This neutral lipid should also equilibrate across the bilayer [Allan *et al* 1979], further enhancing the difference in leaflet areas. This mechanism for the ATP-induced shape changes is thus very much consistent with the bilayer hypothesis, that is, the erythrocyte accommodates the decrease in inner leaflet area by becoming echinocytic.

1.4.4. The Role of Calcium in Erythrocyte Morphology

The intracellular calcium concentration of erythrocytes is less than 10^{-7}M whereas the plasma level is about 10^{-3}M . This gradient across the membrane is maintained by a pumping mechanism dependent on ATP and sensitive to calcium in the micromolar range [Sarkadi 1980]. In erythrocytes, calcium does not appear to be a second messenger as it is in other eukaryotic cells, but may be responsible for cell shape regulation. The intracellular calcium concentration is raised in aged cells or in pathological states such as sickle cell anaemia [Raval & Allan 1986], or artificially, *in vitro*, by the action of extracellular calcium and ionophores [Allan & Michell 1975]. This increase in calcium concentration results in biochemical and morphological alterations in the plasma membrane of the cell. Similar changes are also seen in aged or sickle cells [Raval & Allan 1986].

Calcium-loading leads to the shrinkage of cells due to KCl efflux [Gardos 1958], loss of cytosol-filled microvesicles [Allan *et al* 1976], proteolysis and aggregation of membrane proteins [Lorand *et al* 1976; Allan & Thomas 1981], as well as the breakdown of the polyphosphoinositides by an endogenous phosphodiesterase [Allan & Thomas 1981]. The breakdown of the polyphosphoinositides results in the production of the possibly fusogenic lipid, diacylglycerol, together with a decrease in the area of the inner membrane leaflet. Again, taking the bilayer-couple hypothesis into account, this could explain the morphological changes observed after calcium loading [Ferrell & Huestis 1984].

The influence of calcium on the bilayer structure was also studied by Hope & Cullis (1979). They found that the outer monolayer lipids were unaffected by Ca^{2+} whereas the inner monolayer lipids adopt a hexagonal phase when the intracellular calcium concentration is increased. This transformation in the monolayer configuration was attributed to a lateral segregation of the phosphatidylserine component, allowing the phosphatidylethanolamine to revert to its preferred hexagonal configuration. The change in the stability of the monolayer after calcium-loading is thought to be directly related to the fusion events involved in vesicle formation.

Although the changes in the bilayer that occur after calcium loading can explain both echinocyte and vesicle formation, there are so many other

concurrent modifications to the cell, making it difficult to isolate which effect is primarily responsible for the change in shape.

1.4.5. The Role of the Cytoskeleton in the Control of Erythrocyte Morphology

The lipid bilayer couple model originally proposed by Sheetz and Singer in 1974 to explain amphipath-induced shape changes, and expanded by others to also cover shape changes resulting from lipid breakdown, can explain most instances of erythrocyte shape change, but other relevant factors cannot be ignored.

Studies on patients with hereditary or acquired haemolytic anaemias, have suggested that the abnormal erythrocyte shape found in these patients are linked to defects in the cytoskeleton - a deficiency in a particular cytoskeletal protein or an alteration in the normal protein associations have been associated with morphological changes [Palek & Lux 1983; Liu *et al* 1989].

However, it is possible that in some instances the changes in the cytoskeleton may directly affect the area of the inner bilayer. Cross-linking of skeletal proteins has been shown to alter the association of the skeleton with the bilayer, inducing a shrinkage of the inner leaflet and thus forcing a change in shape. This would suggest that a change in the area of the lipid layer is the

main contributor to the alteration of cell shape, making the Sheetz & Singer hypothesis still relevant for these cases [Mohandas *et al* 1983].

The Role of Ankyrin: The interaction between the cytoskeleton and the lipid bilayer is also thought by others to play an important role in shape and deformability changes of the erythrocyte [Jinbu *et al* 1984]. In their studies human erythrocyte ghosts were subjected to trypsin under conditions where ankyrin was the only skeletal protein to be broken down and the other major cytoskeletal proteins remained intact. Crenated ghosts only changed into discocytes after chlorpromazine or Mg-ATP treatment if they had not been previously treated with trypsin. The number of transformable ghosts paralleled the amount of ankyrin left in the ghosts. They therefore concluded that ankyrin, presumably by acting as the anchor between the cytoskeleton and the membrane, plays a vital role in controlling the shape of the erythrocyte.

The role of spectrin: Spectrin dysfunction or partial deficiency has been closely studied in patients with haematological disorders such as hereditary spherocytosis, hereditary elliptocytosis and pyropoikilocytosis [Liu *et al* 1989]. These studies found that in the erythrocytes from these patients the normal skeletal network was disturbed. The cytoskeletons of normal erythrocytes consist of a hexagonal lattice of junctional complexes cross-linked by spectrin,

but in cells with spectrin deficiency this skeletal architecture is disrupted, since fewer spectrin filaments interconnected the junctional complexes.

The role of phosphorylation: As described previously, dephosphorylation of spectrin after ATP-depletion did not appear to contribute to the change in shape. However the state of phosphorylation of the cytoskeletal proteins may still affect the shape of the erythrocyte.

Although no direct link has been made between phosphorylation and shape change, many studies have shown that an increase in phosphorylation has lead to a decrease in the binding between the cytoskeletal proteins or between the cytoskeleton and the membrane. An increase in the phosphorylation of protein 4.1 leads to a reduced interaction between this protein and spectrin, actin and band 3 [Eder *et al* 1986, Ling *et al* 1987; Danilov *et al* 1990]. This may also explain the calcium-induced shape changes, since in calcium-loaded cells protein 4.1 phosphorylation is increased [Tang 1988].

One can conclude from these studies that the cytoskeleton plays an important role in stabilising the normal shape of the erythrocyte. Clearly, not only modifications to the lipid bilayer, but also changes in the cytoskeleton can influence shape transitions. What is still not fully resolved is whether alterations to the cytoskeleton alone can impose a change in shape or whether these changes are dependent on a contraction in the inner lipid

monolayer. This would make the bilayer the primary factor in the control of erythrocyte shape.

1.4.6. Changes in Erythrocyte Morphology Leading to Endovesiculation or Microvesiculation

Microvesicle release from echinocytic human erythrocytes occurs naturally during the process of ageing [Rumsby *et al* 1977] and in certain disease states such as sickle cell disease [Wagner *et al* 1984; Allan *et al* 1982].

Such vesiculation is not an event unique to the erythrocyte. Membrane shedding in the form of vesicles is a very common physiological phenomenon seen in large number of cells [see review by Beandoin & Grondin 1991].

Vesicle shedding is used by cells to send messengers to other cells, to remove defective parts of the plasma membrane, or secrete intracellular enzymes and hormones. For example, in reticulocytes, transferrin receptors on the plasma membrane are lost through the formation of vesicles [Johnstone *et al* 1987].

In a similar way to echinocytosis, vesicle formation in the human erythrocyte can also be induced *in vitro* under a variety of different conditions such as calcium-loading [Allan *et al* 1976], ATP-depletion [Lutz *et al* 1977], spectrin oxidation [Wagner *et al* 1987] or through the intercalation of certain amphipaths into the outer lipid bilayer [Ott *et al* 1981; Billington & Coleman 1978; Allan *et al* 1989].

Changes in morphology from discocyte to stomatocyte resulting in the formation of endovesicles have been shown to occur when outer membrane lipids are broken down, following treatment with phospholipase C [Allan *et al* 1975]. The formation of intracellular vesicles may also be a clinically important physiological process, as demonstrated by studies which suggest that mature erythrocytes spontaneously form intracellular vesicles both in *in vivo* as well as *in vitro* [Sills *et al* 1988].

In all these vesiculation events the initial step is always a change in cell shape from discocyte to echinocyte or stomatocyte. It therefore appears that this initial change in shape is a prerequisite for vesicle release and that modulators of cell shape also influence the vesiculation process. This was demonstrated by the results of Bütikofer *et al* (1987), which showed that incubation of erythrocytes with stomatocyte-forming amphipaths inhibits the vesiculation induced by a dimyristoylphosphatidylcholine by preventing the cells becoming echinocytic, whereas agents known to induce echinocytes facilitated vesicle formation.

The Sheetz and Singer hypothesis adequately explains the shape changes observed following incubation with an amphipath or a phospholipase, when the area of one monolayer expands or contracts relative to the other. However the mechanism of vesicle formation from echinocytic or stomatocytic cells has only been speculated upon.

Studies of the release of vesicles from ATP-depleted erythrocytes indicated that since EDTA inhibits vesicle release but not the breakdown of polyphosphoinositide, then the polyphosphoinositide breakdown and diacylglycerol production is not the rate-limiting step in the formation of vesicles [Müller *et al* 1981]. These workers concluded that although polyphosphoinositide breakdown and diacylglycerol production was sufficient to cause echinocytosis, vesicle release requires a further process. What this process could be was only discussed in vague terms, although the apparent inhibition by EDTA suggested the possibility of an involvement of bound divalent cations.

Ott (1981) also speculated on the mechanism for vesicle release. He proposed that a perturbation to the inner monolayer lipids could be a prerequisite for the membrane fusion preceding the vesicle release. The long lag time observed prior to vesiculation in dimyristoyl phosphatidylcholine (DMPC)-treated cells was interpreted in terms of the DMPC undergoing "flip-flop" from the outer to the inner monolayer after a few hours. The DMPC in the inner monolayer may then induce a lateral phase separation of phosphatidylserine and phosphatidylethanolamine. As in the case of calcium-treated cells, this separation may lead to phosphatidylethanolamine taking up a hexagonal configuration and this may in turn facilitate membrane fusion and thus vesicle formation [Hope & Cullis 1979]. The link between the transbilayer

movement of DMPC, phase separation and vesicle release is strengthened by the findings that vesicle release is temperature- dependent. No release of vesicles was seen below 20°C, the phase transition temperature for DMPC. It is also worth noting that the rate of transbilayer movement of exogenous lipids is also at a maximum in the temperature range of the gel to liquid-crystalline phase transition of the bilayer [Kruijff & Zoelen 1978].

The phase separation mechanism may also explain the microvesicle release seen with other amphipaths, since it was demonstrated that amphiphilic drugs affect the normal composition of the erythrocyte lipid bilayer [Schneider *et al* 1986]. Incorporation of certain amphipaths into the membrane resulted in the enhancement of the flip rates of exogenous lysophosphatidylcholine and a destabilisation of the asymmetric distribution of phosphatidylethanolamine. Both these events could have a fusogenic effect by disrupting the normal lipid arrangement and thus may assist the formation of vesicles.

Physical studies on model systems support the idea of an involvement between phase separation and vesicle formation. These studies have shown that some degree of phase separation occurs in the membrane, since part of the membrane is pinched off carrying one of the phases as a separate vesicle [Maddox 1993].

The involvement of membrane fusion in the vesiculation process may shed further light on the mechanism of vesicle formation. Several studies have implied that the aggregation of the intrinsic membrane proteins is needed for regions of the lipid bilayer to become fluid enough to fuse [Ahkong *et al* 1975; Billington & Coleman 1978]. Ahkong *et al* suggested that protein aggregation may occur either by disorganising membrane lipids with a micelling agent or by the insertion of a low melting fatty acid or ester.

It is of course unlikely that aggregation of the membrane proteins by itself causes vesiculation, but it is conceivable that a two-step process is involved in vesicle formation, the first being the shape change that brings the two areas of the bilayer close enough together, and the second being the fusogenic step which depends on a change in the lipid environment.

The possible function of the cytoskeleton, particularly spectrin, in vesicle release has also been closely examined. In erythrocyte disorders such as sickle cell anaemia and hereditary spherocytosis, as well as in ATP-depleted, calcium loaded or diamide treated cells, vesiculation was detected together with membrane protein oxidation [Wagner *et al* 1986]. In further detailed studies on the changes in stored erythrocytes, a strong correlation between vesiculation and spectrin oxidation was found. As expected, no correlation between vesiculation and ATP levels was found, although ATP-depletion itself can cause vesicle release [Wagner *et al* 1987]. The oxidation of spectrin

was found to disrupt the interactions between spectrin, actin and protein 4.1, so it is possible that where spectrin is oxidised, the cytoskeletal-lipid bilayer interactions are disrupted resulting in localised areas of damaged membrane which pinch off as vesicles.

1.4.7. Characterisation of Microvesicles from Erythrocytes

The composition of the microvesicle membranes has been generally found to resemble that of the original cell. A notable exception are those membrane proteins that form part of the non-diffusing skeletal complex (e.g. spectrin and actin) which are not found in the microvesicles. Since it has been shown that in echinocytic blebs, the lateral mobility of antigens is not restricted as it is in the rest of the membrane [Gordon & Marquand 1975], this suggests that only those components of the red cell membrane which are free to diffuse in the plane of the bilayer (e.g. lipids and those membrane proteins which are associated with lipid and not with the skeleton) would be expected to be present in the microvesicles. Band 3 does appear among the proteins of the microvesicle membrane so that at least a fraction of this protein must be diffusible. It is well known that band 3 protein can associate (via ankyrin and possibly band 4.1) with the skeletal complex [Bennett & Stenbuck 1980; Pasternack *et al* 1985] but there appear to be too many molecules of band 3 for them all to be bound to ankyrin and band 4.1 [Bennett 1985], so it might

be expected that a fraction of band 3 would be free to diffuse in the lipid bilayer.

1.5. Receptor-Mediated Signal Transduction

In many types of cells extracellular signals are relayed across the plasma membrane by a mechanism in which signalling molecules such as hormones or neurotransmitters, unable to pass through the plasma membrane, bind to cell surface receptors. This induces an increased concentration of intracellular messengers that propagate the signal inside the cell. Binding of agonists to receptors controls either the activity of enzymes such as adenylate cyclase or phospholipases, or results in the opening of ion channels located in the plasma membrane. In this way the intracellular concentration of messengers such as cAMP and Ca^{2+} or inositol trisphosphate can be regulated.

The major families of receptor-induced transmembrane signalling mechanisms are mediated by guanine nucleotide binding proteins (G-proteins) [Dohlman *et al* 1987; Gilman 1987, Cockcroft 1987]. Several experimental observations have supported the involvement of these proteins. For example, since GTP was shown to be a requirement for the hormonal activation of membrane-bound enzymes such as adenylate cyclase, a G-protein appears to mediate this process.

The binding of an agonist to the receptor is believed to precipitate a conformational change in the cytoplasmic domain of the receptor resulting in the binding of GTP to one of the sub-units of the G-protein associated with the receptor. This sub-unit, known as the α -sub-unit, dissociates from its β and γ subunits and activates plasma membrane enzymes such as adenylate cyclase [Birnbaumer *et al* 1990] or phospholipase C [Cockcroft & Gomperts 1985]. This leads to an increase in the concentration of intracellular second messengers, the two most important being cAMP and inositol trisphosphate [Berridge 1985].

1.5.1. The Role of cAMP in Signal Transduction

cAMP is produced from ATP as a result of the activation of adenylate cyclase, an enzyme associated with the plasma membrane. The activity of this enzyme is modulated by stimulatory or inhibitory G-proteins [Gilman 1987]. Activation of a receptor causes binding of GTP to the G-protein, resulting in the activation or inhibition of adenylate cyclase. Activation of adenylate cyclase increases the intracellular cAMP concentration. This leads to the activation of a cAMP-dependent protein kinase (PKA) and the phosphorylation of target proteins [Smith *et al* 1981]. cAMP-regulated responses are switched off via two separate mechanisms:

- 1) when the cAMP concentration falls through the hydrolysis of cAMP by a

phosphodiesterase [Schramm & Selinger 1984]; and 2) by the dephosphorylation of proteins by specific phosphatases.

The function of PKA in the regulation of cellular processes is described in greater detail under section 1.6.2.

1.5.2. The Role of Polyphosphoinositides in Signal Transduction

Animal cells contain three distinct types of inositol phospholipids, the most abundant of which is phosphatidylinositol (PI). Although the polyphosphoinositides are only present in relatively small amounts (usually less than 1% of the total phospholipid), they play a vital role in signal transduction in a wide range of cells. These lipids are the precursors of the intracellular second messengers produced after the stimulation of certain receptors by a variety of agonists, which include neurotransmitters, hormones and growth factors.

PI can be sequentially phosphorylated by specific kinases, thus producing phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP₂), also known as the polyphosphoinositides (PPIs). The PI 4-kinase and PIP 5-kinase have been considered to be separate enzymes since the relative rates of PIP and PIP₂ formation could be altered by manipulation of the ionic conditions. However these kinases have many similarities. Both are stimulated by Mg²⁺ and inhibited by Ca²⁺ and both

are considered to be mainly located in the plasma membrane. PIP 5-kinase has been found to exist in both soluble and membrane-bound forms [Carpenter & Cantley 1990], with the proportion of this kinase in the cytosolic fraction varying greatly according to cell type [Ling *et al* 1989; Imai *et al* 1986; van Dongen *et al* 1984].

The phosphorylation-dephosphorylation reactions are in a state of equilibrium. PIP₂ is degraded to PIP and PIP to PI by Ca²⁺-activated phosphomonoesterases, which have been located in cytosol, plasma membrane and Golgi membrane fractions [Downes & Michell 1985]. In erythrocytes the two phosphomonoesterases have been located in different fractions. PIP phosphomonoesterase is membrane-bound, whereas PIP₂ 5-phosphomonoesterase is found in the cytosol [Mack & Palmer 1988].

The importance of the PPIs in cell signalling in many tissues is reflected by the large volume of papers and reviews written in the past few years [Michell 1975, 1982; Michell *et al* 1981, 1989; Berridge 1984, 1987; Nishizuka 1984 & 1986; Rana & Hokin 1990; Kikkawa *et al* 1989; Majerus 1992]. Cellular responses, which are regulated by the intracellular Ca²⁺ concentration, and controlled by this versatile signalling pathway include secretion and exocytosis, smooth muscle contraction, and modulation of ion conductance, as well as cell growth and proliferation. In order to put the studies described in

this thesis into context, the following paragraphs will provide a brief overview of this complex and already fully documented area.

The transduction of a signal from the outside to the inside of the cell (in those cases which involve PPI breakdown) can be divided in three main components. The first step is the agonist binding to its receptor. This causes a change in conformation of the receptor which then interacts with a guanine nucleotide-binding protein (G-protein) [Cockcroft & Gomperts 1985; Cockcroft 1987]. A membrane-bound polyphosphoinositide specific phosphodiesterase (PPI-PDE) is then activated, catalysing PPI breakdown.

The G-protein involved in this signalling pathway is part of a family of proteins involved in the transduction of signals across the cell membrane [Gilman 1984 & 1987]. This involvement of a G-protein in the inositol-lipid signal transduction pathway was demonstrated in experiments showing that breakdown of PIP_2 occurred when non-hydrolysable guanine-nucleotide analogues (e.g. $\text{GTP-}\gamma\text{-S}$) were added to either permeabilised cells or isolated membranes.

The activation of the PPI-PDE (a phospholipase C) results in the hydrolysis of PIP_2 to form two intracellular messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate [Berridge 1987] (see Figure 1.5). PPI-PDE is believed to be primarily a cytosolic enzyme [Rhee *et al* 1989], but it is also found to be bound to the membrane.

Figure 1.5 Mechanism for the formation of intracellular messengers from the hydrolysis of phosphatidylinositol 4,5-bisphosphate

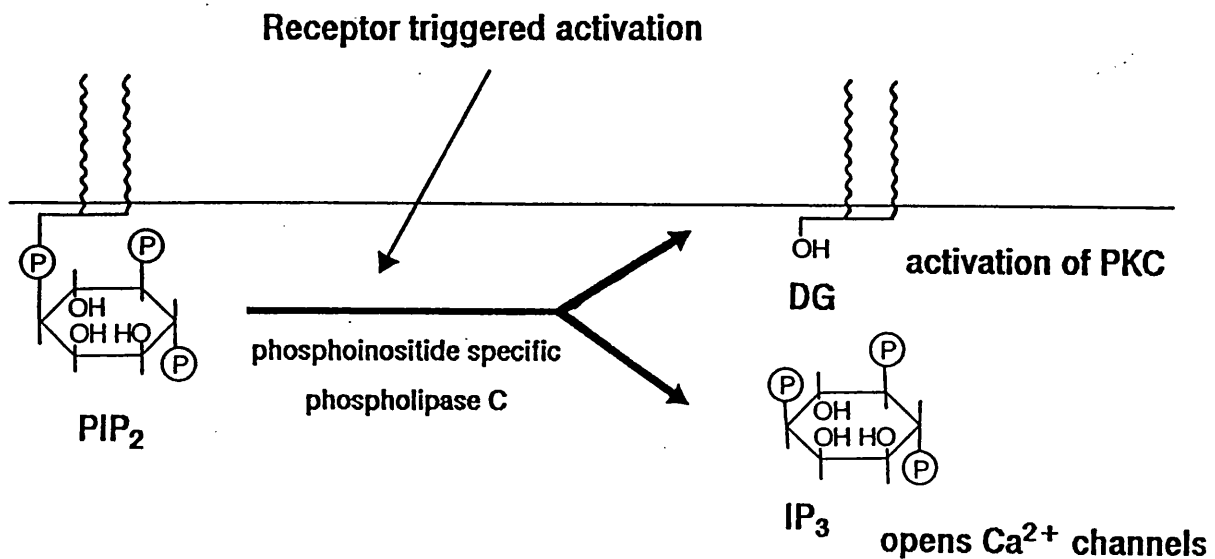
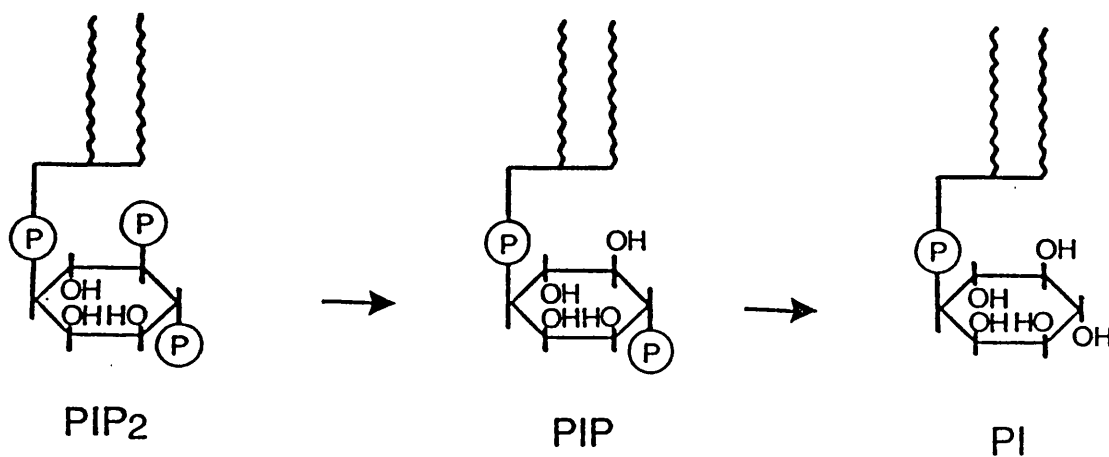


Figure 1.6 Dephosphorylation of Inositol Lipids



PPI-PDE is extremely sensitive to the intracellular Ca^{2+} concentration in the micromolar range, and preferentially degrades PIP_2 .

Diacylglycerol (DAG) activates a calcium-dependent protein kinase (PKC) and this results in the phosphorylation of specific cell proteins [Nishizuka 1984; 1986]. DAG acts to increase the affinity of PKC for Ca^{2+} , fully activating this enzyme without a need for an increase in the base levels of intracellular Ca^{2+} . PKC is only active for a short time - a negative feed-back mechanism comes into effect quickly through the rapid phosphorylation of DAG to phosphatidic acid (PA). Hydrolysis of DAG can also result in the formation of additional messengers, i.e. arachidonic acid, the precursor of prostaglandins, thromboxanes and leukotrienes. Although PKC is only active for a short time, the result of PKC activation may be long-lasting, depending on the state of phosphorylation of the substrate protein.

The main function of **inositol-1,4,5-trisphosphate** (IP_3) is to increase the concentration of cytosolic calcium through the release of calcium from intracellular stores. It does this by acting on a specific receptor, usually on the endoplasmic reticulum, opening the intracellular calcium channels. The involvement of IP_3 was shown in studies where the addition of IP_3 to permeabilised cells or injection into intact cells resulted in calcium release from intracellular stores and the initiation of calcium-dependent physiological responses.

In order to control the calcium levels in the cell and to avoid persistently high concentrations of calcium that may damage the cell, a stringent negative feedback mechanism exists. The intracellular concentration of calcium decreases, due to the active expulsion of calcium from the cells by a calcium-dependent ATPase located on the plasma membrane, and by reuptake of Ca^{2+} into the ER store following the removal of IP₃ by a cytosolic phosphatase. As well as being subject to dephosphorylation, IP₃ can also be phosphorylated giving rise to a number of higher polyphosphates, some of which may have a messenger function of their own [Michell 1986].

Dual Action of the Signalling Pathways:

It is now widely accepted that the two signalling pathways involving DAG and IP₃ can act independently but usually act synergistically in controlling cellular processes [see Nishizuka 1984, 1986; Berridge 1987; Kikkawa *et al* 1989 for reviews]. One example of this co-operative regulation is seen in experiments using platelets. If the intracellular Ca^{2+} concentration is increased with Ca^{2+} plus an ionophore, or if PKC alone is activated with a synthetic DAG (e.g. 1-oleoyl- 2-acetyl glycerol), no serotonin secretion is observed. However when the two stimuli are combined, a large secretion of serotonin is observed. This was also shown in human B cells: no proliferation was observed when cells were incubated with a diacylglycerol or

Ca²⁺/ionophore alone, but the combination of the two did activate cell proliferation [Guy *et al* 1985].

On the whole it appears that the IP₃/Ca²⁺ pathway plays a major role in initiating cellular responses, whereas the function of PKC is to act as a modulator. Indeed, some studies have shown that addition of PKC inhibitors did not prevent normal responses, so in these cases it seems that since PKC is not essential, its role may be to modulate the activity of the primary IP₃/Ca²⁺ pathway [Sha'afi *et al* 1986].

PKC has been found to play a major role in providing a negative feedback control to the IP₃/Ca²⁺ pathway, by removing calcium from the cytosol through the activation of the calcium-dependent ATPase and the Na⁺/Ca²⁺ exchange protein. In other cases PKC can also have an effect on the levels of IP₃ by inhibiting the receptor-mediated hydrolysis of PIP₂ or by stimulating the hydrolysis of IP₃ through the activation of the IP₃ phosphatase.

1.5.3. Polyphosphoinositides in Erythrocytes

Although human erythrocytes are able to metabolise inositol lipids [Hokin & Hokin 1964], unlike avian erythrocytes, they do not appear to possess G-proteins which can activate inositide-specific phospholipase C (G_p) [English *et al* 1992]. However, other types of GTP-binding proteins have been identified [Carty 1990; Damonte 1990; De Flora 1991; Graf *et al* 1992], but the

physiological significance of these proteins in the mature erythrocyte is so far undetermined.

As well as being devoid of G_p , human erythrocytes do not possess agonist-receptors and consequently have no mechanism for receptor-mediated breakdown of PIP_2 . However, their membranes do contain both PIP_2 and PIP (which comprise the bulk of the total inositol lipid), and specific phosphomono-esterases [Mack & Palmer 1985] and kinases [Ling *et al* 1989], which can modify inositol lipids.

Even though human erythrocytes do not appear to contain G_p , erythrocyte PPI - PDE activity was found by Allan & Michell (1978) whilst investigating DAG production when erythrocytes were incubated with Ca^{2+} /ionophore. This enzyme breaks down both PIP_2 and PIP producing DAG and IP_3 or IP_2 . PI however is not hydrolysed [Downes & Michell 1981].

Studies on erythrocytes have provided considerable information on the role of the polyphosphoinositides in membrane function, and this information may be relevant to the role of these lipids in other cells [Allan & Raval 1986; Gascard *et al* 1993]. For example, the role of PKC as a modulator of the Ca^{2+}/IP_3 signalling process was demonstrated in human erythrocytes. In these studies the Ca^{2+} -pump was shown to be phosphorylated, and as a result activated, when cells were incubated with phorbol-12-myristate 13-acetate (PMA), a known stimulator of PKC [Wright *et al* 1993]. Other

experiments, also on erythrocytes, have indicated that PKC may also have some effect on inositol lipid turnover. In these experiments the incubation of cells with PMA lead to an increased content of PIP, but not PIP₂, together with a decrease in PI. This indicates that PI kinase may be activated by a PKC-dependent process [Giraud *et al* 1988].

Polyphosphoinositides may also be important in regulating the interactions between the lipid bilayer and the cytoskeleton in the erythrocyte [Shapiro & Marchesi 1977; Anderson & Lovrien 1984; Gascard *et al* 1993], for instance by mediating the interaction between glycophorin and band 4.1 [Anderson & Marchesi 1985]. The state of phosphorylation of the polyphosphoinositides may be responsible for regulating the affinity of glycophorin for band 4.1 [Anderson & Lovrien 1984]. The involvement of the polyphosphoinositides in the control of cell structure has also been suggested in other cell types. In neurones, the structural properties of the cytoskeletal protein actin may be regulated through its interaction with phosphatidylinositol 4,5-bisphosphate [Forscher 1989].

The interaction between the lipid bilayer and the cytoskeleton mediated by the polyphosphoinositides may be a mechanism by which erythrocyte shape is regulated [Michell 1975; Allan & Thomas 1981; Ferrell & Huestis 1984]. It has also been suggested that membrane fusion might be influenced by polyphosphoinositide degradation because removal of the large negatively-

charged headgroup from these lipids might decrease the charge repulsion between membrane surfaces so enabling them to approach one another more closely [Allan & Michell 1979].

In old cells the ATP levels are reduced and there is a consequent increase in calcium concentration due to a fall in activity of the Ca^{2+} pump. This may induce the breakdown of the polyphosphoinositides by a phosphodiesterase [Allan & Michell 1978], which could lead to an imbalance between the lipid layers causing an alteration in cell morphology and the subsequent elimination of defective cells from the blood.

Ferrell & Huestis (1984b) suggested that a decrease in ATP leads to the dephosphorylation of PIP_2 to PIP and PI (Figure 1.6), and that this change leads to a reduction in the inner lipid leaflet area and an imbalance between the two leaflets of the bilayer that is resolved by echinocytosis.

A link between polyphosphoinositide metabolism and the control of the concentration of free cytosolic calcium has also been made in human erythrocytes. In the cells of patients with hypertension, an increase in the concentration of calcium was observed, together with a decrease in the activity of the kinases involved in PPI formation. These observations are consistent with those seen for non-erythroid cells, where the PPIs may indirectly control the ATP-dependent calcium transport [Remmal *et al* 1988].

1.6. Protein Phosphorylation

1.6.1. The Role of Protein Phosphorylation in Cellular Function

Modification of proteins through phosphorylation is one of the major intracellular mechanism by which cells respond to extracellular stimuli.

Ligand binding to receptors on the cell surface may lead to the activation of protein kinases which transfer a phosphate group from a nucleoside triphosphate (usually ATP) to the serine and threonine residues of specific proteins. Under certain conditions the tyrosine residues can also become phosphorylated.

Phosphorylation of a protein usually, but not always, results in conformational changes which alter the functional properties of the protein.

Protein phosphorylation is fairly complex. For example, a protein can be phosphorylated at multiple sites by a single kinase but not all these sites are functionally equivalent. Different kinases may phosphorylate different sites, leading to distinct changes in the characteristics of the protein.

Such protein alterations can be reversed when the stable ester bond formed between the phosphate and the amino acid is broken by the action of a

protein phosphatase. Thus the activity of a protein can be regulated by the phosphorylation-dephosphorylation cycle [Krebs & Beavo 1979].

The first studies on protein phosphorylation were carried out on skeletal muscle cells. It was shown that adrenaline-induced glycogen synthesis and breakdown was regulated by a phosphorylation-dephosphorylation cycle [Cohen P 1983]. In other cells the receptors for growth factors are also protein kinases that phosphorylate other proteins, initiating the mechanisms for cell division and proliferation. Other processes such as endocytosis, exocytosis, shape regulation and the lateral mobility of membrane proteins may also be influenced by the phosphorylation of the membrane proteins. As described in the previous section, the main regulatory agents of kinase activity are cAMP and calcium.

In erythrocytes, several endogenous kinases and phosphatases have been found which modify the skeletal proteins [Cohen C M 1983; Gratzer 1981]. These enzymes include, amongst others, cAMP-dependent protein kinases, a calcium-dependent protein kinase and a calcium/calmodulin-dependent protein kinase [Boivin 1988].

1.6.2. Cyclic AMP-Dependent Protein Kinase

The cAMP-dependent protein kinase (PKA) is the key component in the regulation of hormonal mechanisms which use cAMP as the second

messenger. This kinase has been encountered in all mammalian cells studied and in most cases is found in the cytosolic fraction of the cell, although both brain and heart cells have been found to contain significant portions bound to the plasma membrane.

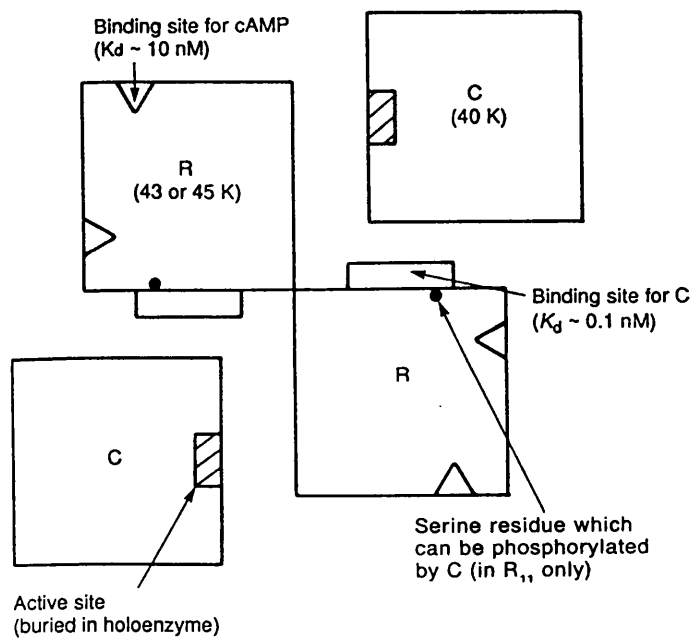
A rise in cAMP results in PKA activation, protein phosphorylation and, according to cell type, the induction of a variety of different responses. For example, in liver cells PKA activation results in glycogen breakdown whereas in endocrine cells it leads to increased hormone secretion. PKA therefore has a broad specificity, that is, it can potentially phosphorylate a large variety of proteins at various sites. However, in reality there is only a limited number of protein sites that are functionally affected by this kinase, since only those serine and threonine residues that are appropriately positioned to interact with the enzyme's active site are phosphorylated.

PKA exists as a tetramer, composed of two types of sub-units. One possesses catalytic activity (known as the C sub-unit) and the other is a regulatory sub-unit (known as R). This gives the enzyme a R_2C_2 structure (see Figure 1.7). The C sub-units appear identical whereas the R sub-units are polymorphic, varying in their relative proportions according to cell type.

A rise in the cytosolic concentration of cAMP leads to the binding of four molecules of cAMP to the regulatory sub-units on the kinase, two on each R sub-unit of the R_2C_2 complex.

Figure 1.7 Diagrammatic Representation of the Structural Features of Protein Kinase A

Taken from Morgan (1989).



This induces a conformational change and the dissociation of the regulatory and catalytic sub- units, with activation of the catalytic sub-units and the phosphorylation of the target proteins [Krebs & Beavo 1979; Smith *et al* 1981].

This whole process is in a state of equilibrium. PKA is inactivated when the R and C sub-units reassociate and cAMP is freed. PKA activity is also regulated by inhibitory proteins which interact with free C sub-units, thus preventing the phosphorylation of the target proteins. Another way to control PKA activity is by the reduction of the concentration of cAMP, achieved through the activation of cAMP phosphodiesterases which hydrolyse cAMP to 5'-AMP.

The mechanism by which PKA is activated, and proteins phosphorylated, is finely tuned. A small change in the concentration of cAMP can lead to a highly amplified and prolonged response, since cAMP also controls a positive feedback mechanism by inhibiting protein phosphatases. PKA also phosphorylates and thereby activates a phosphatase inhibitor protein. This leads to the binding of the inhibitor protein to the phosphatase, inhibiting its activity [Ingebritsen & Cohen 1983].

1.6.3. Calcium-Dependent Protein Kinase

Protein kinase C (PKC) is a regulatory enzyme first discovered over fifteen years ago [Takai *et al* 1979]. PKC has been found in many types of cells and

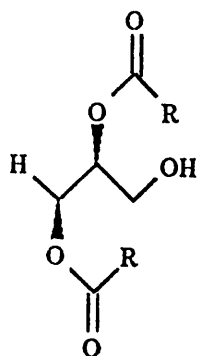
in particular abundance in brain cells [Carpenter *et al* 1987]. This enzyme exists in different forms: several discrete subspecies have been defined by differences in the mode of activation, sensitivity to Ca^{2+} and catalytic activity [Kikkawa *et al* 1989].

This enzyme, thought to be largely present in the cytoplasm in its inactive form, is translocated to the cytoplasmic face of the plasma membrane in a calcium-dependent manner when cells are stimulated [Nishizuka *et al* 1986]. It is activated by diacylglycerol, a product of the receptor-mediated hydrolysis of the polyphosphoinositides [Berridge 1981; Michell 1975; Michell *et al* 1981] (Figure 1.5). Diacylglycerol and the membrane phospholipid phosphatidylserine bind to PKC, increasing the affinity of this enzyme for Ca^{2+} , thereby activating it without a need for an increase in the resting concentration of cytosolic calcium [Nishizuka 1984, 1986].

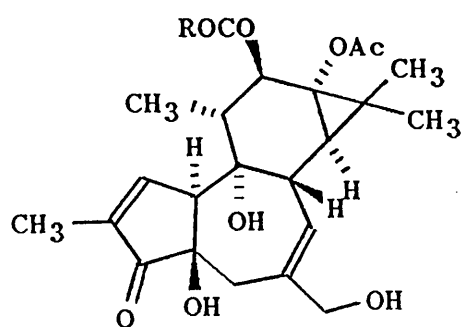
PKC can be activated *in vitro* in the absence of diacylglycerol but in the presence of phosphatidylserine and millimolar Ca^{2+} . Other phospholipids cannot support PKC activation alone but can show inhibition or co-operation [Kaibuchi *et al* 1981; Rando 1985]. PKC can also be inhibited by some cationic amphipathic drugs, possibly by interfering with the interaction of negatively charged phospholipid and the Ca^{2+} -interacting domain [Mori *et al* 1980; Wise 1982].

The activation of PKC by diacylglycerol can be mimicked by the action of other agents such as short chain derivatives of this lipid (e.g. 1-oleyl-2-acetyl glycerol) [Mori *et al* 1982]. The requirement for activation seems to be a 3-hydroxyl group, the two ester groups and the stereochemistry of the glycerol backbone. On the other hand, PKC can also be activated by the tumour-promoting phorbol esters acting on the cytosolic surface of the membrane [Hecker 1971; Castagna *et al* 1982; Nishizuka 1983]. Phorbol myristate acetate (PMA) (Figure 1.8) permanently activates PKC at very low concentrations (1000-fold lower than DAG) by increasing its affinity for Ca^{2+} and promoting the association of the cytosolic enzyme with the membrane. The extent of the structural similarity between diacylglycerol and PMA is not clear and still needs to be resolved [Ganong *et al* 1986; Jeffrey & Liskamp 1986].

Figure 1.8 The Structure of phorbol myristate acetate



Diacylglycerol



Phorbol Ester

1.7. Summary

The review of the work done up to date on the mechanisms of erythrocyte shape changes and vesicle release indicates that there are still some areas of uncertainty. It is still not clear how much influence the cytoskeleton has on the morphology of the erythrocyte, or for that matter whether perturbation in the bilayer alone is sufficient to cause vesiculation. What is clear is that since shape changes and vesicle release occur under such different conditions, one theory alone is unlikely to explain all instances of shape change or vesicle formation.

The work in this thesis should increase our understanding of the influence of the lipid membrane and cytoskeleton on the morphology of the erythrocyte.

The effect of amphipaths on erythrocyte morphology are examined by studying the relationship between the extent of binding of an amphipath (in this case merocyanine 540) and echinocytosis and vesiculation. We wanted to closely analyse particular cases of echinocytosis and vesiculation to test the theory that extreme modifications to the area of one of the lipid monolayers relative to the other, is sufficient to induce vesicle release, without the need for cytoskeletal alterations.

We also thought that it should also be possible to obtain a quantitative measure of the proportion of band 3 molecules that were freely diffusing (i.e.

not bound to skeletal proteins) by determining the relative amount of band 3 which partitioned into microvesicles produced under mild conditions.

Furthermore, such measurements might reveal differences in the extent of attachment of band 3 under various conditions of microvesicle release.

Similar considerations would also apply to other components of the bilayer portion of the membrane such as glycophorin and polyphosphoinositides which may also have interactions with the membrane skeleton [Anderson & Marchesi 1985].

Further work on the effect of phosphorylating the cytoskeletal proteins may also shed some light on what effect phosphorylation has on cell shape or the interactions between membrane components. Studying the composition of vesicles from cells which have been pre-exposed to kinases may also provide information on the effect of phosphorylation on the lateral diffusion of the intrinsic membrane proteins.

2. MATERIALS AND METHODS

2.1. Materials

Sphingomyelinase (SMase), Lucifer Yellow, phospholipase A₂ (PLA₂) from bee venom, phorbol myristate acetate (PMA), Merocyanine 540, dimyristoyl phosphatidylcholine (DMPC), ionophore A23187, cAMP, dibutyryl-cAMP, Triton-X100, N-2-hydroxyethyl- piperazine-N¹-2-ethanesulphonic acid (Hepes), 3-[N-morpholino]-propanosulphonic acid (Mops), tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Company.

³²P was obtained as phosphorus-32-orthophosphate in dilute hydrochloric acid from Amersham International.

Merck 20x20 cm silica gel HL60 plates (BDH) were used for thin layer chromatography (TLC). Fuji X-ray films were used for radioautography.

Other reagents were obtained from Sigma, BDH or Aldrich.

2.2. Microscopy

Changes in cell morphology were followed by light microscopy using a Leitz microscope at a magnification of x400. Fluorescence was detected using a Perkin Elmer Fluorimeter.

2.3. Preparation of Cells

Fresh human blood was obtained either from known, healthy volunteers using EDTA as anticoagulant or from the Blood Transfusion Service on the day of collection. The erythrocytes were sedimented by centrifugation (3000rpm, 5min) in a MSE Centaur Centrifuge, washed twice with 150mM NaCl with the removal of the buffy coat and once with 10mM Hepes-NaOH, 150mM NaCl, 0.1mM EDTA pH=7.4 (Hepes buffer).

2.4. Preparation of Ghosts

Ghosts were prepared by lysing the cells with ice-cold lysis buffer, 20mM tris-HCl, 2mM EDTA pH=7.3. Membranes were sedimented in a Sorvall RC2-B centrifuge at 15,000 rpm (25,000g) for 10min. Washing was repeated only once prior to polyphosphoinositide extraction, but otherwise two or three times until ghosts were pale pink.

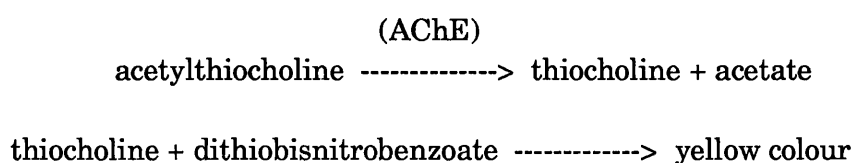
2.5. Measurement of Lysis

Lysis was quantified by measuring the absorption at 418nm of the supernatant after sedimentation of the cells, using an LKB spectrophotometer. Reference values were obtained by taking a known number of cells, lysing them in water, and measuring the absorption spectrum.

Figure 2.1 shows such a reference graph. In this case different volumes (5-160 μ l) of a 1% suspension were added to the spectrophotometer cell containing water (1.5ml). The absorption was plotted against the haematocrit, giving a straight line. The percentage lysis in the experimental samples was calculated from reference values obtained from cells used in the experiment.

2.6. Acetylcholinesterase Analysis

Acetylcholinesterase (AChE) activity was determined according to Ellman *et al* (1961) with slight modifications. AChE activity is measured by following the increase in absorbance at 412nm of the yellow colour produced when thiocholine, produced from the enzymatic breakdown of acetylthiocholine, reacts with dithiobisnitrobenzoic acid to produce 5-thio-2-nitro-benzoic acid:

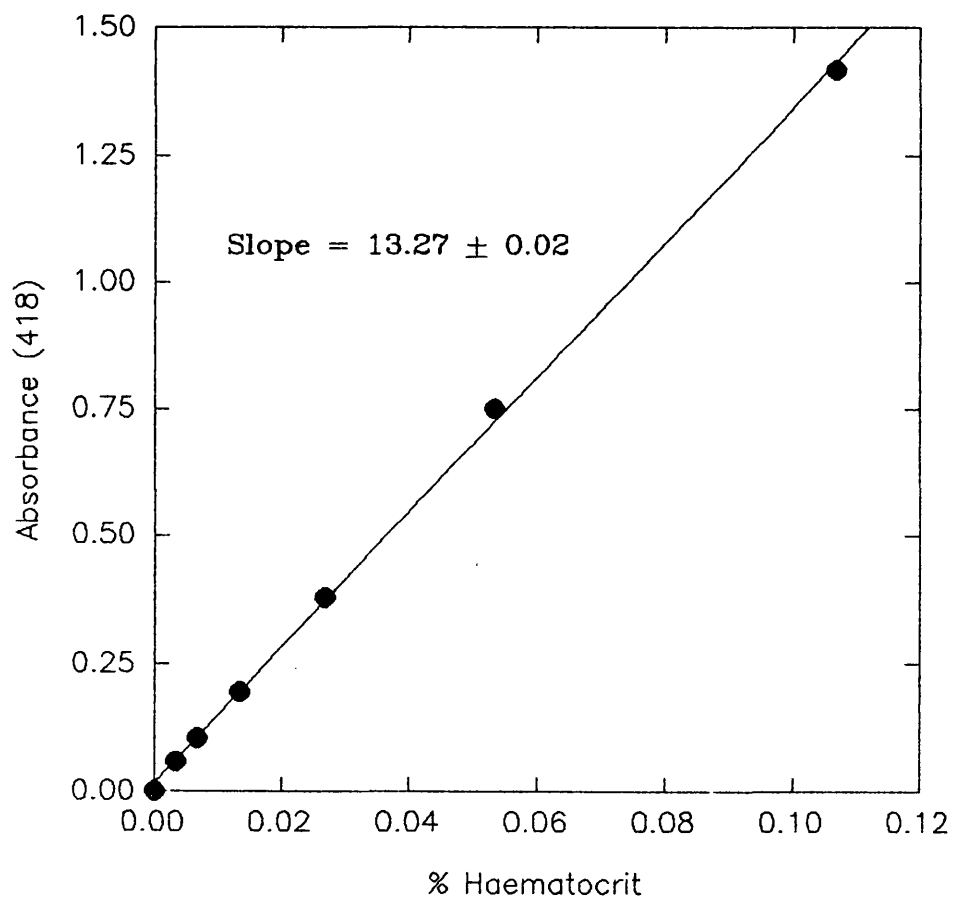


A plot of the rate of increase in absorbance over time is linear, the slope being proportional to the amount of enzyme present in the sample. Since the molar extinction coefficient of the yellow anion is known (1.36×10^4), the rate of reaction can be converted to absolute units:

$$\text{Rate (moles/l per min)} = \frac{\Delta \text{ absorbance per min}}{1.36 \times 10^4}$$

Figure 2.1 Lysis Measurement: Plot of Absorbance against Haematocrit

Different volumes (5-160 μ l) of a 1% haematocrit erythrocyte cell suspension were added to the spectrophotometer cell containing water (1.5ml) and the absorbance measured at 418nm.



The results obtained from a typical experiment using four control samples gave the figure of 8.85×10^{-16} moles of substrate hydrolysed per min per red blood cell (see Table 2.1) - this compares favourably with the results obtained by Ellman *et al* of $(1.08 \pm 0.16) \times 10^{-15}$.

The reaction rates were recorded using a LKB Biochrom Ultrospec 4050 at 412nm and an Enzyme Kinetics program on a BBC computer (Figure 2.2). The relative amount of AChE present in a sample containing an unknown quantity of cells was calculated using control samples containing a known quantity of cells. For a typical experiment the amount of enzyme was calculated by relating it to the average value obtained from five control samples. The value for a sample with no cells, was subtracted from each result.

Erythrocyte samples (5-20 μ l at 1% haematocrit) were added by means of a micropipette to a photocell containing buffer (750 μ l, 0.1M phosphate buffer pH=8), 0.5mM acetylthiocholine iodide (5 μ l, 75mM solution) and 0.33mM dithiobisnitrobenzoic acid/phosphate buffer pH=7 (25 μ l, 10mM solution). The mixture was stirred using a pipette and left to stabilise for 2 minutes before recording the reaction rates over 10 minutes (Figure 2.2)

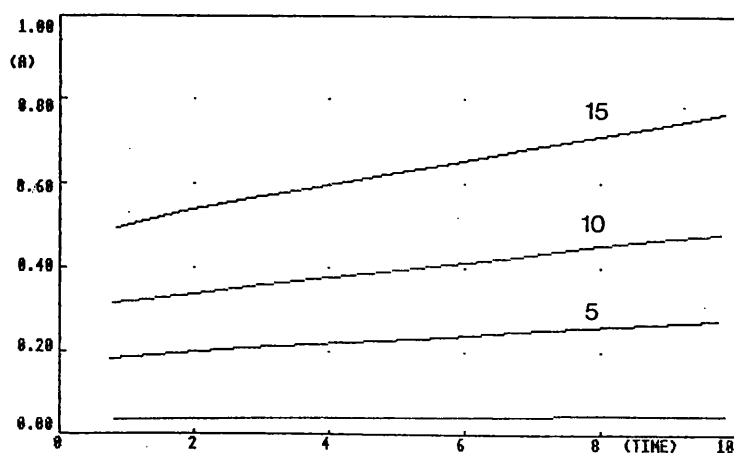
Table 2.1 Measurement of Acetylcholinesterase Activity

Samples (5, 10, 15 and 20 μl) of an erythrocyte suspension at 1% haematocrit were added to the substrate solution (total volume 800 μl , containing 0.5 mM acetylthiocholine iodide) as described in the text. The number of cells in each sample was calculated, assuming that 1 μl of packed cells contains approximately 1×10^7 cells. The results for moles of substrate hydrolysed per minute (rate) were plotted against number of red cells in the sample, giving a straight line. The slope (corrected for concentration and volume) gives the moles of substrate hydrolysed per min per cell, which in this case equals $8.85 \pm 0.03 \times 10^{-16}$.

Number of cells	Δ absorbance per min	Rate
0.5×10^6	0.007	5.15×10^{-7}
1.0×10^6	0.015	1.10×10^{-6}
1.5×10^6	0.025	1.84×10^{-6}
2.0×10^6	0.029	2.13×10^{-6}

Figure 2.2 Example of Acetylcholinesterase Analysis Plot

Figure shows the output for three different samples of erythrocytes, plus a control sample.



2.7. Lipid Analysis

Erythrocyte ghost samples were resuspended in 150mM sodium chloride (0.5ml) and the membranes were extracted by the addition of methanol/chloroform (1.9ml, 2:1 v/v). Phase separation was achieved by adding 0.6ml each of chloroform and water, vortexing and centrifugation (1500rpm, 5 minutes) [Bligh & Dyer 1959]. The lower organic layer was dried first under reduced pressure and then under a stream of nitrogen. The lipids were redissolved in chloroform (50µl) before being separated on silica gel 60 TLC plates run in chloroform, methanol, acetic acid, water (75:45:12:2). Spots were visualised with iodine, and in some experiments scraped from the plate and assayed for phosphorus as described below. Since blank areas of silica gave significant amount of absorbance, the results were corrected by subtracting the value obtained for the same area of blank silica [Mitchell *et al* 1986].

2.7.1. Polyphosphoinositide Analysis

Polyphosphoinositides were extracted in methanol/chloroform as above, but with addition of concentrated hydrochloric acid (30µl). Acid is needed for the extraction of polyphosphoinositides bound to proteins [Michell 1982]. Phase separation was achieved either by adding sodium chloride solution/chloroform as before or 0.1M hydrochloric acid/chloroform (using acid for the phase

separation made no difference to the amount of lipid recovered). Lipid separation was carried out on TLC plates that had been impregnated with potassium oxalate (1%) in methanol/water (2:3 v/v) and left to dry overnight, using chloroform, acetic acid, methanol, acetone and water (40:12:13:15:7) as the solvent system. Radioactive spots were visualised by autoradiography. Scraped spots were assayed for phosphorus and radioactive sample counted in water using a 6880 Searle Liquid Scintillation Counter.

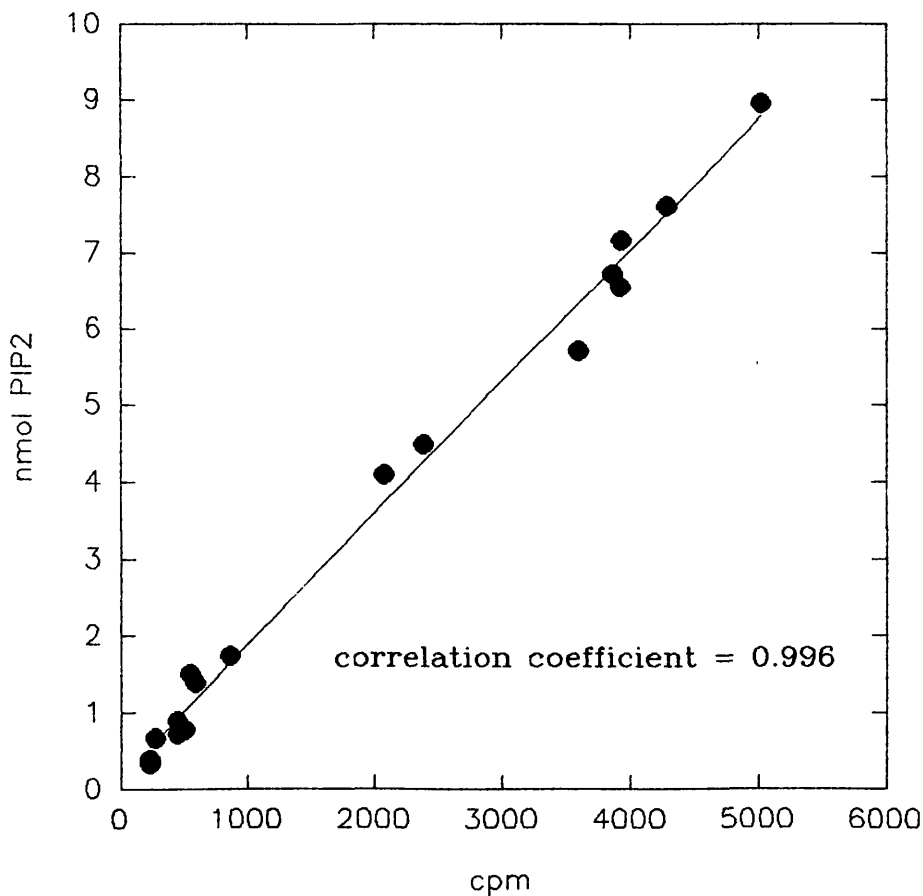
TLC analysis of ^{32}P -labelled microvesicles and ghosts confirmed previous results that the only erythrocyte lipids to be labelled with ^{32}P were those with monoester phosphate groups, i.e. PIP, PIP₂ and phosphatidic acid (PA) [Hokin & Hokin 1964].

The specific activity of PIP₂ (cpm/nmol PL) was calculated from plots of nmol phospholipid against counts per minute (Figure 2.3). Labelling of PIP₂ reached equilibrium after incubation overnight with ^{32}P [Gascard *et al* 1989]. The specific activity of PIP₂ was found to be between 80 and 85% that of ATP. Figure 2.3 shows that the amount of radioactivity found in PIP₂ (specific activity) was constant in control cells, cells treated with MC540, DMPC, Ca^{2+} /A23187 or iodoacetamide (ATP-depletion), and also constant in the microvesicles from treated cells.

Figure 2.3 Correlation of Mass and Radioactivity for Phosphatidylinositol 4,5-Bisphosphate

Cells were labelled at 37°C with ^{32}P ($\sim 20\mu\text{Ci}/\text{ml}$ cells) for over 16 hours prior to incubation with MC540, DMPC, $\text{Ca}^{2+}/\text{A23187}$, or iodoacetamide (ATP-depletion). The phospholipids from the control cells, treated cells and microvesicles were separated by TLC and the PIP_2 spots scraped from the plates. These samples were analysed for phosphorus and radioactivity. Results from the phosphorus analysis (nmol P_i) were plotted against the cpm. The specific activity of PIP_2 can be calculated from the slope, taking into account that the amount of P_i corresponds to the three phosphates, and that only two of the phosphates are labelled.

A similar correlation between P_i and cpm was seen in three other experiments.



It is therefore acceptable to take counts per minute as a measure of amount of lipid present - measuring radioactivity rather than phosphorus is quicker and simpler. It was also more accurate, particularly in samples containing small amounts of phosphorus.

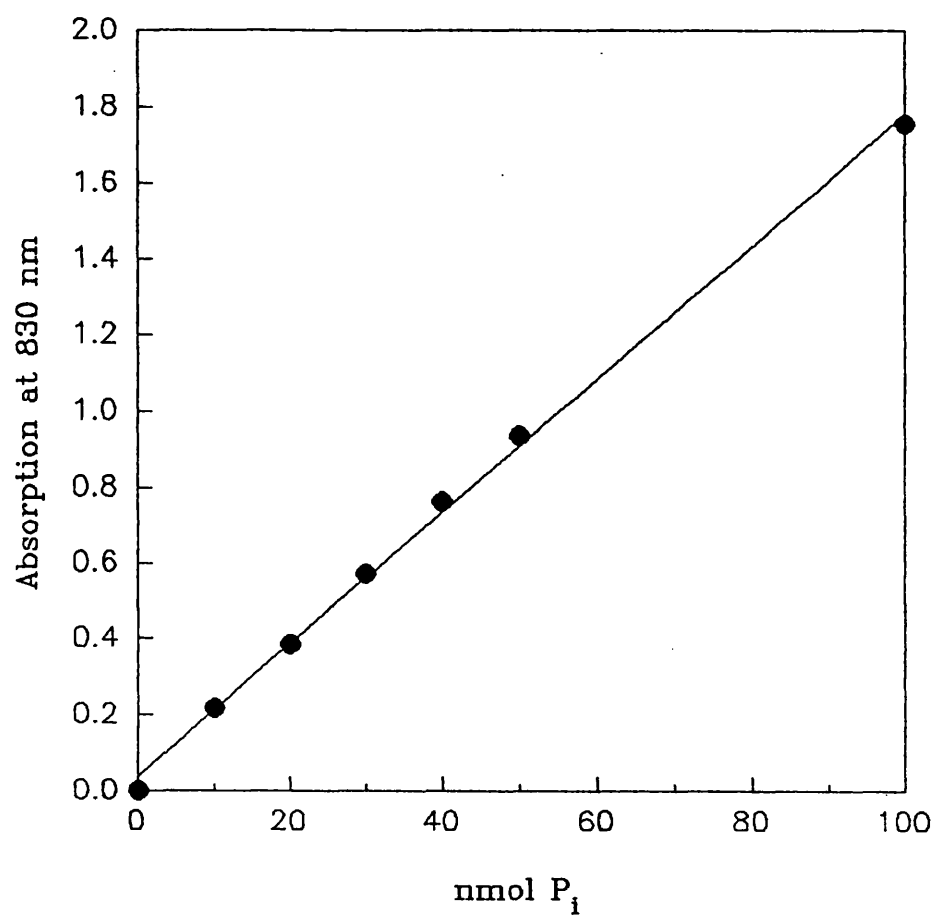
The amount of P_i for the PIP spots was calculated from the counts, assuming that the specific activities of PIP to PIP_2 are in the ratio of 1:2 [Mitchell *et al* 1986]. The amount of PIP was calculated in this way because a clean separation of this lipid from other phospholipids (particularly lyso-phosphatidylcholine) was not achieved.

2.8. Phosphate Analysis

Phosphorus was assayed by a micro-adaptation of the method of Bartlett (1959). Lipids were extracted as described above and digested for one hour at 180°C in 70% perchloric acid (200µl) plus a drop of 0.25% ammonium molybdate. Water (1.5ml), 10% ascorbic acid (200µl) and 5% sodium molybdate were added and the solution was heated at 60°C for 15 minutes. The absorption of the solution at 830nm was read on a Cecil 272 Ultraviolet Spectrometer. The same procedure was followed for solutions containing known amounts of phosphorus (0-100 nmoles P_i). A plot of absorption against nmoles of phosphorus gave a straight line (Figure 2.4). This plot was used to calculate the quantity of phosphorus from the absorption values.

Figure 2.4 Phosphate Analysis

Plot of absorption at 830nm against nmol phosphorus.



2.9. Polypeptide Analysis

Cell or vesicle polypeptides were analysed by SDS-polyacrylamide gel electrophoresis following the method of Laemmli (1970) on a BioRad Minigel apparatus at 200V. Peptides on known molecular weights were used as markers.

Sample buffer was added to cell samples (in a ration of 2 : 1, total volume ~ 150µl) and heated at 95°C for 10 min. The sample buffer was composed of 0.5M tris-HCl pH=6.8 (4ml), glycerol (1.6ml), 10% SDS (6.4ml), 2-β mercapto-ethanol (1.6ml) and 0.05% bromophenol blue (0.8ml).

Gels were prepared by combining the following solutions:

	<u>Main Gel</u>	<u>Stacking Gel</u>
0.75M tris-HCL (pH=8.8)	5ml	--
0.25M tris-HCl (pH=6.8)	--	4ml
20% SDS	50µl	40µl
ammonium persulphate	50µl	50µl
TEMED	5µl	5µl
30% acrylamide, 0.8% bisacrylamide	variable	1ml
distilled water	variable	3ml

The ratio of acrylamide plus bisacrylamide to water was varied to give for different density gels. The running buffer consisted of glycine (28.8g), Tris (6g), SDS (1g) in water (1L) pH=8.3.

Gels were stained with Coomassie Brilliant Blue (0.05% w/v) for several hours and destained with 50% methanol/acetic acid (4:1 v/v). Glycophorins were detected using Periodate/Schiff Reagent according to the procedure of Zacharius *et al* (1969):

1. The gels were immersed in sodium metaperiodate (1%) in acetic acid (3%) for 50 minutes.
2. Washed with water for at least one hour.
3. Immersed in Schiff's Reagent (fuchsin-sulphite) in the dark for 50 minutes.
4. Washed with sodium bisulphite (0.5%).

Radioactive polypeptides were located by autoradiography. Quantification of bands on gels was achieved by scanning with a BioRad 1650 Scanning Densitometer linked to a Shimadzu C-R3A Integrator.

2.10. Preparation of ^{32}P -Labelled Erythrocytes

A suspension of erythrocytes at 20% haematocrit was incubated at 37°C in Hepes buffer (10mM Hepes-NaOH, 150mM NaCl, 0.1mM EGTA pH=7.4) with Mg^{2+} (1mM), glucose (10mM) and ^{32}P . In the experiments where the

labelling of the membrane proteins was of interest, the cells were incubated for 1-2 hours with approximately 50-100 μ Ci per ml of packed cells, and the amount of radioactivity was quantified by scanning the radioautographs of electrophoresis gels. In the experiments where the polyphosphoinositides were measured by TLC, the cells were incubated overnight with 15-25 μ Ci per ml of packed cells.

2.11. Treatment of Erythrocytes with MC540

2.11.1. Binding of MC540 to Erythrocytes

The amount of binding of MC540 to cells was measured according to Allan *et al* (1989). Erythrocytes at 2% haematocrit, in Hepes buffer (10mM Hepes-NaOH, 150mM NaCl, 0.1mM EDTA pH=7.4) were incubated at 37°C with varying amounts of MC540 (0-30 μ M). After 5 minutes the samples were centrifuged at 3000rpm and the supernatant solution was removed. The pellet was resuspended in buffer and a sample was added to n-butanol. The amount of MC540 was measured from its fluorescence emission at 580nm. The emission spectra were measured in a Perkin-Elmer Fluorescence Spectrometer using an excitation wavelength of 540nm.

A standard reference graph was plotted by measuring the emissions at 580nm from butanol solutions containing known amounts of MC540 (0 - 100nM).

2.11.2. Microvesicle Release in Erythrocytes Treated with MC540

Studies measuring the extent of microvesicle release were carried out on cell suspensions of 2% haematocrit. Using low haematocrits avoids the problems associated with using large amounts of MC540. The dye becomes increasingly insoluble at higher concentration and binds to the glass tubes used in the experiments. Higher haematocrits (5 or 10%) were used in experiments where a large yield of vesicles was needed, but the exact amount of vesicle formation was not being measured. In the experiments where the PIP₂ and PIP content in the microvesicles was being measured, a 2% haematocrit sample was impractical, since it was difficult to obtain enough vesicles for phosphorus analysis, without using very large sample volumes. Because of this, and because in these experiments the exact amount of vesicle release was not being measured, the haematocrit was raised to 5% and the concentration of MC540 raised proportionally (i.e. instead of incubating a 2% haematocrit sample with 25µM MC540, a 5% haematocrit sample was incubated with

62.5 μ M). In the experiments to measure changes in phospholipid asymmetry (incubation with PLA₂ and SMase), 10% haematocrit was used.

Erythrocytes were incubated with MC540 (2-30 μ M) usually for 2 minutes (but 10 minutes in some experiments) at 37°C. In the time course of microvesiculation experiments, erythrocytes were incubated with MC540 (20 μ M) for 0-10 minutes at 37°C. In the temperature dependence experiments the incubation temperature was varied from 0 to 45°C. Aliquots were taken at appropriate times, the reaction was quenched by the addition of an equal volume of ice-cold buffer and the cells were sedimented (1500rpm, 5 minutes). The supernatant was assayed for AChE activity and extent of lysis. 100% AChE activity and lysis was calculated from readings for an aliquot taken from the incubation mixture at the beginning of the experiment.

Microvesicles were obtained from the supernatant by centrifugation at 40,000rpm (100,000g) for 1hr. SDS- PAGE, TLC and phosphorus analysis was carried out on the microvesicles and the ghosts from MC540-treated cells as described previously.

2.12. Treatment of Erythrocytes

2.12.1. Treatment with Dimyristoyl Phosphatidylcholine

A suspension of dimyristoyl phosphatidylcholine (DMPC) was prepared by sonicating the dry lipid at a concentration of 0.5mg/ml Hepes buffer using an MSE sonicator at an amplitude of 14 μ m for 15 minutes. Erythrocytes (20% haematocrit) were incubated in this buffer for up to 2.5 hours [Ott *et al* 1978]. In the time course experiments, the incubation was terminated rapidly by the addition of ice-cold buffer. After incubation the cells were sedimented and the AChE activity and extent of lysis was measured as in the MC540 experiments.

Because of DMPC contamination total phospholipid release could not be measured directly, but was calculated after separating the lipids by TLC and measuring phosphatidylserine and phosphatidylethanolamine, assuming that these phospholipids represent 43% (16% plus 27%) of the total phospholipid [Nelson 1972].

2.12.2. Treatment with Calcium and Ionophore

Cells at 20% haematocrit in Hepes buffer (10mM Hepes-NaOH, 150mM NaCl, pH=7.4) were incubated at 37°C with CaCl₂ (1mM) and A23187 (5 μ M)

for 20-25 minutes. The incubation was terminated by the addition of EGTA (4mM) [Allan & Thomas 1981]. Treated cells were isolated by centrifugation at 3000rpm and microvesicles isolated by centrifugation of the supernatant at 15,000 rpm. In the experiments where the activity of the polyphosphoinositide phosphodiesterase was measured, cells, ghosts or microvesicles were treated as described above. Treated ghosts were isolated after lysis of treated cells by centrifugation at 15,000 rpm.

2.12.3. Preparation of ATP- Depleted Cells

Erythrocytes at 20% haematocrit in Hepes buffer were incubated at 37°C with iodoacetamide (5mM) in the absence of glucose for 24hrs [Lutz *et al* 1977, Bütikofer & Ott 1985]. The cells were sedimented and the microvesicles isolated by centrifugation as described previously.

2.12.4. Ageing of Erythrocytes in Vitro

Whole blood was stored for 10-15 weeks under sterile conditions in citric acid/sodium citrate/dextrose at 4°C. The cells were sedimented at 3000rpm and the supernatant spun at 15,000rpm to isolate the microvesicles. Because the extent of lysis was high, the microvesicle samples were contaminated with ghosts. However, a clear separation between the pellet of red

microvesicles and the overlaying pale ghosts made possible the removal of the ghosts by careful aspiration.

2.12.5. Treatment with Sphingomyelinase

The sphingomyelinase preparation (as a solution in 50% glycerol, 0.25M phosphate buffer) had a protein concentration of 0.24mg/ml with an enzyme activity of 160IU/mg (1IU equals 1mmol of substrate degraded per min at 37°C). Washed cells at 10% haematocrit in Mops buffer (20mM Mops-NAOH/150mM NaCl, pH=7.1) plus 1mM MgCl₂ and 10mM glucose) were incubated with sphingomyelinase (10µg/ml) at 37°C for 15-20 minutes [Allan & Walklin 1988].

Preparation of endovesicles:

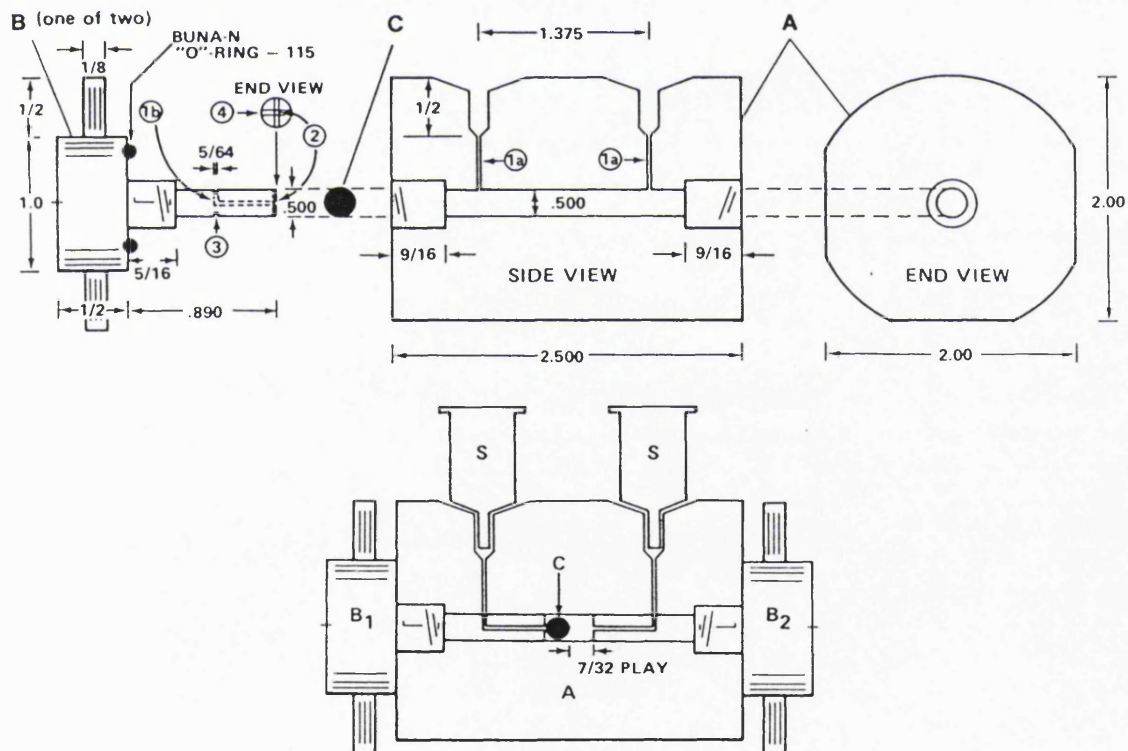
Packed cells (1ml) were incubated at 37°C for one hour with sphingomyelinase (5µl) and Lucifer yellow (20µl) for 20 min. In some experiments, Percoll (50µl) was added to the incubation mixture. Changes in cell morphology were followed by light microscopy. Cells were washed with buffer at 37°C, sedimented, and resuspended in buffer and pushed through a "cell cracker" (Figure 2.5).

Breakage of the erythrocytes was accomplished using a ball-bearing homogenizer similar in design to that originally used by Balch and Rothman (1985) to break vesicular stomatitis virus-infected CHO clone 15B cells.

Figure 2.5 Design of a Ball-Bearing Homogenizer (Cell Cracker)

From Balch and Rothman (1985).

The cell suspension (approximately 5ml) was drawn up into a syringe (marked S) and the apparatus assembled in an ice bucket as shown. This suspension was forced repeatedly back and forth past the stainless-steel ball bearing by alternatively pushing down one of the two syringes. Cell breakage was achieved by performing this operation approximately ten times.



The cell suspension was forced back and forth past the ball bearing at least ten times at 0°C. The resultant suspension was analysed under a microscope in order to ascertain that the cells had been broken.

In the Percoll experiments the suspension was centrifuged on a 20-50% sucrose gradient at 30,000rpm in a Kontron Ultracentrifuge for one hour. In the other experiments, the suspension was filtered through 0.45µm and 0.2µm filters under nitrogen pressure and the filtrates were centrifuged at 40,000rpm for one hour. The resultant pellets were analysed by microscopy and SDS- PAGE.

2.12.6. Treatment with Phospholipase A₂

Washed erythrocytes at 10% haematocrit in Mops buffer (20mM Mops-NAOH/150mM NaCl, pH=7.1) were incubated with phospholipase A₂ (from bee venom, 10µg/ml) plus Ca²⁺ (1mM) at 37°C for 15min.

2.12.7. Treatment with Phorbol Myristate Acetate

Labelled erythrocytes, 10% haematocrit in Hepes buffer (10mM Hepes- NaOH, 150mM NaCl, pH=7.4) were incubated, at 37°C for two hours, with 100nM PMA (added as 0.1mM solution in ethanol). Cells were sedimented and washed with lysis buffer as described previously. SDS-PAGE analysis of the ghosts was used to quantify the increase in protein phosphorylation.

2.12.8. Treatment with cAMP and dbcAMP

Erythrocytes at 20% haematocrit were incubated at 37°C in Hepes buffer (10mM Hepes-NaOH, 150mM NaCl, pH=7.4) plus Mg^{2+} (1mM) and glucose (10mM) with the addition of either cAMP (100 or 500 μ M) or dbcAMP (100 μ M). In most experiments the cells were pre-labelled with ^{32}P in order to measure the extent of protein phosphorylation by SDS-PAGE and radioautography.

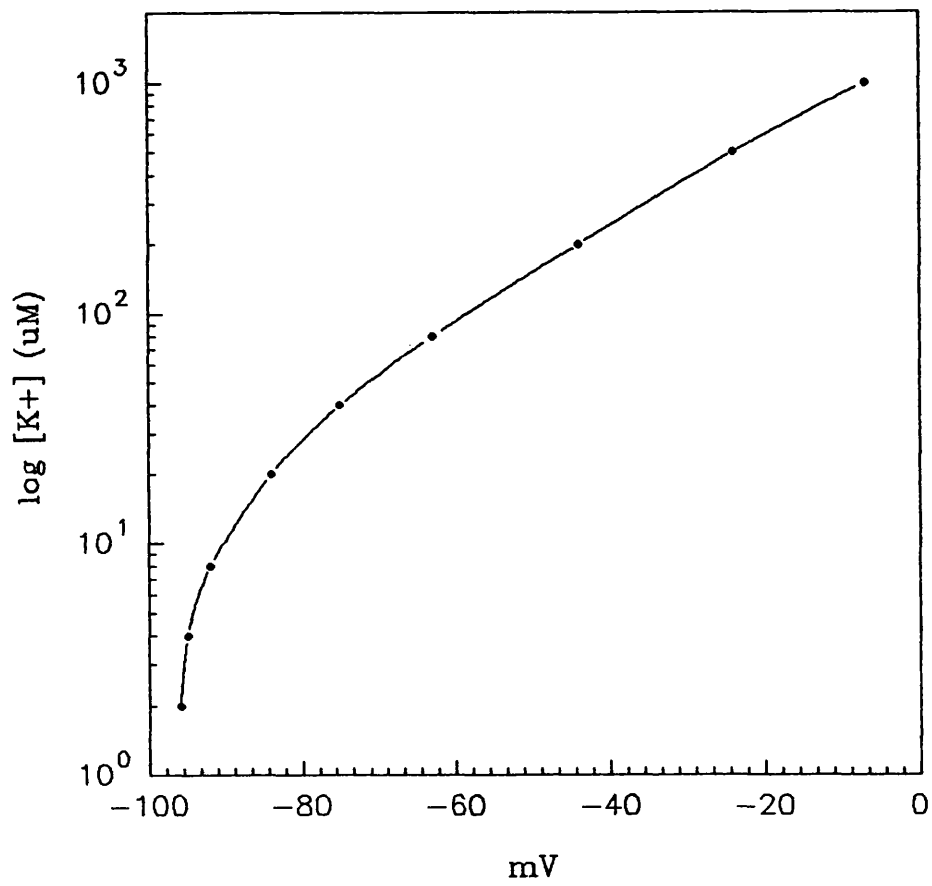
2.13. Measurement of K^+ Channel Activity after Incubation with cAMP

Erythrocytes were incubated for 16 hours at 37°C in Hepes buffer at 20% haematocrit with Mg^{2+} (1mM), glucose (10mM) and gentamycin (2 μ l/ml) with or without cAMP (50 μ M). The next day the cells were washed twice with fresh buffer and resuspended to the same haematocrit. An aliquot of the cell suspension (100 μ l) was added to NaCl (400 μ l) and the Ca^{2+} - dependent K^+ channels were activated by the addition of A23187 (1 μ M) followed by Ca^{2+} (100 μ M).

K^+ efflux from the cells was measured using a potassium electrode. In each experiment measurements were standardised by plotting $\log[K^+]$ against mV for solutions of known K^+ concentration (see Figure 2.6).

Figure 2.6 Measurement of K^+ Efflux

Plot of $\log[K^+]$ against mV.



From this curve subsequent extra cellular K^+ concentrations were calculated.

The total intracellular K^+ concentration was measured by lysing the cells with 0.1% Triton X100.

2.14. Measurement of ATP

ATP content of erythrocytes was analysed by high performance liquid chromatography (HPLC) using a method similar to that described by Anderson & Murphy (1976).

Sample preparation:

Methanol/chloroform (1.9ml, 2:1 v/v) was added to erythrocyte samples at 20% haematocrit (0.5ml). Water (0.5ml) and chloroform (0.5ml) was added to this mixture and the sample was vortexed for one minute prior to centrifugation (1500rpm, 5 minutes). 1ml of the top aqueous layer was taken and dried down in a rotary evaporator (Savant Speed Vac Concentrator) under reduced pressure. The dried sample was subsequently resuspended in 100 μ l Hepes buffer.

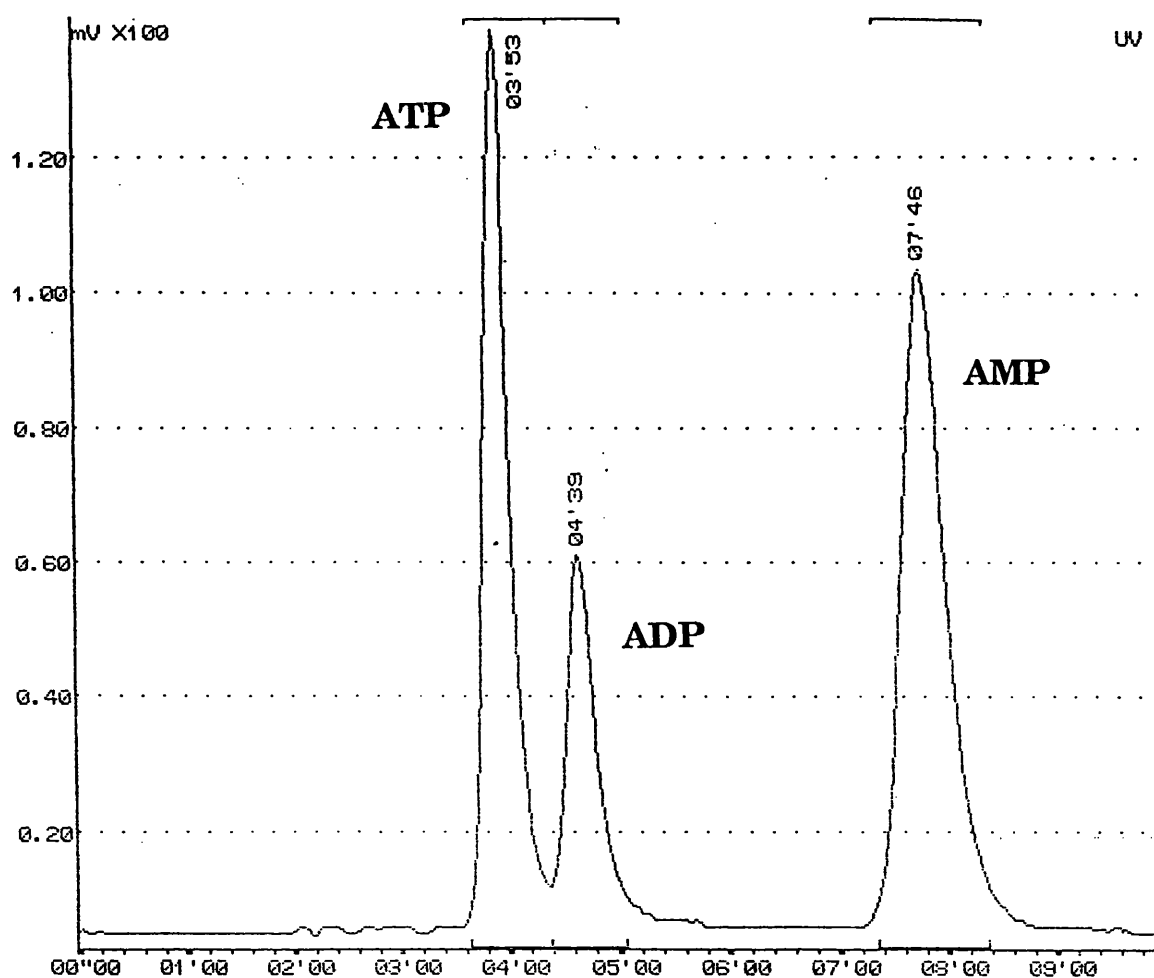
HPLC:

Chromatography was carried out using Waters equipment with a micro C18 reversed phase column. Analysis was done at room temperature with phosphate buffer (0.05M, pH=6.0) as the solvent system at a flow rate of 2ml/min at 430psi. UV absorbing eluents were detected using a UV flow cell

(254nm) connected to the column. 5µl samples were injected into the chromatograph. Figure 2.7 shows the HPLC trace for control samples of AMP, ADP and ATP.

Figure 2.7 Nucleotide Separation by HPLC

Plot of UV absorbance over time, showing ATP, ADP and AMP.



3. SHAPE CHANGES AND VESICLE FORMATION IN HUMAN ERYTHROCYTES

3.1. Merocyanine 540 in the Study of Echinocytosis and Microvesicle Release

3.1.1. Introduction

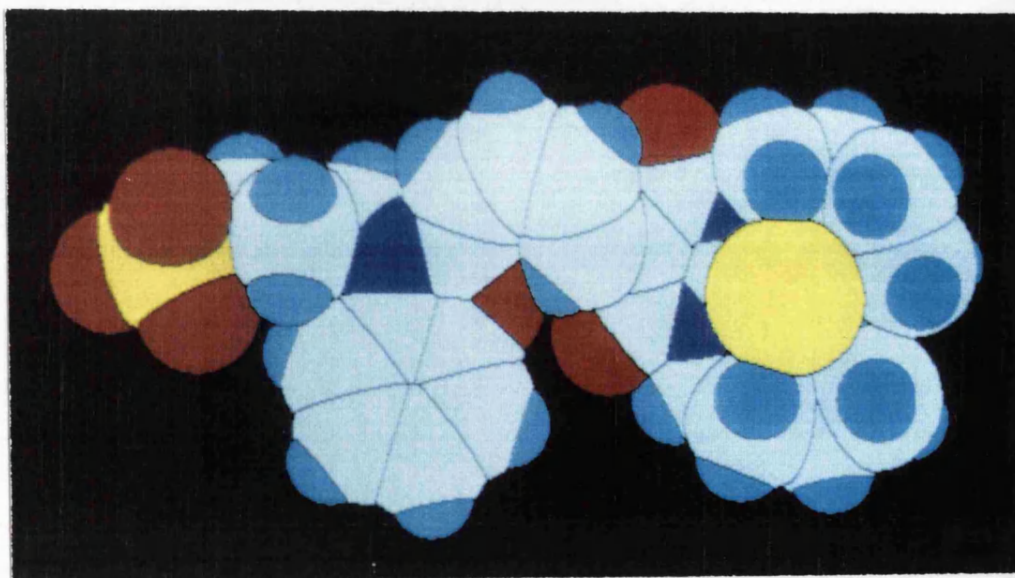
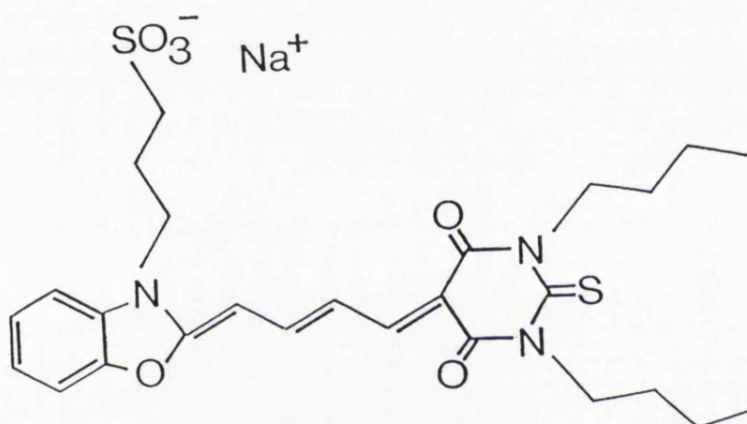
Merocyanine 540 (MC540) is a fluorescent anionic amphipathic molecule (Figure 3.1) which has been used as a probe to monitor structural and functional changes in biological membranes [Lelkes & Miller 1980]. Later studies have used MC540 to detect differences in phospholipid packing domains within lipid vesicles and to sense minor changes in the arrangement of phospholipids in biological membranes [Stillwell *et al* 1993].

The fluorescence spectrum of MC540 has been shown to be dependent on the solvent-dye interactions and particularly on the polarity of the solvent.

MC540 is unusual because its fluorescence maximum is shifted towards the red in less polar media, whereas with most fluorophores it is shifted towards the blue. For example, in experiments where the environment of the dye was altered by adding an aqueous solution of MC540 to an excess of phospholipid or erythrocyte membrane, the emission maximum was shifted from the

Figure 3.1 Structure of Merocyanine 540

Colour photograph taken from a computer display of the molecule created using Desktop Molecular Modeller (Oxford Electronic Publishing). From such molecular models the dimensions of hydrophobic part of the molecule could be measured - this was estimated at $18.5 \times 9 \times 4.5 \text{ \AA}$ [Allan *et al* 1989].



aqueous value of 565nm to 583nm, a value similar to n-decanol [Allan *et al* 1989]. This suggests that the environment experienced by MC540 within a biological membrane has a similar polarity to n-decanol.

Early studies suggested that this fluorescent molecule bound selectively to unsaturated phospholipids (such as phosphatidylethanolamine and phosphatidylserine) and therefore only bound to cells that had lost their original phospholipid asymmetry, that is, only to cells where the inner phospholipids phosphatidylethanolamine and phosphatidylserine had migrated to the outer leaflet [Schlegel *et al* 1980; Williamson *et al* 1983; Verhoven *et al* 1992]. Later studies [Raval & Allan 1984, Allan *et al* 1989] discounted this theory by showing that the binding of dye to lysed cells was twice that to whole cells and therefore that MC540 binds equally well to inner and outer leaflet lipids.

The permanent negative charge on the MC540 molecule (see Figure 3.1) prevents it from crossing the cell membrane and it was proposed that MC540 selectively enters the outer membrane leaflet of whole cells, with the hydrophobic part of the molecule associating with the fatty acid chains of the membrane phospholipids and the hydrophilic part (the sulphonate group), interacting with the aqueous medium [Allan *et al* 1989]. The intercalation of MC540 between the fatty acid chains should result in a relative expansion of the area of the outer lipid leaflet. As proposed by the Sheetz and Singer

bilayer couple hypothesis (see General Introduction), such an expansion of the outer relative to the inner lipid layer explains the formation of echinocytes when erythrocytes are incubated with MC540.

When cells are incubated with higher concentrations of MC540, membrane material is pinched off in the form of vesicles. Examination of the microvesiculation of erythrocytes induced by amphipathic molecules such as MC540 should give new information on the mechanism of microvesicle release and membrane fusion. Unlike the microvesiculation induced by increased intracellular calcium [Allan & Thomas 1981], or ATP-depletion [Lutz *et al* 1977], MC540 treatment is not expected to lead to the degradation of band 4.1 or the polyphosphoinositides. It was thought that it might be possible to confirm that modifications in the cytoskeleton or lipid breakdown are not really necessary for microvesicle release to occur.

In this section, the morphological changes that occur when erythrocytes are treated with MC540 have been related to changes in MC540 binding, as well as to the time course, dose response and temperature dependence of microvesicle release. Possible alterations in the phospholipid asymmetry in MC540-treated cells and the resultant microvesicles were also investigated, in order to determine whether changes in lipid asymmetry accompany amphipath-induced microvesicle release. This is a possibility, as it has been suggested by others that factors affecting the lipid bilayer such as incubation

with amphiphilic drugs [Schneider *et al* 1986] or sickling of sickle cells [Franck *et al* 1985] may alter the lipid asymmetry. On the other hand, studies by Raval & Allan (1984) demonstrated that Ca^{2+} /ionophore-treated cells and the derived microvesicles retained their lipid asymmetry.

Microvesiculation produced by this method may not require a phospholipid 'flip-flop' since it occurs in parallel with a breakdown in cytoskeletal protein and a formation of diacylglycerol, a possible modulator of fusogenic events [Allan & Michell 1979].

The effect of another amphipath, dimyristoyl phosphatidylcholine (DMPC) on erythrocytes has been studied previously in great detail [Ott *et al* 1981, Bütikofer P & Ott P 1985; Bütikofer *et al* 1987; Weitz *et al* 1982]. In considering the possible mechanism for the release of microvesicles from DMPC-treated erythrocytes, it was postulated that the slow movement (flip-flop) of DMPC to the cytoplasmic side of the bilayer induces the lateral phase separation of phosphatidylserine and phosphatidylethanolamine, this separation being a proposed prerequisite for membrane fusion [Ott *et al* 1981]. Here we compare MC540-induced to DMPC-induced microvesicle release, in order to examine whether MC540 behaves in a similar way to DMPC, and to answer the question of whether a similar mechanism can be responsible for the formation of microvesicles for both these amphipaths.

3.1.2. Treatment of Erythrocytes with MC540

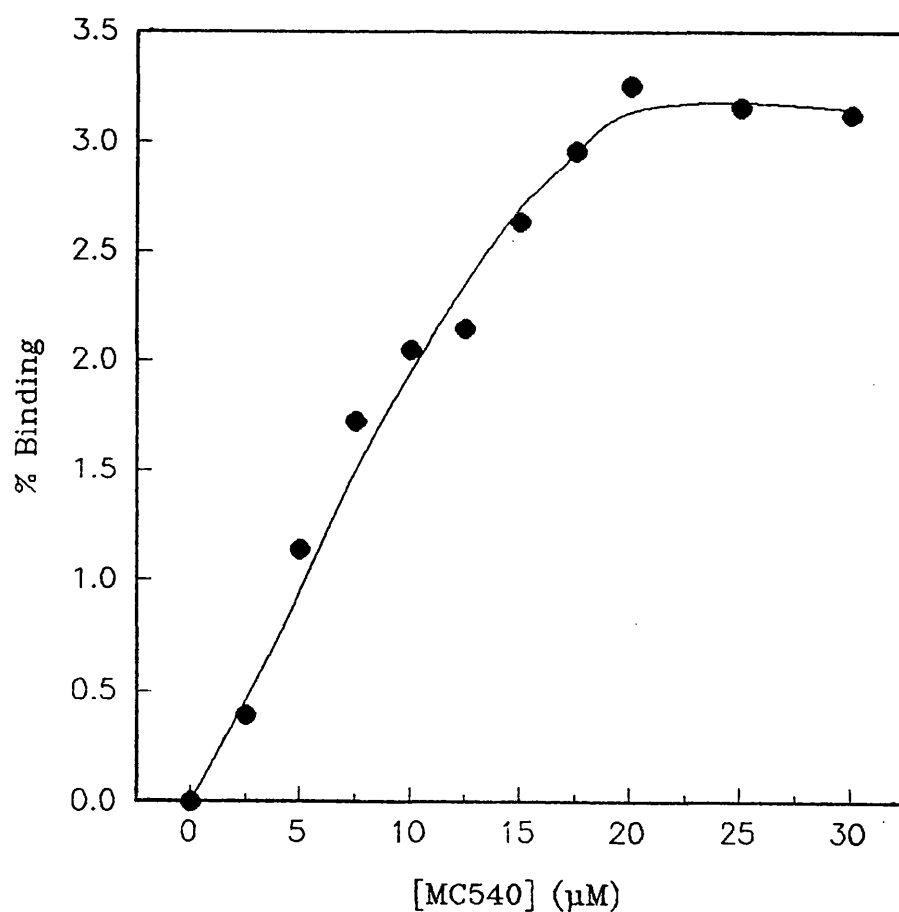
When erythrocytes were treated with increasing concentrations of MC540, a transformation of cell morphology took place from discocyte through echinocyte to spherocyte. The final stages of these shape changes were associated with the release of microvesicles from the cell membrane. The extent of microvesicle release was quantified by measuring the acetylcholinesterase (AChE) activity and amount of phospholipid that remained in the supernatant after the sedimentation of the MC540-treated erythrocytes. The released material was isolated from the supernatant after centrifugation at 40,000rpm for one hour.

3.1.3. Binding of MC540 to Erythrocytes

Binding of MC540 to erythrocytes at 37°C increased with concentration but appeared to saturate at approximately 20µM (Figure 3.2). By quantifying the fluorescence emission, the number of molecules of MC540 in the outer leaflet of the membrane could be calculated. The maximum amount of MC540 bound to cells at 37°C was about 3.2 moles dye per 100 moles of phospholipid. From molecular models (Figure 3.1) the cross-sectional area of the MC540 molecule was estimated to be 0.4nm^2 (40\AA^2) [Allan *et al* 1989].

Figure 3.2 Binding of MC540 to Erythrocytes

Cells (at 2% haematocrit) were incubated at 37°C with varying concentrations of MC540 for 10 minutes. The erythrocytes were sedimented and binding of dye was measured by resuspending an aliquot of cells in butanol and measuring the fluorescence as described under Methods. The amount of phospholipid in the cells was measured by phosphate analysis. % binding equals number of moles of MC540 bound per 100 moles of phospholipid.



A reasonable assumption is that MC540 partitions into the outer lipid layer with the negatively charged headgroup towards the outside of the membrane and the rest of the molecule sitting parallel to the phospholipid chains. An approximate calculation of the outer leaflet expansion can be made when the binding of MC540 is maximal, assuming that an erythrocyte has a surface area of $138\mu\text{m}^2$ [Daleke & Huestis 1989] and contains 4.3×10^{-16} moles of phospholipids (2.6×10^8 molecules) [Nelson 1972]. The binding of 3.2 moles of dye to 100 moles of phospholipid is equivalent to the binding of 8.3×10^6 molecules of MC540 per cell and leads to an expansion of the surface area by $3.3\mu\text{m}^2$ or 2.4%.

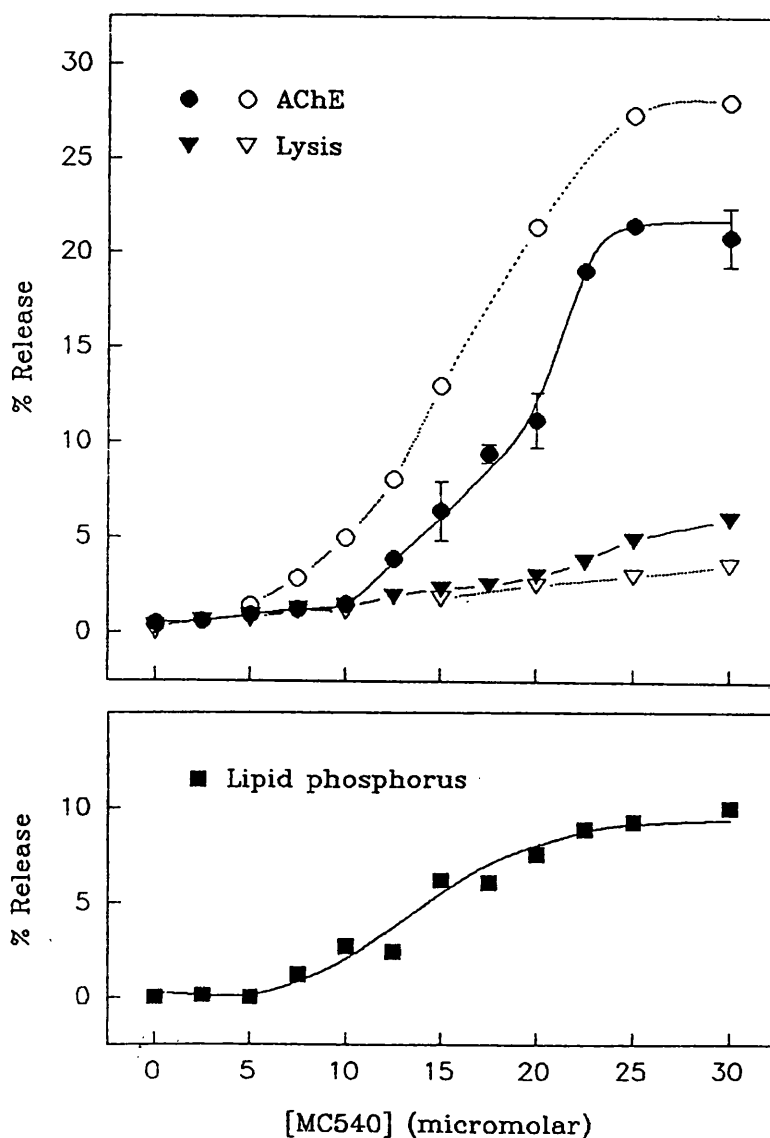
3.1.4. Microvesicle Release in Erythrocytes Treated with MC540: Dose Dependence, Time Course and Temperature Dependence

22.5-25 μM MC540, the concentration range for maximum MC540 binding, was also the concentration range over which maximum phospholipid and AChE release occurred (Figure 3.3). Half-maximal phospholipid and AChE release occurred in the 15-17.5 μM range.

The amount of AChE release was higher and lysis was minimised with a short incubation time.

Figure 3.3 Dose Dependence of Microvesicle Release from MC540-Treated Erythrocytes

Cells (at 2% haematocrit) were incubated at 37°C with varying concentrations of MC540. Incubations were terminated after 2 minutes (open symbols) or 10 minutes (closed symbols). The cells were sedimented and the supernatant was assayed for AChE activity, phospholipid release and amount of lysis. Figures for % AChE and phospholipid release were corrected for lysis. 100% values were calculated from aliquots of the total incubation mixture at the beginning of the experiment (average of five samples). Results for AChE release after 10 minutes are the means \pm S.E. from four experiments. Other figures are from two experiments carried out with duplicate samples.



The time-course showed that maximum release of AChE occurred after approximately two minutes and tended to taper off with time (Figure 3.4).

The extent of lysis rose with concentration of MC540 and with time but in all cases was below 5% (Figures 3.3 and 3.4).

Experiments on the temperature dependence of microvesicle formation demonstrated that there was little microvesicle release below 10°C and maximum release between 35°C and 40°C (Figure 3.5). Above 40°C there was a decrease in both the amount of AChE and phospholipid released and at these higher temperatures the extent of lysis was elevated.

3.1.5. Protein Composition of Microvesicles from MC540-Treated Erythrocytes

SDS-PAGE analysis of the microvesicles from MC540- treated cells showed that these microvesicles contained mainly band 3 and glycophorin in addition to cytosolic proteins. In some cases, small amounts of spectrin and band 4.1 could be detected, but the relative amounts were very small and varied between experiments, suggesting that this was due to variable ghost contamination in the samples.

Figure 3.4 Time Course of Microvesicle Release from MC540-Treated Erythrocytes

Cells (at 2% haematocrit) were incubated with MC540 (20 μ M) at 37°C for up to 10 minutes. Aliquots were taken at different times, the incubation was terminated by addition of ice-cold buffer and the cells were sedimented. AChE activity in the supernatant and extent of lysis were measured as described previously. Shown are the results from a representative experiment.

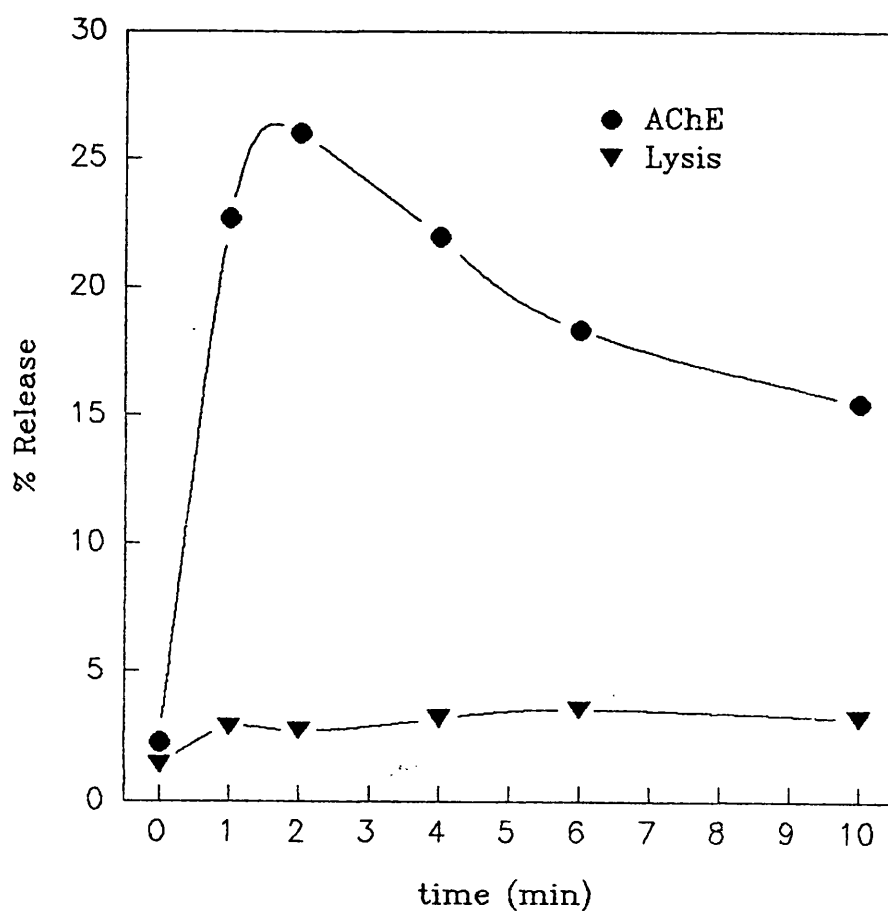
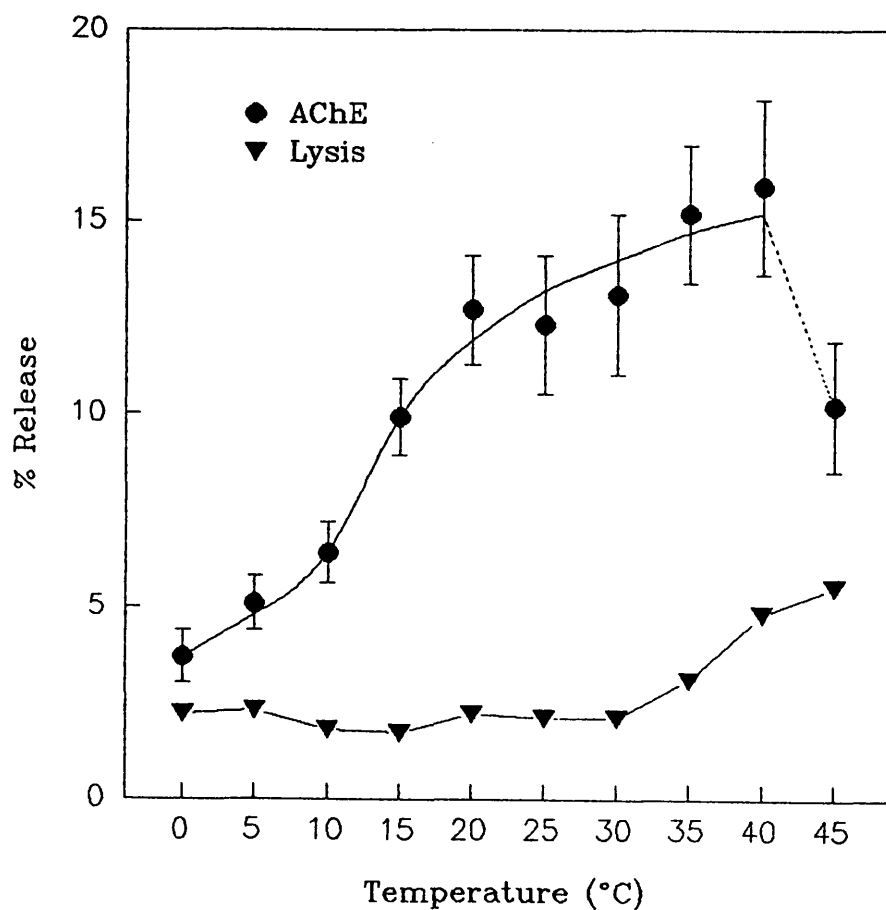


Figure 3.5 Temperature Dependence of Microvesicle Release from Erythrocytes Treated with MC540

Erythrocytes (at 2% haematocrit) were incubated with MC540 (25 μ M) for 2 minutes at various incubation temperatures. % AChE release and % lysis were measured as described previously. Figures shown are the means of five experiments \pm standard error.



Since the microvesicles only contained those membrane proteins associated with the lipid bilayer and little (if any) of the membrane skeletal proteins, it appeared that the skeletal proteins were not free to diffuse into the vesicles.

From densitometric scans of the gels (Figure 3.6), the relative amounts of each protein in the treated cells and microvesicles could be quantified. The results on MC540 microvesicles showed that the ratio of band 3 to phospholipid in the microvesicles was less than 50% of that in ghosts.

Similarly, the major glycoprotein, glycophorin A (the only glycoprotein that could be easily measured) was depleted in the microvesicles to the same extent.

However, AChE and phospholipid analysis showed that the specific activity of AChE relative to phospholipid in the microvesicles is 2-3 times higher than in the original cells (Table 3.1), confirming the results obtained by others on microvesicles derived by various treatments [Billington & Coleman 1978; Ott *et al* 1981; Allan & Thomas 1981; Wagner 1987]. There was no change in the total activity of this enzyme. As expected, the residual cells showed decreased activity, equivalent to the activity found in the microvesicles, and the total activity (microvesicles plus treated cells) added up to 100%. To ascertain whether MC540 has an effect on the activity of AChE, treated and untreated cells were compared, and no difference was found.

Figure 3.6 Densitometric Scans of Polypeptide Pattern Obtained by SDS-PAGE of Erythrocyte Membranes and Microvesicles from MC540-Treated Cells

Erythrocytes were incubated with MC540 as described previously. The polypeptide composition of a) cytosol; b) membranes and c) microvesicles were analyzed by SDS-PAGE (10% w/w gel). Bands were visualised using Coomassie stain (shown here) or Schiff's reagent, and scanned as described under Methods.

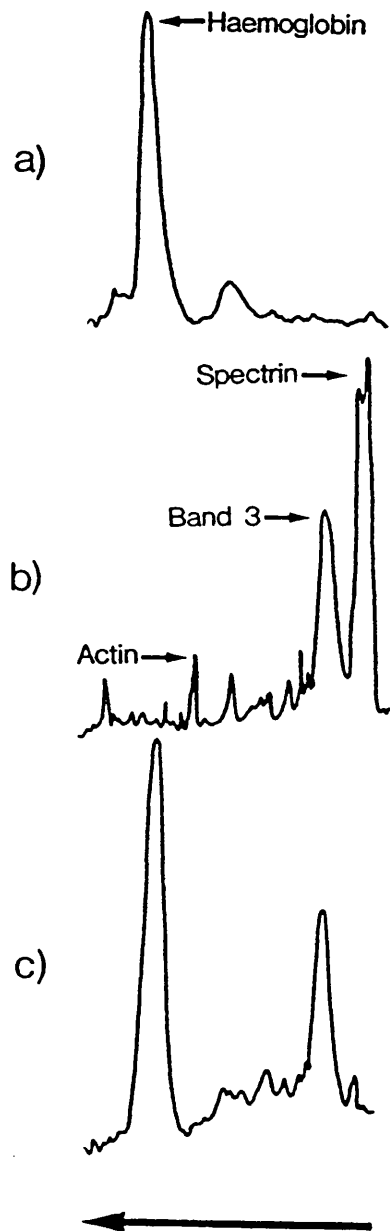


Table 3.1 AChE Activity in Control Cells, MC540- Treated Cells and Microvesicles

Erythrocytes (at 2% haematocrit) were incubated with MC540 for 2 minutes. The cells were sedimented and the supernatant, containing the microvesicles was separated from the treated cells. The activity of AChE and the phosphate (P_i) content was measured in samples containing: 1) untreated erythrocytes (control), 2) MC540-treated cells and 3) microvesicles, as described under Methods. Shown is the specific enzyme activity relative to phospholipid (with the result for control cells and the total enzyme activity set at 100%). Results are for ten samples, expressed as means \pm S.D. from three experiments (two dose response experiments and one temperature dependence experiment) using different samples of cells. Variations in the concentration of MC540, or temperature, had no effect in on AChE/ P_i ratio.

	Control Cells	MC540 Cells	Micro- vesicles
Specific AChE Activity	100	89 ± 3	240 ± 25
Total AChE Activity	100	89	10

A strong correlation between AChE release and phospholipid release was found for microvesicles formed by treating erythrocytes with MC540 (Figure 3.7). The same result was obtained for microvesicles obtained by various methods [Ott *et al* 1981; Wagner *et al* 1987]. AChE release, which was quick and simple to analyse, was therefore taken as a quantitative measure of microvesicle release in most experiments.

3.1.6. Polyphosphoinositide Phosphodiesterase Activity in Microvesicles and MC540-Treated Erythrocytes

When intact erythrocytes were incubated with A23187 and millimolar Ca^{2+} , or erythrocyte ghosts were incubated with millimolar Ca^{2+} , approximately 50% of the polyphosphoinositides were hydrolysed. These results are in agreement with those of Allan & Michell (1978), which showed that an increase in the calcium concentration leads to the activation of the polyphosphoinositide phosphodiesterase (PPI-PDE) and the breakdown of the polyphosphoinositides.

Addition of MC540 to ghosts, prior to incubation with Ca^{2+} /A23187, had no effect on this enzyme activity. Moreover, when the microvesicles from the MC540-treated cells were incubated with A23187 and Ca^{2+} , the polyphosphoinositide content remained the same, i.e. there was no evidence for PPI-PDE activity in the microvesicles (Figure 3.8).

Figure 3.7 Correlation Between Percentage AChE Release and Phospholipid Release in Microvesicles from MC540-Treated Erythrocytes

Erythrocytes (at 2% haematocrit) were incubated with 0-30 μ M MC540 for 2 minutes at 37°C as described under Methods. The cells were sedimented and AChE activity and amount of phospholipid were measured in the supernatant. 100% activity, phospholipid release or lysis were taken from the total incubation mixture at the beginning of the experiment. % AChE activity and % phospholipid release were corrected for % lysis which was never above 6%. Results are from a representative experiment.

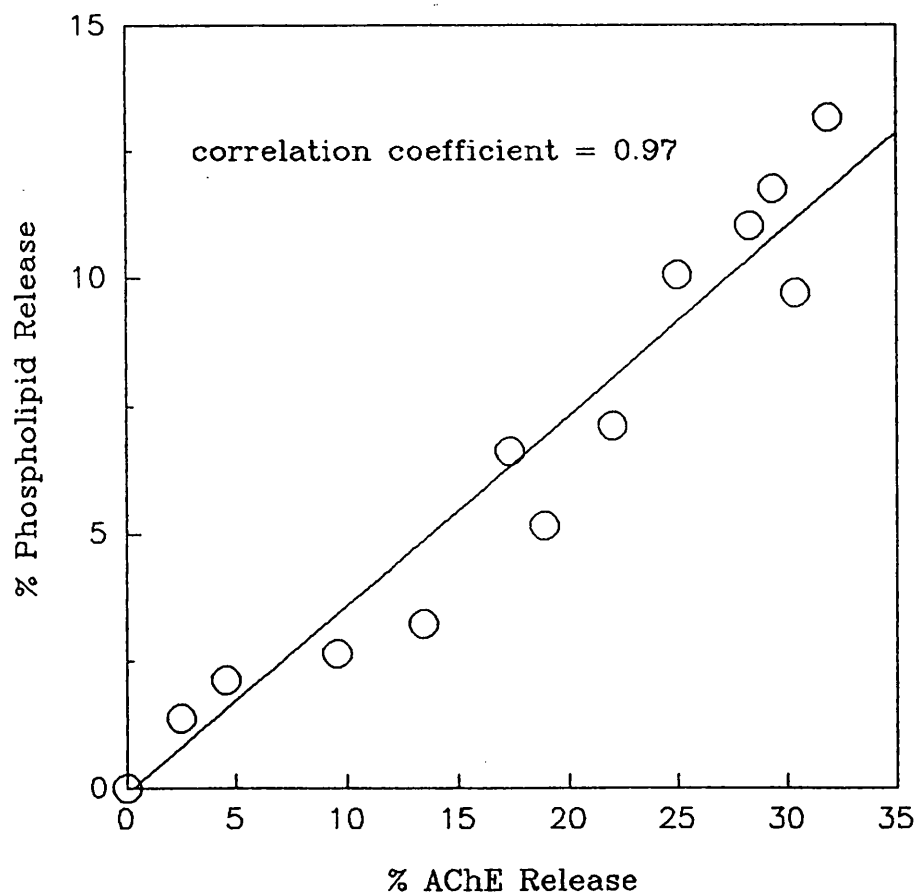
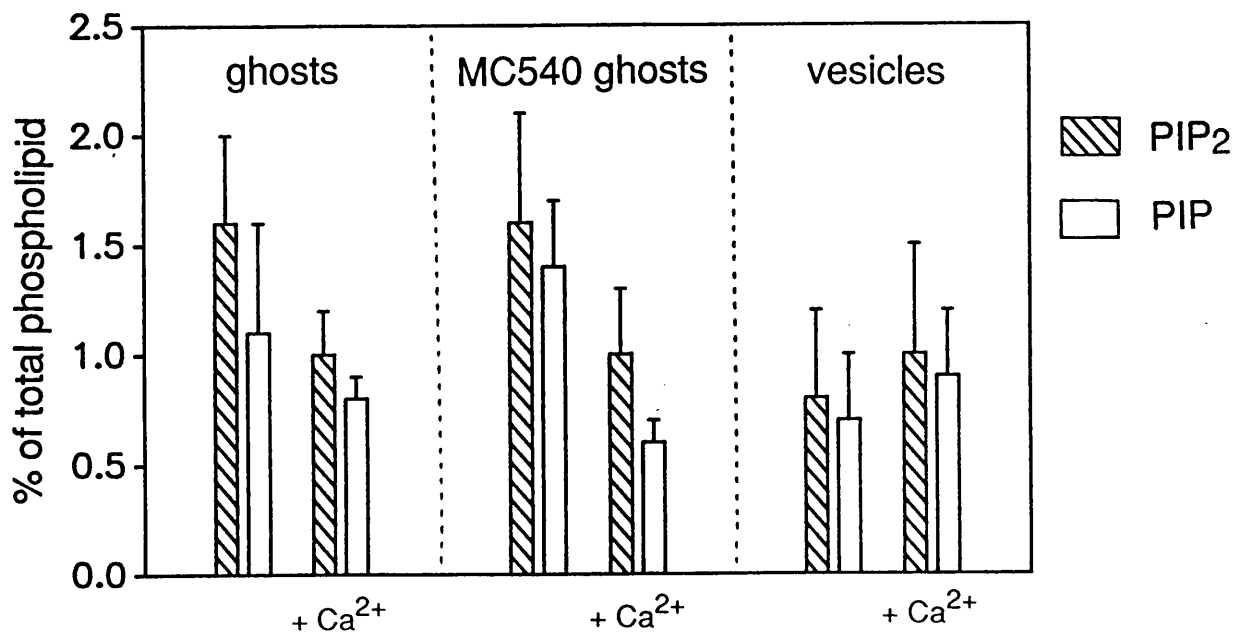


Figure 3.8 Effect of Ca^{2+} Treatment on Levels of Polyphosphoinositides in Normal Erythrocyte Membranes, Membranes from MC540-Treated Cells and Microvesicles

Cells were labelled with ^{32}P for 16 hours (15-25 $\mu\text{Ci/ml}$ cells) and incubated at 5% haematocrit with MC540 (62.5 μM) at 37°C for 2 minutes. Microvesicles and ghosts were isolated as described under Methods. The effect of polyphosphoinositide phosphodiesterase activity was observed by incubating ghosts from normal erythrocytes or ghosts from MC540-treated cells, and microvesicles with Ca^{2+} (1mM) and A23187 (5mM) for 20 minutes at 37°C before lipid extraction. Phospholipids were separated by TLC and spots were scraped, assayed for phosphorus and radioactive spots were assayed for radioactivity. The amount of polyphosphoinositides was calculated from the counts as described under Methods (see Figure 2.3) and, for some samples the amount of PIP_2 was also measured by phosphorus analysis to confirm that there was no difference in specific activity in the ghosts or microvesicles. Results are the means \pm S.D. of four experiments done in duplicate.



Since the ghosts from MC540-treated cells showed a normal reduction in the amount of polyphosphoinositides after incubation with calcium, the apparent lack of enzyme activity in the microvesicles was not due to the inhibition by MC540.

3.1.7. Phospholipid Composition of Microvesicles from MC540-Treated Erythrocytes

The phospholipid composition of the microvesicles, the parent erythrocytes and the MC540-treated erythrocytes was very similar, with little variation in the composition of the major lipids (Table 3.2). This result was as expected, since the major phospholipids are thought to be generally mobile within the plane of the membrane and would be expected to pass easily into any buds formed.

The amount of phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 4-phosphate (PIP) in the ghost samples was found to be approximately 1.6% and 1.2% of total phospholipid respectively. This compares well with figures given by other workers of approximately 1.5% and 0.9% respectively [Ferrell & Huestis 1984; Mitchell 1986; Christensen 1986].

The microvesicle polyphosphoinositide content was reduced to approximately 0.8% for PIP₂ and PIP, but the level of phosphatidylinositol (PI) remained constant at 1%.

Table 3.2 Phospholipid Composition of Untreated Erythrocyte Ghosts, Ghosts from MC540- Treated Cells and Microvesicles.

Phospholipids were extracted in chloroform/methanol and analysed by TLC as described under Methods. Spots were scraped from the plates and assayed for phosphorus. Results represent mole per cent of total phospholipid, shown as means \pm S.D. from four experiments, each carried out with duplicate samples.

PE phosphatidylethanolamine
 PS phosphatidylserine
 PC phosphatidylcholine
 SM sphingomyelin
 PI phosphatidylinositol
 PIP phosphatidylinositol 4-phosphate
 PIP₂ phosphatidylinositol 4,5-bisphosphate

Lipid	Control ghosts	MC540 ghosts	Vesicles
PE	29.9 \pm 0.3	28.8 \pm 0.9	28.8 \pm 1.9
PS	15.6 \pm 1.9	14.0 \pm 0.5	12.9 \pm 2.0
PC	28.4 \pm 0.7	29.2 \pm 1.3	30.2 \pm 3.3
SM	23.2 \pm 1.4	25.0 \pm 1.8	23.8 \pm 3.4
PI	1.0 \pm 0.3	1.1 \pm 0.3	1.0 \pm 0.1
PIP	1.2 \pm 0.4	1.4 \pm 0.3	0.8 \pm 0.3
PIP₂	1.6 \pm 0.4	1.6 \pm 0.3	0.8 \pm 0.4

Thus the relative reduction in the proportion of the polyphosphoinositides in the microvesicles was not due to the dephosphorylation of PIP₂ or PIP.

This analysis of the polyphosphoinositide composition indicated that the microvesicles were depleted of the polyphosphoinositides by more than 50% relative to other phospholipids. This suggests that only about half of these lipids are free to diffuse into the microvesicles. In other words, the polyphosphoinositides are not free to diffuse evenly within the membrane.

When cells were incubated overnight with ³²P, the labelling of PIP₂ approached equilibrium with added radioactive label. The specific activity of the two labelled phosphates in PIP₂ was over 80% that of the ATP β, γ phosphates. This agrees with the results of others showing that the labelling of the polyphosphoinositides reached 78-85% that of ATP after a 23 hour incubation [Gascard *et al* 1989].

3.1.8. Phospholipid Asymmetry in MC540-Treated Cells and Microvesicles

Previous studies demonstrated that when whole cells are incubated with phospholipase A₂ under conditions where lysis was negligible, more than half of the phosphatidyl-choline (PC), but little phosphatidylethanolamine (PE) or phosphatidylserine (PS) was broken down. In the case of ghosts more than 80% of the PC and PE and 60% of the PS was broken down [Zwaal *et al* 1975;

Raval & Allan 1984]. Since only the lipids that are accessible to the phospholipase are broken down, these results were used to demonstrate the phospholipid asymmetry of the plasma membrane. The asymmetry of sphingomyelin (SM) distribution was shown by incubating cells and ghosts with sphingomyelinase. In both cases most of this lipid is degraded, leading to the conclusion that SM is found predominantly in the outer leaflet [Allan & Raval 1987].

The experiments here agree with these previous results. When control cells or MC540-treated cells were incubated with phospholipase A₂, about half of the PC was broken down to lyso-PC, but little change was seen in the content of PE or PS of the cells. No variation of this result was seen as the concentration of MC540 was raised (Figure 3.9). When the cells were treated with sphingomyelinase, about 80% of sphingomyelin was broken down (Figure 3.10). In the same experiments, ghosts from MC540-treated cells were incubated either with phospholipase A₂ or sphingomyelinase. About 90% of the PE and PC and SM were degraded by these enzymes. The original asymmetry in the cells does not appear to be altered by MC540 and this dye does not affect the activity of phospholipase A₂ or sphingomyelinase.

Figure 3.9 Degradation of Phospholipids in Control and MC540-Treated Cells and Microvesicles

Erythrocytes at 10% haematocrit in MOPS- saline were incubated for 15min at 37°C in the presence of phospholipase A₂ (10µg/ml) and CaCl₂ (1mM) with or without addition of MC540. A fraction of the incubation mixture was taken for measurement of lysis and the rest extracted with methanol/chloroform for TLC analysis. Lysis was between 1-8%, rising with increasing MC540 concentration. The amount of each lipid (PE, PC and SM) remaining was calculated as a percentage of original total lipid. The results are from a representative experiment (similar results were obtained in two further experiments under the same conditions).

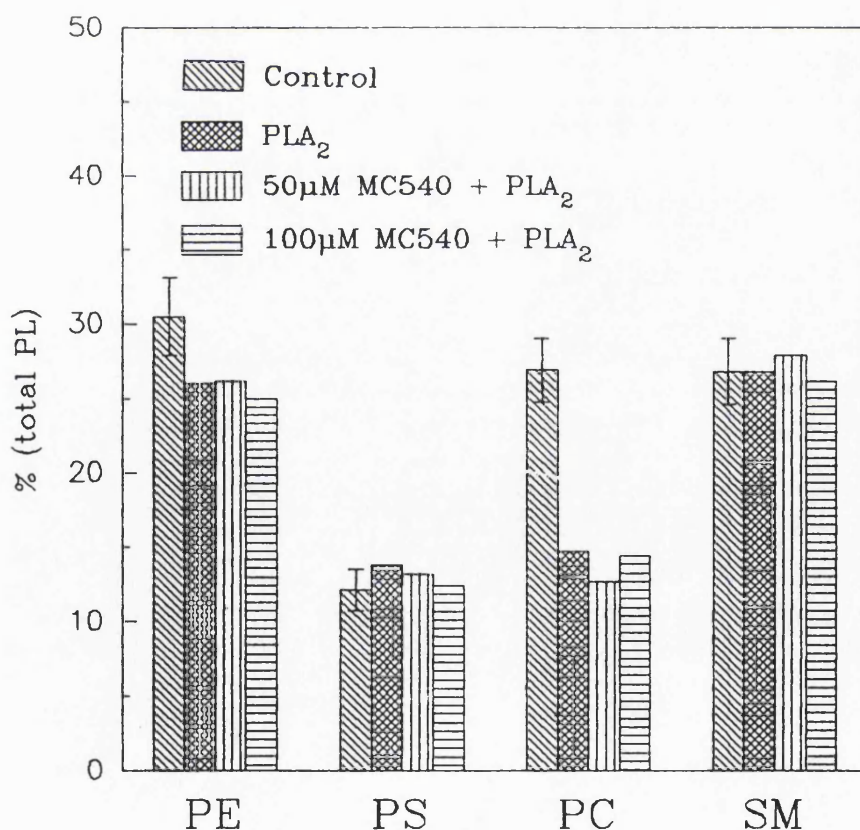
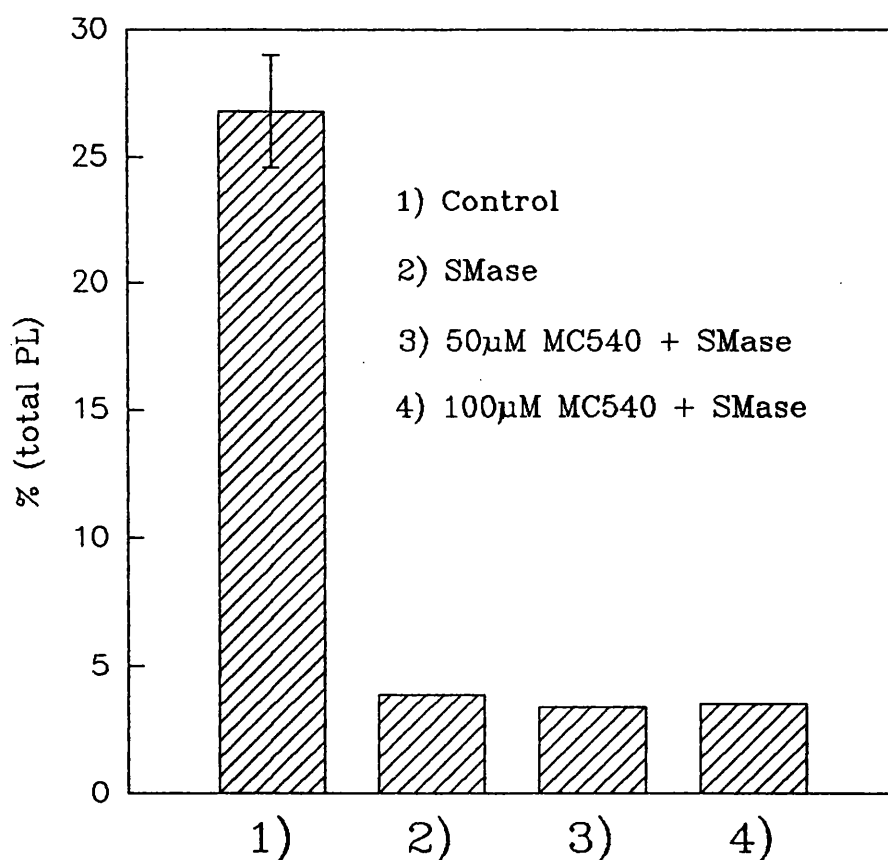


Figure 3.10 Degradation of Sphingomyelin in Control Erythrocytes and MC540-Treated Erythrocytes by Sphingomyelinase

Erythrocytes at 10% haematocrit in MOPS- saline were incubated for 15min at 37°C in the presence of sphingomyelinase (10µg/ml) with or without MC540. A fraction of the incubation mixture was taken for measurement of lysis and the rest extracted with methanol/chloroform for TLC analysis. Lysis was between 1-8%, rising with increasing MC540 concentration. The amount of sphingomyelin remaining was calculated as a percentage of original total lipid. The results are from a representative experiment (similar results were obtained in two further experiments under the same conditions)



3.2. Segregation of Proteins and Lipids into Microvesicles from Human Erythrocytes

3.2.1. Introduction

Microvesicles can be obtained *in vitro* from human erythrocytes under a variety of different conditions. These include treatment with MC540, or other amphipaths such as dimyristoylphosphatidylcholine (DMPC) [Ott *et al* 1981], calcium loading [Allan *et al* 1976], ATP- depletion [Lutz *et al* 1977] or storage for several weeks [Greenwalt & Dumaswala 1988].

Microvesicles obtained by these methods were closely analysed and were found to contain only a certain proportion of the integral membrane proteins but none of the skeletal proteins. This is as expected, since macromolecular complexes cannot diffuse freely, so that the cytoskeleton stays behind during the vesiculation process [Ott *et al* 1981, Allan & Thomas 1981; Wagner 1987].

It can reasonably be assumed that microvesicles contain a sample of the freely-diffusing components of the original cell, including the major phospholipids and some of the membrane proteins. In other words, only those lipids and proteins which are not involved in macromolecular interactions with the cytoskeleton are free to diffuse and are found in the microvesicles.

A quantitative measure of the lipid and protein composition of microvesicles may provide valuable information about the lipid and protein associations in the original cell, and can give a value for the proportion of any component which is attached to the skeleton. Protein-protein and protein-lipid interactions may change when erythrocytes are subjected to certain treatments, and these changes may be reflected in the proportion of different proteins and lipids that are found in the microvesicles.

As shown in the previous section, microvesicles from MC540-treated cells were depleted of those proteins (band 3 and glycophorin) and lipids (polyphosphoinositides) that had been shown by others to be associated with the cytoskeleton [Tsuji *et al* 1988; Nigg *et al* 1980; Anderson & Marchesi 1985]. The following experiments aimed to discover whether the composition of microvesicles from cells subjected to various treatments is altered, and if so, whether this is a reflection of the changes in the association between the cytoskeleton and the lipid bilayer brought about by these treatments.

Incubation of erythrocytes with DMPC causes microvesiculation in a similar manner to MC540. With both of these amphipathic molecules, it is the increase in the area of the outer lipid leaflet rather than changes in the cytoskeleton, that is believed to cause the echinocytosis and microvesiculation. The mechanism for microvesicle release in each case may be

very similar and therefore it is to be expected that the lipid and protein composition of microvesicles obtained by the two methods would be alike.

On the other hand, incubation of erythrocytes with Ca^{2+} /ionophore, ATP-depletion or storage are likely to produce changes in the phosphorylation of the cytoskeletal proteins and the breakdown of the polyphosphoinositides, as well as inducing some specific proteolytic changes. The composition of the microvesicles could potentially reflect alterations in protein-protein and protein-lipid interactions associated with these changes.

Incubation of erythrocytes with Ca^{2+} and the ionophore A23187 leads to cell shrinkage and to the breakdown of the cytoskeletal proteins band 4.1 and ankyrin [Allan & Thomas 1981], possibly leading to the uncoupling of the cytoskeleton from the bilayer. Such changes in the cytoskeleton may affect the interaction of band 3 and glycophorin with the cytoskeleton and an increase in these proteins in the microvesicles would confirm this.

Ca^{2+} -ionophore treatment also activates the phosphoinositidase C [Allan & Michell 1978] leading to the hydrolysis of approximately 50% of the polyphosphoinositides [Downes & Michell 1981]. Previous studies on labelled cells have shown that this enzyme does not distinguish between metabolically active and inactive pools of polyphosphoinositides [King *et al* 1987]. One explanation for this may be that Ca^{2+} causes the dissociation of these lipids from the cytoskeleton. If this is the case, all the polyphosphoinositides may be

free to diffuse within the bilayer and one would expect the microvesicles from Ca^{2+} -treated cells to contain the same proportion of polyphosphoinositides as the residual cells.

ATP-depletion results in the dephosphorylation of cytoskeletal proteins [Brenner & Korn 1979] and the polyphosphoinositides [Ferrell & Huestis 1982]. Dephosphorylation of the cytoskeletal proteins may alter the interaction between the membrane components prior to microvesicle release and therefore change the composition of the microvesicles. In ATP-depleted cells about 50% of the polyphosphoinositides are converted to phosphatidylinositol. By quantifying the content of these lipids in the microvesicles and assuming that the microvesicles will only contain lipid that is free to diffuse in the bilayer (i.e. unbound), it may be possible to estimate the amount of bound and unbound lipid present in the cells after ATP-depletion.

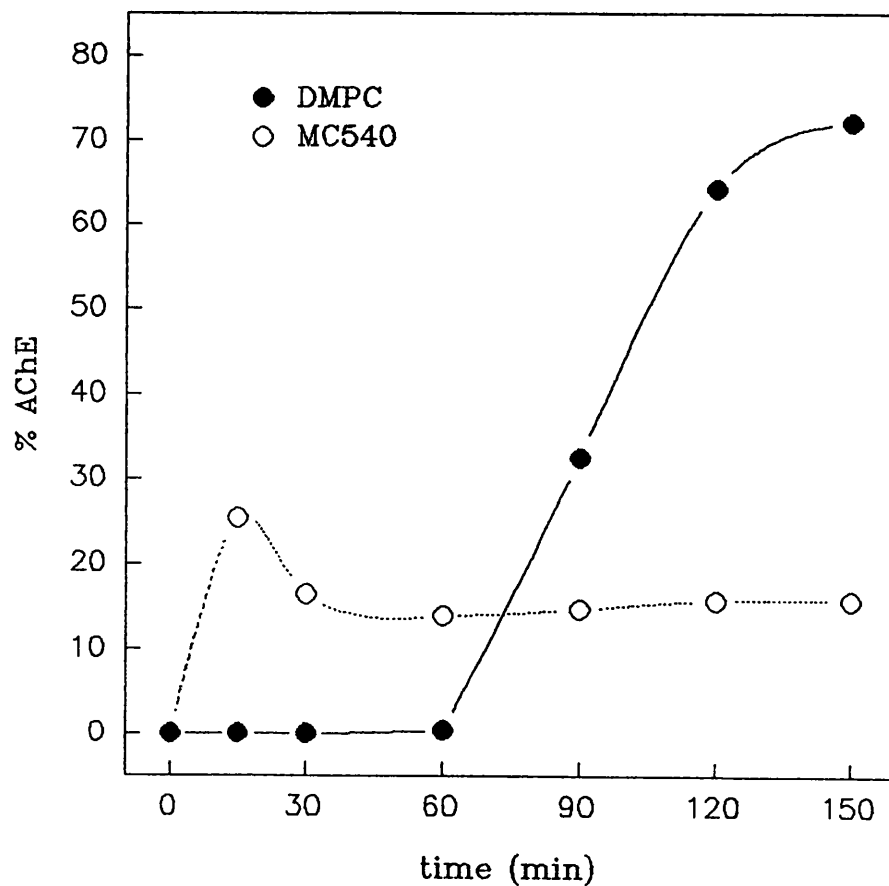
Changes during storage may also involve the breakdown of membrane proteins and of the polyphosphoinositides [Dumaswala *et al* 1986]. Again, the analysis of the microvesicles should provide further information about the membrane interactions in aged cells.

3.2.2. Microvesicle Release from Human Erythrocytes after DMPC Treatment, Calcium/Ionophore Treatment, ATP Depletion and Storage

DMPC treatment: Confirming the results of Ott *et al* (1981) and Bütikofer and Ott (1985), incubation of erythrocytes with DMPC in a glucose-free medium showed a relatively long lag period of about 60 minutes before any microvesicles were released. In the experiments presented here, maximum release of microvesicles was after 150 minutes, when 75% of the total AChE activity could be detected in the supernatant after cell sedimentation, the extent of lysis was however still below 6%. This release of AChE after DMPC treatment contrasts with MC540 where the effect was rapid and microvesicles were released within minutes (Figure 3.11). The amount of AChE (and phospholipid) released after DMPC treatment was two to three fold higher than with MC540 treatment. Over 25% of the phospholipid was released in the form of vesicles after DMPC treatment. It is difficult to conceive such a loss in membrane lipid without cell lysis, but this is explained by the results of Ott *et al* (1981). Both the treated cells and the resulting microvesicles contain over 15% more phosphatidylcholine than the control cells (accounted for by associated DMPC), therefore the apparent 25% of phospholipid release in reality only signifies an approximate 10% release of the original cell lipid.

Figure 3.11 Time Course of Microvesicle Release from Erythrocytes Treated with MC540 or DMPC

Cells were incubated with MC540 (20 μ M) or DMPC (0.5mg/ml buffer, ~1mM) at 37°C. Aliquots were taken at different times, and the incubation was terminated by addition of ice-cold buffer. The cells were sedimented and extent of lysis and AChE activity in the supernatant was measured.



As shown previously, both with MC540 (Section 3.2.3) and DMPC [Ott *et al* 1981] the ratio of AChE relative to lipid was 2-3 times higher in the microvesicles relative to the cells, so that in both cases the microvesicles were enriched in AChE.

Calcium ionophore treatment: Incubation of human erythrocytes with the ionophore A23187 (5mM) and increasing concentrations of Ca^{2+} , at 37°C for 20 minutes, confirmed earlier studies showing that maximal microvesicle release occurs at a Ca^{2+} concentration of 1mM, and declines at higher concentrations of Ca^{2+} [Allan *et al* 1982; Allan & Thomas 1981].

Approximately 15% of the AChE and 5-6% of the phospholipid was released in the microvesicles.

ATP depletion: Incubation of erythrocytes for 24 hours with 5mM iodoacetamide in the absence of glucose at 37°C results in ATP depletion [Lutz *et al* 1977] and the release of approximately 20% of the total AChE into the medium in microvesicles.

Ageing in vitro: Keeping whole blood under blood bank conditions at 4°C for 10 to 15 weeks leads to the formation of microvesicles. Because the extent of lysis was high, the microvesicle sample was washed several times, with careful removal of the ghost contamination by aspiration.

3.2.3. Membrane Protein Composition of Microvesicles from Human Erythrocytes

Based on results from SDS-PAGE (Figure 3.12), microvesicles released after DMPC treatment, Ca^{2+} /A23187 treatment, ATP-depletion or after storage had the same protein composition as the microvesicles from MC540-treated cells. The microvesicles from all the treatments contained only band 3 and glycophorin in addition to cytosolic proteins [Lutz *et al* 1977; Weitz *et al* 1982]. However, the amounts of band 3 and glycophorin relative to phospholipid in microvesicles was substantially less than in either the original cells or the residual treated cells. Thus for MC540, DMPC, Ca^{2+} /A23187-treatment or storage microvesicles the amounts of band 3 and glycophorin were between 40- 50% of the values observed in ghosts from untreated cells (Figure 3.13). Higher values (60-70%) were seen in microvesicles from ATP-depleted cells, and this result was the same whether iodoacetamide or deoxyglucose was used for ATP-depletion, although iodoacetamide gave a somewhat higher microvesicle yield. A substantially lower value (20%) for band 3 but not for glycophorin was observed in the case of microvesicles from stored cells.

Figure 3.12 SDS-PAGE of Erythrocytes and Microvesicles from DMPC- and Ca^{2+} /Ionophore-Treated Cells, ATP-Depleted Cells and Stored Cells

Erythrocytes were treated with DMPC, Ca^{2+} /A23187, or iodoacetamide/ deoxyglucose (ATP-depletion) at 37°C , or aged by storing at 4°C as described under Methods. The cells were sedimented and the micro-vesicles obtained from the supernatant by high-speed centrifugation. Ghosts from control cells and microvesicles were analysed by SDS-PAGE on 10% gels stained with Coomassie blue. Glycophorins were visualised with Periodate/Schiff's reagent.

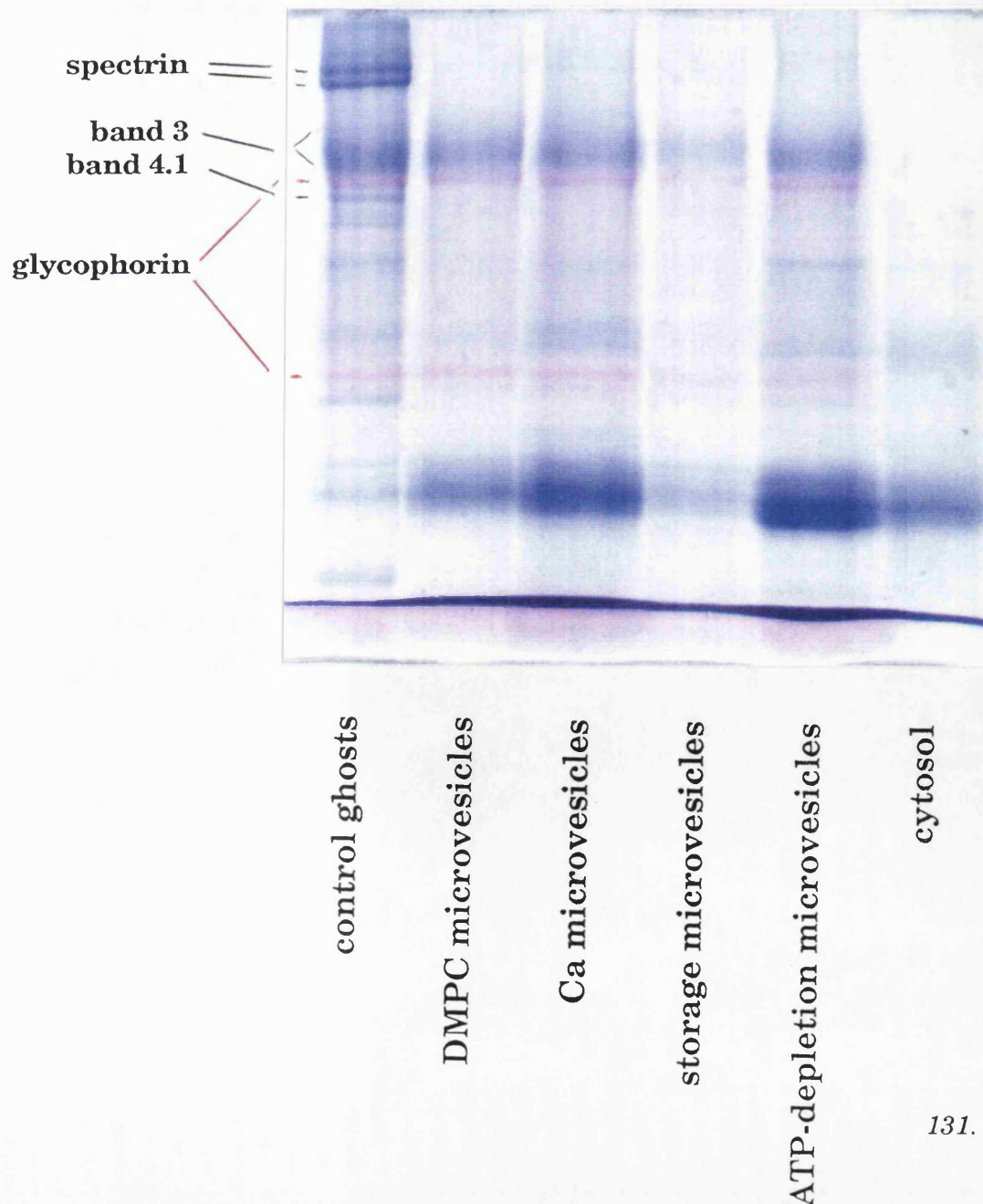
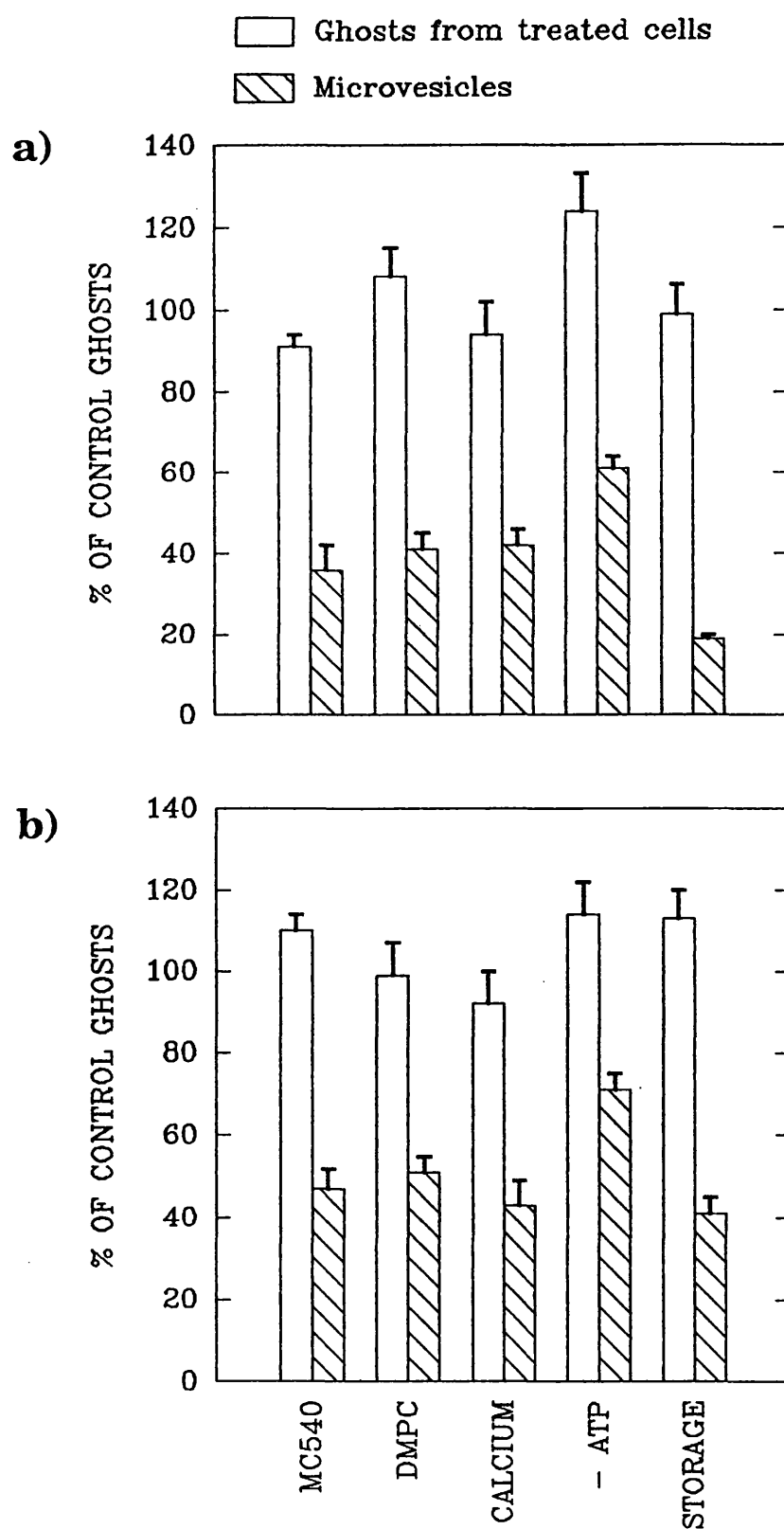


Figure 3.13 Segregation of a) Band 3 and b) Glycophorin into Microvesicles

The ratio of band 3 and glycophorin concentration (derived from gel scans) to phospholipid was measured for control ghosts from untreated cells, and ghosts and microvesicles from cells treated with MC540, DMPC, Ca^{2+} /A23187 or iodoacetamide/deoxyglucose (ATP-depletion) and from cells aged by storage. Values for the control ghosts are taken as 100%. Results are expressed as means \pm standard error taken from four experiments (seven in the case of MC540) carried out in duplicate. The relative amounts of band 3 and glycophorin in the microvesicles from the different treatments were compared using analysis of variance. Microvesicles from ATP-depleted cells contained significantly more band 3 and glycophorin than the other microvesicles ($P < 0.01$) and the microvesicles from aged cells contained less band 3 than those from the other treatments ($P < 0.01$).

(See next page)

Figure 3.13 Segregation of a) Band 3 and b) Glycophorin into Microvesicles



In contrast to the relative depletion of band 3 and glycophorin, a 2-3 fold enrichment of AChE relative to phospholipid was observed in all the microvesicle preparations, similar to that described previously [Bütikofer *et al* 1989; Allan *et al* 1980]. Total activity of this enzyme was not changed since there was an equivalent decrease in activity in the residual cells.

3.2.4. Membrane Lipid Composition of Microvesicles from Human Erythrocytes

As shown with the MC540-treatment, the overall phospholipid composition of the microvesicles, the parent erythrocytes and the treated erythrocytes was very similar, with little variation in the relative amounts of the major lipids.

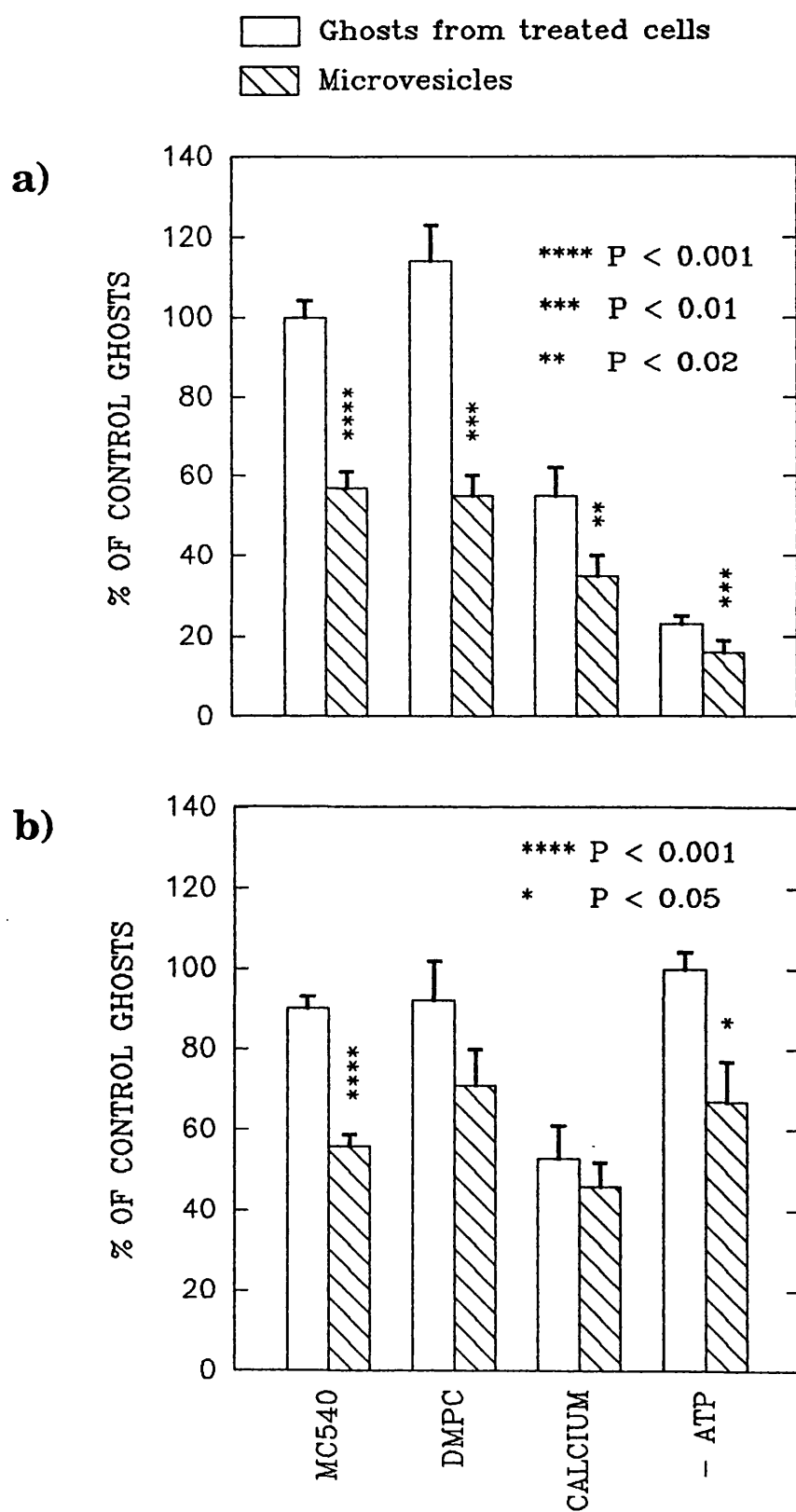
However, the amounts, relative to phospholipid, of PIP and particularly PIP₂, were markedly reduced in the microvesicles from MC540 treatment compared with the original cells (Figure 3.14). In microvesicles from DMPC treated-cells the PIP₂ levels were similarly reduced but PIP levels were reduced to a lesser extent. PIP₂ levels in microvesicles, relative to treated cells, were reduced by approximately 43% after MC540 treatment and 51% after DMPC treatment.

Figure 3.14 Content of a) Phosphatidylinositol 4,5-Bisphosphate and b) Phosphatidylinositol 4-Phosphate in Ghosts from Treated Cells and Microvesicles

Phospholipids were extracted and analysed as described under Methods. The amount of PIP₂ and PIP in the ghosts and microvesicles from cells treated with MC540, DMPC, Ca²⁺/A23187 or iodoacetamide/deoxy-glucose (ATP-depletion) were measured from the radioactive counts and phosphate analysis taking the values for the control ghosts as 100%. The results represent mole % of total phospholipid and are shown as mean \pm standard error from four experiments (seven for MC540) done in duplicate. Significant differences between treated cells and vesicles were shown using paired t-tests: * P<0.05; ** P<0.02; *** P<0.01; **** P<0.001.

(See next page)

Figure 3.14 Content of a) Phosphatidylinositol 4,5-Bisphosphate and b) Phosphatidylinositol 4-Phosphate in Ghosts from Treated Cells and Microvesicles



The level of PI remained constant at 1% in ghosts and microvesicles so that the reduction in the polyphosphoinositides was not due to their dephosphorylation. Neither was there any evidence for breakdown of these lipids by endogenous phospholipase C since there was no increase in either diacylglycerol or phosphatidate, relative to total phospholipid, in the ghosts or microvesicles (except with calcium treatment [Allan & Thomas 1981]).

In cells which had been depleted of ATP or treated with Ca^{2+} /A23187 the amount of PIP_2 was considerably reduced. Due to dephosphorylation, the ATP depleted cells only contained 23% of this lipid relative to control cells. The Ca^{2+} /A23187 treatment, which activates polyphosphoinositide phosphodiesterase by increasing intracellular Ca^{2+} [Allan & Michell 1978] resulted in the breakdown of 45% of PIP_2 , leaving 55% in the treated cells.

However, the microvesicles from these pre-treated cells showed a very similar relative decrease in the content of the polyphosphoinositides. Again only a fraction of these lipids was free to segregate into the microvesicles (see Figure 3.14). In the case of the microvesicles from ATP-depleted cells the PIP_2 content was reduced from 23% in the treated cells (relative to controls) to 16% in the microvesicles - a 30% reduction. In the Ca^{2+} /A23187-treated cells the reduction was from 55% to 35% - again a similar reduction of 36%.

3.3. Endovesiculation of Erythrocytes Following Treatment with Sphingomyelinase C

3.3.1. Introduction

Incubation of human erythrocytes with exogenous sphingomyelinase leads to the breakdown of the outer leaflet lipid sphingomyelin to give ceramide. The breakdown of this lipid is accompanied by changes in the morphology of the cells from discocytes to stomatocytes to spherocytes and an internalisation of membrane lipid in the form of endovesicles [Allan *et al* 1975; Allan & Walklin 1988]. Because ceramide can cross the membrane easily, there is a net loss of lipid from the outer leaflet and a net gain to the inner leaflet area. Therefore it is likely that the removal of the phosphorylcholine headgroup leading to the generation of ceramide and the subsequent decrease in outer leaflet area relative to inner leaflet may be responsible for the increased membrane curvature leading to endocytosis.

Attempts were made to separate these endovesicles from the rest of the cell membrane so that their lipid and protein composition could be analysed.

These vesicles could then be compared with the exovesicles produced by the action of amphipaths, Ca^{2+} /A23187 or ATP-depletion.

3.3.2. Treatment of Erythrocytes with Sphingomyelinase C

Light microscopy confirmed previous reports that treatment of human erythrocytes with sphingomyelinase produced changes in cell morphology. The cells first became cup-shaped (stomatocytic) and subsequently areas of the membrane invaginated, making the cells spherocytic. The cell suspension was kept at 37°C to avoid excessive lysis, as previous workers had shown that phospholipase C-treated cells show extensive lysis below 30°C [Allan & Walklin 1988; Bernheimer 1974; Ikezawa *et al* 1980].

3.3.3. Isolation of Endovesicles from Erythrocytes Treated with Sphingomyelinase

The separation of endovesicles from the parent cell was needed, before an analysis of the lipid and protein composition of endovesicles could be made. The cells were ruptured using a cell cracker (see Methods) after treatment with sphingomyelinase. The resulting suspension was passed through an ultrafilter, with the expectation that only the cytosol and the vesicles would go through the filter and the membrane of the ruptured cell would stay behind. The vesicles might then be isolated by centrifugation.

The strongly fluorescent compound Lucifer Yellow was incorporated inside the endovesicles by including it in the medium before incubation with the

sphingomyelinase. This helped the detection by microscopy of any fluorescent vesicles inside the cells or any free vesicles that were obtained after filtration.

The major problem was that although fluorescent vesicles could be seen in the lysate, they were still attached to the membrane and very difficult to remove. A few free vesicles could be identified by their rapid Brownian motion, but most were motionless, presumably due to a membrane attachment. In some experiments, Percoll of a defined density was also added to the external medium prior to incubating the cells with sphingomyelinase. It was thought that any vesicles freed after the cells had been broken could be separated on the basis of their known density, using centrifugation on a sucrose gradient. However, vesicles free from ghost contamination could not be obtained by this method.

According to the electron micrographs presented in the paper by Allan *et al* (1975) most the endovesicles appeared to be between 0.1 and 0.4 μ m in diameter. It may therefore be possible to use filtration in order to isolate free vesicles from the rest of the cell membrane. Filtration of the broken cell suspension through a 0.45 μ m filter and centrifugation of these filtrates gave small amounts of free vesicles. These were sedimented to give small pellets enriched in band 3 and depleted in spectrin but the yield from this process was never sufficient to give a convincing gel pattern after SDS-PAGE. AChE analysis of these filtrates showed a two-fold increase after addition of Triton-

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X100 showing that this enzyme was partly latent. This suggests that inside-out vesicles were present, but again these results were not sufficiently reproducible to make the procedure worthwhile.

3.4. DISCUSSION

3.4.1. Interaction of MC540 with Human Erythrocytes

As shown in this thesis and in previous studies [Allan *et al* 1989] the interaction of MC540 with human erythrocytes causes morphological changes similar to those seen with other amphipathic molecules such as dimyristoyl phosphatidylcholine (DMPC) [Ott *et al* 1981] or glycocholate [Billington & Coleman 1978]. The effect of this interaction is a shape transformation to echinocyte and, at higher concentrations of MC540, to spherocyte. This amphipath-induced shape transformation is not thought to be dependent on the metabolic depletion of the cell since studies using DMPC showed that amphipath-induced microvesicle release from erythrocytes is not conditional on the reduction of the ATP levels in the cell [Ott *et al* 1981], although ATP depletion speeds up the process.

The binding studies (Figure 3.2) showed that at 37°C erythrocytes bind approximately 3.2 moles of dye per 100 moles of phospholipid. The amount bound to cells was measured in the cells after sedimentation, and would therefore not include the amount bound to any released microvesicles. These measurements may therefore under-estimate the amount bound to the cells prior to vesiculation, particularly if MC540 is more concentrated in the

microvesicles. This may in part explain why the figures quoted here are lower than estimates reported previously by others for outer leaflet expansion in echinocytosis [Allan *et al* 1989]. However, since the microvesicles represent less than 10% of the total lipid released, the released microvesicles would have to contain an unlikely large amount of MC540 in order to significantly affect the estimate of MC540 bound to the sedimented cells. In these experiments it was impossible to measure the amount of MC540 bound to microvesicles, since residual MC540 was always found after high speed centrifugation of the solution without added cells.

As discussed by Allan *et al* (1989) the result for the amount of MC540 bound to cells conflicts with the observations by Schlegel *et al* (1980) which suggested that normal erythrocytes do not bind MC540. This contradiction can be explained by the fact that different experimental conditions were employed. We used serum- free medium, whereas Schlegel *et al* used a medium containing albumin and lipoprotein which compete with the binding of MC540 to cells.

Because of its charged nature, MC540 is unable to cross the cell membrane and it partitions into the outer lipid leaflet, probably oriented so that the hydrophobic part of the molecule lies parallel with the fatty acid chains of the membrane phospholipids, with the hydrophilic part interacting with the phospholipid headgroups [Allan *et al* 1989]. This interaction of MC540 with

erythrocytes results in an increase of the area of the outer lipid leaflet relative to the inner leaflet. This causes the localised bending of the membrane as predicted by the bilayer couple hypothesis of Sheetz and Singer (1974). A consequence of this membrane expansion is the change in cell morphology leading to echinocytosis. An extreme change in morphology, plus the disruption of the normal lipid environment caused by high concentrations of amphipath, has been shown to lead to the release of microvesicles.

3.4.2. Microvesicle Release from MC540-Treated Erythrocytes

The time course experiments and the dose response experiments gave very puzzling results. In the dose response experiment (Figure 3.3) an incubation of 2 minutes gave a higher AChE release than an incubation of 10 minutes. This was also clearly shown in the time course experiment (Figure 3.4). AChE release reaches a maximum, relatively quickly, after 2 minutes. After this time AChE release appears to decrease with time. No obvious explanation could be found for this phenomenon. A possible suggestion is that vesicles could begin to "stick" to each other and to the treated cells after a few minutes. Fewer microvesicles would therefore be left in the supernatant after centrifugation and thus less AChE would be measurable. Another possibility is that the microvesicles are unstable and lyse to produce a residue which is difficult to sediment.

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The temperature dependence of microvesicle release with MC540 is similar to that with DMPC [Ott *et al* 1981] and shows that microvesicle release occurs above 20°C. This may be due to a temperature-dependent decrease in membrane lipid fluidity which affects membrane fusion.

Another possible explanation is that more MC540 partitions into the cells as the lipid membrane becomes more fluid with increased temperature - more MC540 would mean an increase in membrane bending and this could assist vesicle formation. Other studies have correlated changes in the fluid state of the membrane with increased temperature with an elevated partitioning of the probe into the lipid phase [Langner & Hui 1993]. However, as shown in that study, there is a relationship between fluidity and MC540 partitioning at low MC540 concentrations. At higher MC540 concentrations the fraction of MC540 in the lipid phase is not dependent on membrane fluidity. The results detailed in the paper by Allan *et al* (1989) confirm that MC540 binding, at the concentrations used, does not seem to depend on temperature. The amount of maximal binding at 20°C and 37°C was similar, being 3.6 and 3.2 molecules of MC540 per 100 molecules of phospholipid.

By contrast with DMPC [Ott *et al* 1981], there was no time-lag before the release of microvesicles after incubation of erythrocytes with MC540. Ott *et al* rationalised the lag-time before microvesicle release in terms of the DMPC undergoing 'flip-flop' to the inner monolayer, causing lateral phase separation

of phosphatidylserine (PS) and phosphatidylethanolamine (PE). This phase separation might be necessary for membrane fusion [Hope & Cullis 1979]. A different, but very plausible explanation is that the lag-time is dependent on the rate of entry of amphipath into the outer lipid leaflet. DMPC may need to be in a free monomeric form rather than in a micellar form in order to interact with the erythrocyte membrane. Although DMPC was shown to intercalate into the outer lipid leaflet within minutes [Ott *et al* 1981] it may take much longer for enough DMPC to build up in the membrane to cause microvesiculation. MC540 may form monomers more easily, therefore partition into the membrane more readily and thus produce vesicles more rapidly. The main determinant of microvesicle release may be the amount of amphipath in the outer bilayer and the expansion it causes.

Another explanation for the rapid effect of MC540 could be that it causes a loss in asymmetry and this may influence the microvesiculation process. This is a reasonable proposition in the light of other studies which demonstrated that factors affecting the lipid bilayer alter the phospholipid asymmetry. It has been shown that amphiphilic drugs may alter the transbilayer distribution and mobility of the membrane phospholipids [Schneider *et al* 1986], and that the aminophospholipids (PE and PS) of microvesicles obtained by shearing cells under pressure partly lose their asymmetric distribution and become susceptible to reaction with external

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trinitrobenzenesulphonate and phospholipase (PLA₂) [Scott *et al* 1984].

Sickled erythrocytes also showed the presence of negatively charged phospholipids on the outer surface of the membrane [Lubin *et al* 1981]. On reoxygenation of the cells an enhanced transbilayer mobility of phosphatidylcholine (PC) was observed [Franck *et al* 1985]. Spicules released by these authors from sickle cells during repetitive sickling also showed negatively charged phospholipids on the surface, although this was not confirmed by Raval & Allan (1984) using PLA₂ treatment. Schlegel *et al* (1980) also suggest that MC540 binds more strongly to PS and PE, therefore stabilising these lipids in the outer leaflet of the bilayer and encouraging loss of asymmetry. No evidence could be found by us for the suggestion that MC540 binds preferentially to the inner leaflet lipids, since the binding to ghosts was almost exactly twice that to cells [Allan *et al* 1989], suggesting that binding to inner leaflet was about the same as binding to outer leaflet. The notion that MC540 may cause a change in phospholipid asymmetry was discounted by the results in this thesis using exogenous phospholipases to study the phospholipid distribution of the treated cells. The results shown here suggest that microvesicle release does not necessarily involve the loss of lipid asymmetry.

It is of course possible that the inner leaflet phospholipids do flip to the outer leaflet but that these lipids are not broken down by PLA₂ because they are

somehow shielded by the MC540 molecules in the outer leaflet. This is unlikely since in ghosts from MC540-treated cells, where MC540 had been shown to be present in both lipid leaflets, the breakdown of PE and PS by PLA₂ was no different to the breakdown seen in control cells.

The results here showing no changes in lipid asymmetry also agree with those of Raval & Allan (1984). They treated erythrocytes with Ca²⁺/ionophore and showed that the subsequent microvesicles retained their phospholipid asymmetry. In this case a different mechanism might be responsible. Microvesiculation caused by this procedure may not require a phospholipid 'flip-flop' since it coincides with a breakdown in cytoskeletal proteins and a formation of diacylglycerol, conceivably a modulator of fusogenic events [Allan & Michell 1979; Siegel *et al* 1989].

Microvesicle release from MC540-treated cells does not involve the formation of diacylglycerol. A more likely mechanism for this microvesicle release may be that the intercalation of MC540 into the outer lipid leaflet results in an imbalance between the two leaflets, leading to extreme bending of the membrane sufficient to induce microvesiculation. A similar mechanism could also apply in the case of calcium treatment. An increase in the breakdown of the polyphosphoinositides, which follows the rise in intracellular calcium concentration, causes a decrease in the area of the internal leaflet and an

imbalance between the two leaflets and this could explain the resultant microvesicle release [Ferrell *et al* 1985].

3.4.3. Segregation of Proteins into Microvesicles

The content of band 3 relative to phospholipid in the microvesicles from amphipath (MC540 and DMPC) or calcium-induced microvesicles is about 40% of that in the original cells. These results are consistent with the concept that over half of this protein is bound to the cytoskeleton and is unable to diffuse into the microvesicles. Studies which measured the rotational diffusion of band 3 also showed that more than half of the band 3 is bound to the cytoskeleton, either to ankyrin or band 4.1 and glycophorin [Nigg & Cherry 1980; Tsuji *et al* 1988]. It is not surprising that there should be free Band 3 since the number of Band 3 molecules (even if they are dimers) exceeds the number of ankyrin and band 4.1, that is more than the possible number of attachment sites (see Table 2.1). The amount of glycophorin A in the microvesicles is also reduced, to about 45% that of the original cell.

The similar values for the proportions of band 3 and glycophorin A which can diffuse into microvesicles suggest that comparable proportions of both proteins are attached to the cytoskeleton. The existence of a complex formation between band 3 and glycophorin A [Nigg *et al* 1980; Pinto da Silva & Nicholson 1974] could explain the result that both of these proteins are

depleted by the same degree, as the restricted diffusion of one would affect the other. The attachment of Band 3 to ankyrin may cause indirect immobilisation of glycophorin.

On the other hand, the microvesicles from ATP-depleted cells were previously found to contain the same amounts, relative to total phospholipid, of band 3 and glycophorin as the original cells [Lutz *et al* 1977], indicating no barrier to the free diffusion of these proteins into microvesicles after energy depletion. This would suggest that energy depletion inhibits binding of band 3 and glycophorin to the cytoskeleton. The results in this thesis imply that a fraction of these proteins are still bound to the cytoskeleton, even after metabolic depletion, but that the amount of this interaction is reduced since the microvesicles from ATP-depleted cells contain significantly more band 3 (61%) and glycophorin (71%) than the microvesicles prepared by other methods. This implies that ATP-depletion and possibly the dephosphorylation of cytoskeletal proteins affects the binding of band 3 and glycophorin A to the membrane skeleton.

The microvesicles from stored cells also showed a different protein composition from those generated by amphipath treatment (Figure 3.15). The microvesicles from stored cells contained a significantly lower amount of band 3 (19%) but similar amounts of glycophorin to other microvesicles.

Band 3 may be degraded during the long storage period, but this explanation is less likely because the stored cells contain comparable amounts of Band 3 to those found in fresh control cells. It may be that Band 3 aggregates during storage, possibly due to the formation of -S-S- links, causing less of it to be mobile.

3.4.4. Acetylcholinesterase Content in Microvesicles

In contrast with the transmembrane proteins, which are depleted in the microvesicles, acetylcholinesterase (AChE) is enriched in the microvesicles. This enzyme is thought to be linked to the cell surface via a phosphatidylinositol-glycan linkage [Low & Saltiel 1988, Cross 1990] and the phosphatidylinositol anchor should be freely diffusing in the plane of the membrane. It is therefore reasonable that any enzyme linked to it would also be freely diffusing. This enrichment of AChE in the microvesicles has also been seen by others in microvesicles obtained by procedures such as Ca^{2+} -loading [Allan & Thomas 1981], treatment with DMPC [Ott *et al* 1981] or spectrin oxidation [Wagner *et al* 1987].

A similar increase is also seen for another phosphatidylinositol-linked membrane protein, the decay accelerating factor [Bütikofer *et al* 1989], leading to suggestions that changes in the membrane structure prior to microvesiculation result in the concentration of the phosphatidylinositol-

linked proteins into the domain of the membrane which then buds off. It is possible that more AChE can diffuse into the microvesicle membrane because other membrane proteins such as band 3 and glycophorin are depleted in the microvesicles thus allowing more space for mobile protein to diffuse. Put in another way, the decrease in packing pressure due to the removal of half of the protein from the bilayer favours movement mobile phosphatidylinositol-linked proteins out of the residual cells and into the microvesicles.

3.4.5. Polyphosphoinositide Phosphodiesterase Activity in Microvesicles

The activity of polyphosphoinositide-specific phosphodiesterase (PPI-PDE) in erythrocytes and microvesicles from MC540-treated cells was measured by incubation with Ca^{2+} /ionophore. An increase in Ca^{2+} concentration activates this enzyme [Allan & Michell 1978] leading to the breakdown of the polyphosphoinositides (PPIs). Although breakdown of the PPIs in the cells occurred normally, the content of PPIs in the Ca^{2+} -treated microvesicles was the same as the untreated microvesicles, i.e. no activity of the phospholipase could be detected in the microvesicles. Since the lack of activity is not due to suppression by MC540, these results suggest that this enzyme is not free to partition into the microvesicles because it is bound to the cytoskeleton or to another protein which itself has a cytoskeletal attachment. It seems less

likely that the enzyme is present in the microvesicles but is unable to attack the mobile pool of the PPIs there. Certainly, the mobile pool is attacked in cells exposed to Ca^{2+} /ionophore.

A possible explanation for the reduction of PPIs, and apparent lack of PPI-PDE, in the microvesicles is that this phospholipase is activated during microvesiculation, hydrolysing all accessible substrate. It is unlikely that this calcium-dependent enzyme would be activated in such a way. Even if it was, a reduction in the PPIs would also be seen in the treated cells after vesiculation - this is not the case. Additionally, no increase in either diacylglycerol or phosphatidate was observed after incubation with MC540. This means that there is no evidence for breakdown of the PPIs during the process of vesiculation.

3.4.6. Segregation of Polyphosphoinositides into Microvesicles

Since the main erythrocyte phospholipids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and sphingomyelin are thought to diffuse freely in the plane of the membrane, and thus should diffuse freely into the microvesicles as they bud, any microvesicles should contain a representative sample of these lipids. This was the case in the microvesicles from MC540, DMPC, Ca^{2+} /ionophore treated or ATP-depleted cells where the composition of the major phospholipids was very similar to that of the original cells.

The only phospholipids to behave differently are the polyphosphoinositides (PPIs). The microvesicles from MC540 and DMPC treated cells only contain approximately half of the relative to the total phospholipid content, and since the reduction was not due to dephosphorylation or breakdown by a phospholipase, this implies that only half of the PPIs are free to diffuse into the microvesicles. In the case of Ca^{2+} -treatment or ATP- depletion the polyphosphoinositide 4,5-bisphosphate (PIP_2) content is reduced in the cells, but again the microvesicles only contained a fraction of the PIP_2 content relative to the treated cells.

It may not be a coincidence that similar proportions of Band 3, glycoporphin A and PIP_2 are found in the microvesicles from amphipath, Ca^{2+} treated or ATP-depleted cells, but rather a reflection of the interactions between these proteins, PIP_2 , and the membrane cytoskeleton. It is possible that the primary interaction of both glycoporphin and PIP_2 is with band 3 and that the restriction in the diffusion of band 3 by an interaction with the skeleton determines the distribution of the other components.

The results here are consistent with the suggestion that the PPIs may be associated with the cytoskeletal proteins, perhaps mediating interactions between band 4.1 and glycoporphin [Anderson & Marchesi 1985]. Studies using ^{31}P nuclear magnetic resonance (^{31}P -NMR) showed that four molecules of inositol phospholipid are tightly bound to each glycoporphin

molecule [Yeagle & Kelsey 1989]. If it is assumed that the most likely lipid to be associated with the glycoprotein is PIP₂, these studies suggest that approximately 40% of PIP₂ is bound to glycoprotein. There are approximately 2.6×10^8 molecules of phospholipid in each cell. PIP₂ accounts for 1.5% of total phospholipid, i.e. there are 3.9×10^6 molecules of PIP₂ per cell. Since 4×10^5 molecules of glycoprotein are present in each cell [Bennett 1985], 1.6×10^6 molecules per cells (or 41%) of PIP₂ could be bound to glycoprotein.

The restricted mobility of the PPIs in the membrane fits with the idea of metabolic pooling of the PPIs [Müller *et al* 1986; King *et al* 1987; Gascard *et al* 1993]. King *et al* incubated cells with ³²P under conditions which facilitated the rapid entry of ³²P into the cells and therefore rapid labelling to equilibrium. They showed that at steady state (5-7 hours), the maximum specific radioactivity of the PPIs was only 30% of the specific radioactivity of the gamma-phosphate of ATP and that therefore a substantial fraction of the total was in a metabolically more inert pool. When the Ca²⁺-dependent polyphosphoinositide phosphodiesterase (PPI-PDE) was activated, the PIP₂ content was halved, but the specific activity of the lipid remained the same. They rationalised the results in terms of the existence of at least four metabolic pools of lipid: 1) Metabolically active and broken down by PPI-PDE; 2) metabolically active and not broken down by PPI-PDE; 3) metabolically

inactive and broken down by PPI-PDE; 4) metabolically inactive and not broken down by PPI-PDE.

Another, less likely, explanation given by King *et al* is not that four metabolic pools exist but only two, one metabolically inert and the other active, with those PIP₂ molecules not associated with protein 4.1 being labelled. The specific activity remains the same after PPI-PDE activation because the high concentration of Ca²⁺ used might lead to the breakdown of band 4.1 protein [Allan & Thomas 1981], causing the dissociation of bound PIP₂ and hence the mixing of metabolic pools.

Because of the long period of incubation which was used in the experiments described in this thesis, the PPIs were labelled almost to equilibrium with ATP, so that no differences could be measured between the specific radioactivity of the PIP₂ which partitioned into the microvesicles and that which did not.

Nevertheless, the results lend credence to the concept of multiple compartmentalisation. When the cells are incubated with Ca²⁺ and ionophore about half of PIP₂ is broken down. It is possible that the fraction of PIP₂ which is not broken down is the fraction which is linked to the cytoskeletal proteins and that Ca²⁺-treatment does not disturb this interaction. The other half is degraded because it is unbound and freely diffusing in the membrane and therefore available to the phosphodiesterase.

Shape Changes and Vesicle Formation - Discussion

If a non-association with the cytoskeleton was the only criterion for both degradation by the phosphodiesterase and diffusion into the microvesicles, one would expect the microvesicles from Ca^{2+} /ionophore-treated cells to contain none of the PIP_2 . This cannot be the case, since the microvesicles contain significant amounts of PIP_2 . The treated cells were found to contain 55% of PIP_2 (relative to total phospholipid) compared to control cells and the microvesicles contained 35% of the PIP_2 . In other words, more than 60% of the PIP_2 in the Ca^{2+} -treated cells is free to segregate into the microvesicles.

Similarly, in the experiments of King *et al* only a fraction of lipid is labelled (possibly that not linked to the cytoskeleton), but the phospholipase treatment does not distinguish between labelled and unlabelled lipid.

In the case of the ATP-depleted cells, most of the PIP_2 is dephosphorylated, leaving 23% of this lipid intact. The microvesicles are still depleted of the PIP_2 , but the difference is less marked. In this case 16% (compared to controls) of the PIP_2 relative to total phospholipid is found in the microvesicles. Since more than two thirds of the PIP_2 pool in the ATP-treated cells is free to diffuse into the microvesicles, this leads to the conclusion that ATP-depletion may disrupt the association between PIP_2 and cytoskeleton.

4. MEMBRANE PROTEIN PHOSPHORYLATION IN HUMAN ERYTHROCYTES

4.1. Alteration of Erythrocyte Membrane Properties after cAMP-Activated Protein Phosphorylation

4.1.1. Introduction

When erythrocytes are incubated in the presence of cAMP the phosphorylation state of several of the skeletal proteins is enhanced through the activation of cAMP-dependent protein kinases (PKA) [Thomas *et al* 1979]. In erythrocytes several of these kinases exist, and they are found either in the cytoplasm or associated with the cell membrane [Plut *et al* 1978].

Although human erythrocytes contain insignificant amounts of adenylate cyclase and cAMP-phosphodiesterase (the enzymes required for the synthesis and breakdown of cAMP), cAMP-dependent phosphorylation [Rodan *et al* 1976, Thomas *et al* 1979] is believed to play a role in the regulation of membrane fluidity [Kury & McConnell 1975] and is believed to influence the structural properties of the cell [Thomas *et al* 1979, Husain-Chishti 1988; Husain-Chishti *et al* 1989]. Erythrocyte deformability has also been shown to

be reduced following exposure to cAMP [Kury & McConnel 1975; Tsukamoto *et al* 1980].

Thomas *et al* (1979) further speculated about the role of cAMP *in vivo*. In certain disease states or within certain organs, erythrocytes may be exposed to significant concentrations of cAMP. As the intracellular concentration of cAMP is increased, probably as result of its entry via the anion transport channel (Band 3), the cells' flexibility is altered and this may lead to their destruction.

In other cell types, cAMP-dependent phosphorylation regulates a variety of different membrane-dependent functions, such as aggregation and differentiation, secretory processes, and cellular permeability [Rubin & Rosen 1973]. In yeast strains, cAMP-dependent phosphorylation has been found to influence the metabolism of the polyphosphoinositides (PPIs). In this case the PPI synthesis was enhanced through the activation of the phosphatidylinositol kinase and phosphatidylinositol 4- phosphate kinase. Increased production of phosphatidylinositol 4,5-bisphosphate (PIP₂) could lead subsequently to an enhanced production of 1,4,5-tris-phosphate and diacylglycerol, the messengers that trigger cell proliferation [Kato *et al* 1989].

In mammalian cells an effect of cAMP on PPI metabolism has also been shown. If lymphocytes or platelets are incubated with ³²P and cAMP, incorporation of radioactivity into the lipids is increased by more than 60%.

This change was shown to be due to an increase in the net synthesis of the PPIs [Enyedi *et al* 1983]. Other effects on PPI metabolism, in this case inhibition, have been shown by the results of Takenawa *et al* (1986). Their study suggests that prostaglandin E₂ suppressed inositol phospholipid metabolism through an elevation in intracellular cAMP.

cAMP-dependent phosphorylation has also been shown to alter the gating properties of the ion-channels in excitable cells [Ewald *et al* 1985] and pancreatic duct cells [Gray *et al* 1990]. Since erythrocytes also contain K⁺-channels [Gardos 1958], it is conceivable that cAMP may also have some effect on the activity of these channels. This possibility is explored in experiments described in the following pages.

Other effects of increased cAMP on the erythrocyte are also examined in the studies presented here. For example, it is unclear what effect cAMP has on the inositide metabolism of erythrocytes. Although Endeyi *et al* also carried out their experiments on erythrocytes, they did not convincingly show the same results for erythrocytes as for lymphocytes or platelets.

Because of the relative simplicity of the erythrocyte, it also provides a convenient model for the study of the effects of cAMP-dependent phosphorylation in intact cells, since it is possible to raise the intracellular concentration of cAMP by incubating erythrocytes with extra-cellular cAMP. Results presented here will hopefully go towards increasing the

understanding of the role of cAMP, not only in erythrocytes but in other cell types.

4.1.2. Phosphorylation of Membrane Proteins by cAMP-

Activated Kinase

When erythrocytes are incubated in the presence of ^{32}P , radioactivity is incorporated into lipids such as phosphatidic acid and the polyphosphoinositides as well as several of the membrane proteins. SDS-PAGE analysis shows that most of the labelling occurs on spectrin and proteins in the band 3 region. As shown previously [Thomas *et al* 1979; Hosey & Tao 1976], addition of cAMP during the incubation of whole cells with ^{32}P does not significantly change the labelling of spectrin or band 3 (Figure 4.1) but leads to the enhanced labelling of several additional membrane proteins, notably bands 4.1, 4.9 and 7. As shown in Figure 4.1 maximum labelling of these proteins occurs after 16 hours.

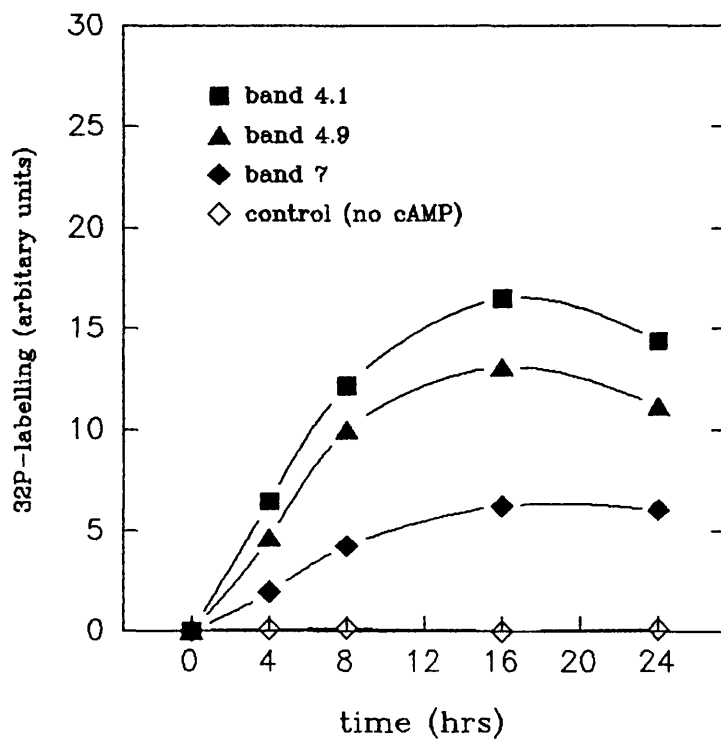
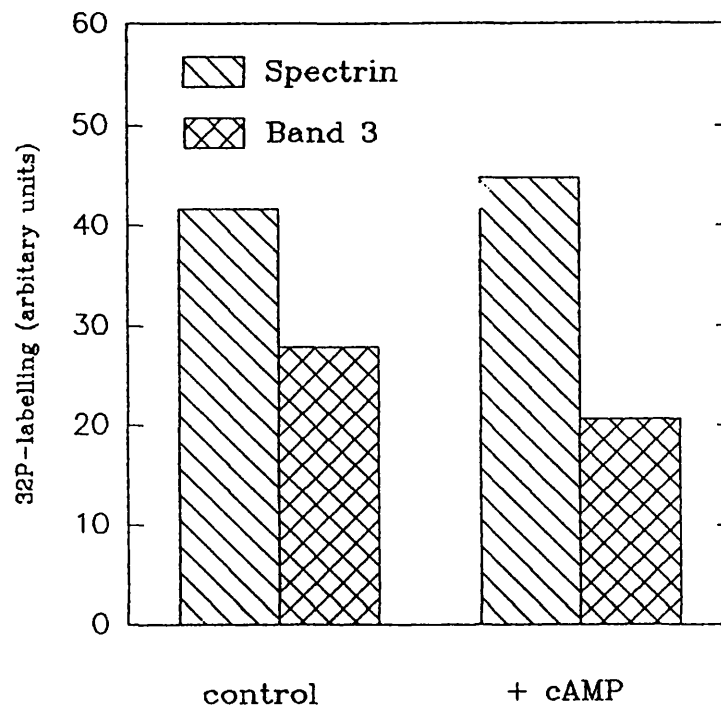
Similar results were obtained when dibutyryl cAMP (dbcAMP) is used, but since this nucleotide enters the cell more rapidly than cAMP, maximum protein labelling is achieved after four hours.

Figure 4.1 Membrane Protein Phosphorylation in Erythrocytes Treated with cAMP

Erythrocytes (at 20% haematocrit) were incubated at 37°C with ^{32}P (~ 50 $\mu\text{Ci/ml}$ packed cells) for up to 24hrs with or without 100 μM cAMP. Cells were lysed and membrane samples (15 μl) were analysed by SDS- PAGE (15% w/w) as described under Methods. ^{32}P -labelling was quantified by scanning the radio autographs or by scintillation counting of excised spots. a) Labelling of spectrin and band 3 after 24hrs in control cells and cells treated with cAMP. b) Increase in ^{32}P -labelling for bands 4.1, 4.9 and 7 with time for cells treated with cAMP. No increase in labelling for these proteins (apart from spectrin and band 3) was seen in control cells.

(see next page)

Figure 4.1 Membrane Protein Phosphorylation in Erythrocytes Treated with cAMP



4.1.3. Changes in the Ca²⁺-Dependent K⁺-Channel Activity

Produced by cAMP

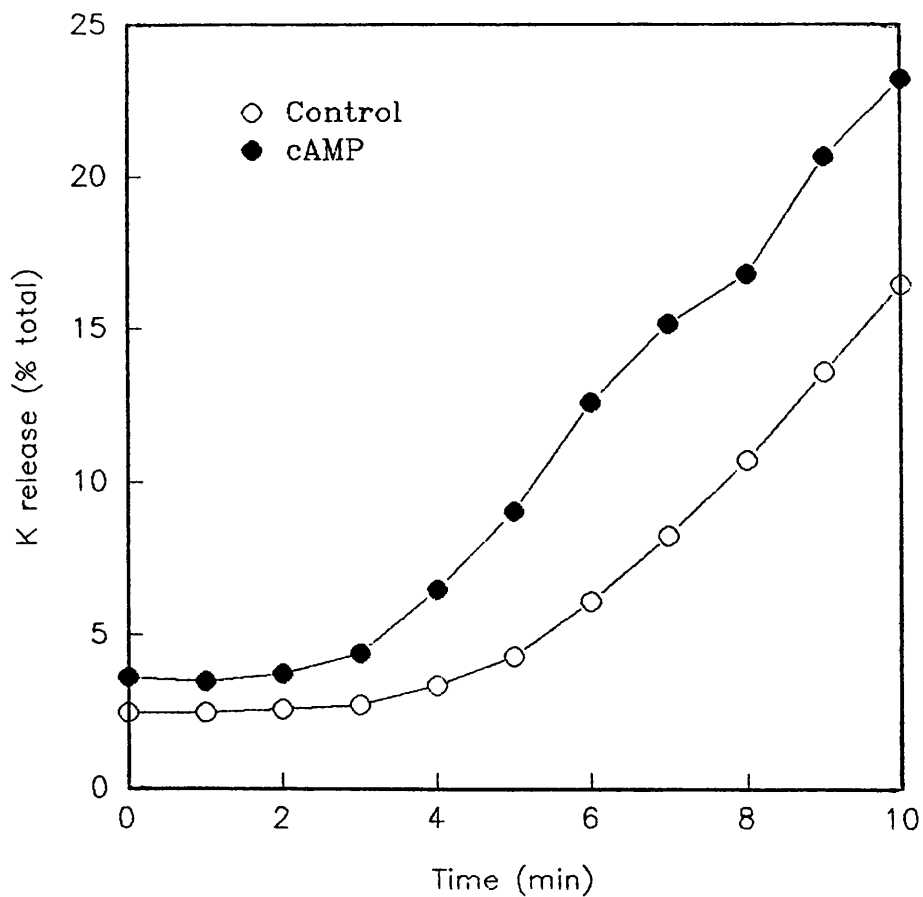
The addition of calcium and an ionophore such as A23187 to erythrocytes results in a large efflux of intracellular K⁺ [Kirkpatrick *et al* 1975]. This is believed to occur via a K⁺-gating mechanism activated by an increase of intracellular Ca²⁺ in excess of 0.1μM.

Figure 4.2 shows the Ca²⁺-activated K⁺ efflux in normal and cAMP-treated erythrocytes for a representative experiment. cAMP treatment itself did not lead to efflux of K⁺ from the cells (not shown), but increased the K⁺ efflux once the Ca²⁺-dependent K⁺ channels are activated. In two other experiments similar results were obtained. After 5 minutes, the K⁺ efflux in the cAMP-treated cells was 35-40% higher than in the untreated cells.

These results shown here for erythrocytes are consistent with other studies on neurones [Ewald *et al* 1985] and pancreatic duct cells [Gray *et al* 1990] which showed that the Ca²⁺-dependent K⁺ channels in both these cell types are regulated by cAMP-dependent phosphorylation.

Figure 4.2 Ca^{2+} -Dependent K^{+} Efflux in Normal and cAMP-Treated Erythrocytes:

Erythrocytes (at 20% haematocrit) were incubated overnight at 37°C with or without 500 μM cAMP. K^{+} efflux was activated by the addition of 100 μM Ca^{2+} and 1 μM A23187. The amount of K^{+} release was measured using a K^{+} electrode, standardised against K^{+} solutions of known concentration (see Methods). 100% efflux was measured after lysing the cells with 0.1% Triton X100. Results are from a representative experiment.



4.1.4. Changes in Phosphatidylinositol 4,5-Bisphosphate

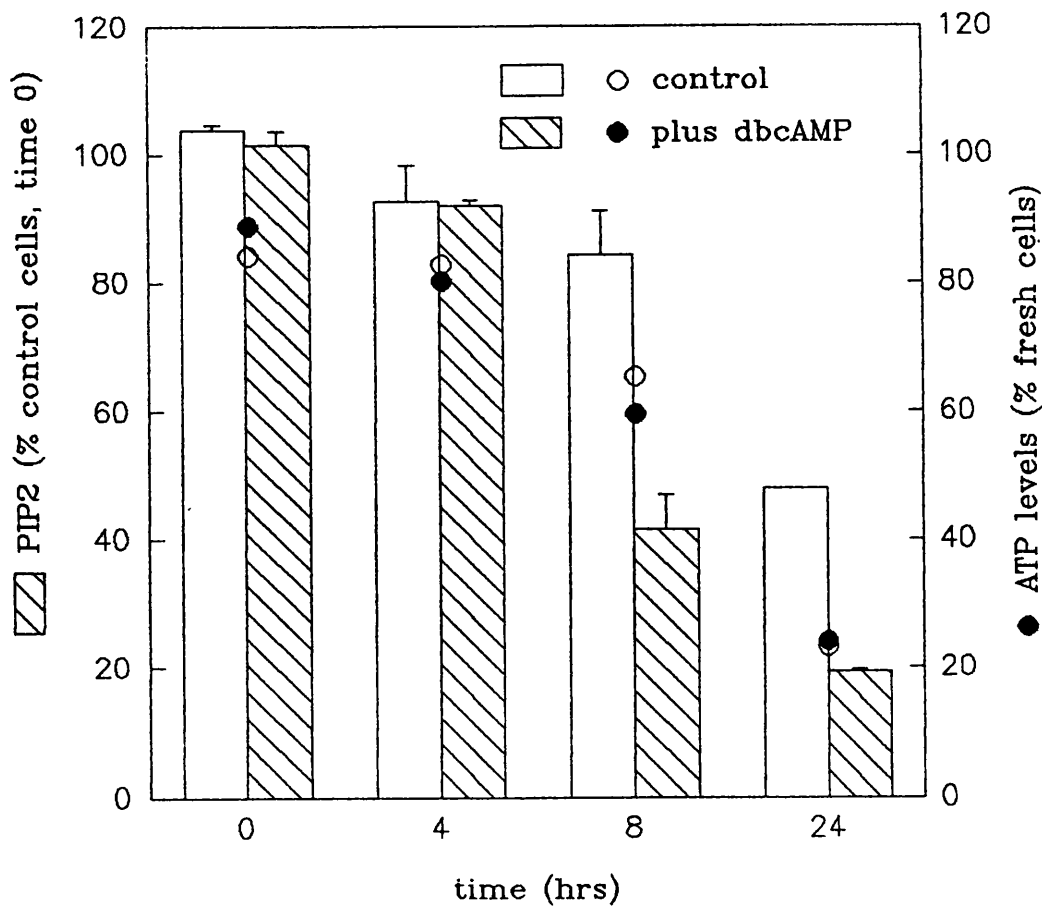
Content in dbcAMP-Treated Cells

The effect of cAMP on the inositol lipids was examined by first incubating erythrocytes overnight with ^{32}P to label the polyphosphoinositides to equilibrium and then subsequently incubating with dbcAMP for up to 24 hours.

When erythrocytes were incubated in the presence of dbcAMP for two hours, a slight increase in the levels of PIP_2 was observed. When the incubation period was increased to over eight hours, the levels of PIP_2 were substantially reduced in the cells treated with dbcAMP (Figure 4.3). Although a reduction in ATP levels could lead to the dephosphorylation of PIP_2 , this lipid was not broken down to the same extent in the control cells. There was no difference in ATP levels between the treated and control cells (Figure 4.3). After 24 hours the ATP levels had fallen by over 70%, and the PIP_2 content in both the control and treated cells had fallen substantially, but PIP_2 was still broken down to a greater extent in the treated cells.

Figure 4.3 Content of Phosphatidylinositol 4,5- Bisphosphate and ATP in Control Erythrocytes and Erythrocytes Treated with dbcAMP

Erythrocytes were incubated overnight at 37°C with ^{32}P . The labelled cells were resuspended to 20% haematocrit in a medium containing ^{32}P , at the same specific activity as before, and incubated with or without 100 μM dbcAMP for up to 24 hours. The phospholipids were extracted and the amounts of PIP_2 were measured from the radioactive counts, taking the values for the cells at time 0 as 100%. ATP levels were measured by HPLC as described under Methods, taking the ATP levels in fresh cells as 100%. Results for the PIP_2 analysis are means \pm standard error from three experiments carried out in duplicate. The ATP levels are from a representative experiment.



4.2. Effect of Membrane Protein Phosphorylation on Microvesicle Release and on the Protein and Polyphosphoinositide Composition of Microvesicles

4.2.1. Introduction

Although little direct evidence has been found to support the idea that phosphorylation has an effect on the mechanical properties of normal erythrocytes, several studies have shown that the skeletal proteins are affected by their phosphorylation state [Cohen *et al* 1990]. The phosphorylation of membrane proteins may therefore be one of the mechanisms by which the interaction between these proteins is controlled and thereby the erythrocyte's shape and deformability is regulated.

The phosphorylation of protein 4.1 leads to a five-fold decrease in its affinity for spectrin and a decrease in its ability to promote the spectrin-actin interaction [Eder *et al* 1986; Ling *et al* 1988]. Since the complex between spectrin, actin and protein 4.1 is the basis of the cell's cytoskeleton, any changes in this complex may lead to a destabilisation of the cytoskeleton, thus causing morphological changes and facilitating microvesicle release.

Previous studies on the effect of protein kinase C-induced phosphorylation found no apparent changes in erythrocyte morphology [Raval & Allan 1985].

It was of interest to examine more closely whether cAMP-dependent phosphorylation results in any morphological changes or alters microvesicle formation. Since the phosphorylation site on protein 4.1 is dependent on the type of protein kinase activated [Horne *et al* 1985], some differences may be apparent after either protein kinase C or cAMP-dependent kinase-induced phosphorylation.

cAMP induces the phosphorylation of the 10 and 16 kDa chymotryptic domains whereas activation of protein kinase C leads to phosphorylation only within the 16 kDa domain on Band 4.1. The 10 kDa domain is thought to contain the spectrin-actin binding site [Correas *et al* 1986]. Changes in spectrin-actin binding may change membrane interactions, morphology and alter microvesicle release, so it may be that the activation of PKA results in morphological alterations not seen with the activation of PKC.

In the previous chapter it was shown that microvesicles are depleted of membrane protein relative to phospholipid by approximately 50%. This result suggests that half of these protein components are associated with the cytoskeleton and therefore not able to diffuse during the microvesiculation process. If phosphorylation of the cytoskeletal proteins leads to decreased associations between cytoskeletal and membrane proteins, one would expect an increased amount of the integral membrane proteins in the microvesicles.

4.2.2. Microvesicle Formation in cAMP and Phorbol Treated Erythrocytes

Erythrocytes were incubated with either dbcAMP or PMA in order to induce the increased phosphorylation of the membrane proteins by activation of PKA or PKC respectively. No changes in the normal morphology of these erythrocytes were apparent under the light microscope.

The erythrocytes were subsequently incubated in the presence of dimyristoyl phosphatidylcholine (DMPC), resulting in the release of microvesicles. The extent of microvesicle release was measured by analysing the AChE content in the supernatant after sedimentation of the treated erythrocytes. As shown in the previous chapter, a strong correlation exists between the amount of AChE release and amount of phospholipid release. No differences could be detected in the amount of AChE release in the control cells and in the cells treated with dbcAMP and PMA. The amount of AChE release was very similar in all three cases, with approximately 30% of the total AChE found in the microvesicles.

These results infer that the phosphorylation of the membrane proteins does not lead to any apparent changes in erythrocyte morphology, nor does it promote or hinder amphipath-induced microvesicle release.

4.2.3. Protein and Polyphosphoinositide Composition of Microvesicles from cAMP and PMA Treated Erythrocytes

As shown previously, microvesicles from erythrocytes with increased protein phosphorylation, were obtained by incubating erythrocytes with cAMP or PMA and subsequently with DMPC. These microvesicles were isolated and analysed by SDS-PAGE and TLC in order to measure the protein and lipid composition of the microvesicles and the parent cells. The results shown in Figure 4.4, suggest that phosphorylation had very little effect on the composition of the microvesicles. In both the microvesicles from the control cells and the "phosphorylated" cells, there was no significant difference in the segregation of integral proteins and polyphosphoinositide which occurs during the microvesiculation process.

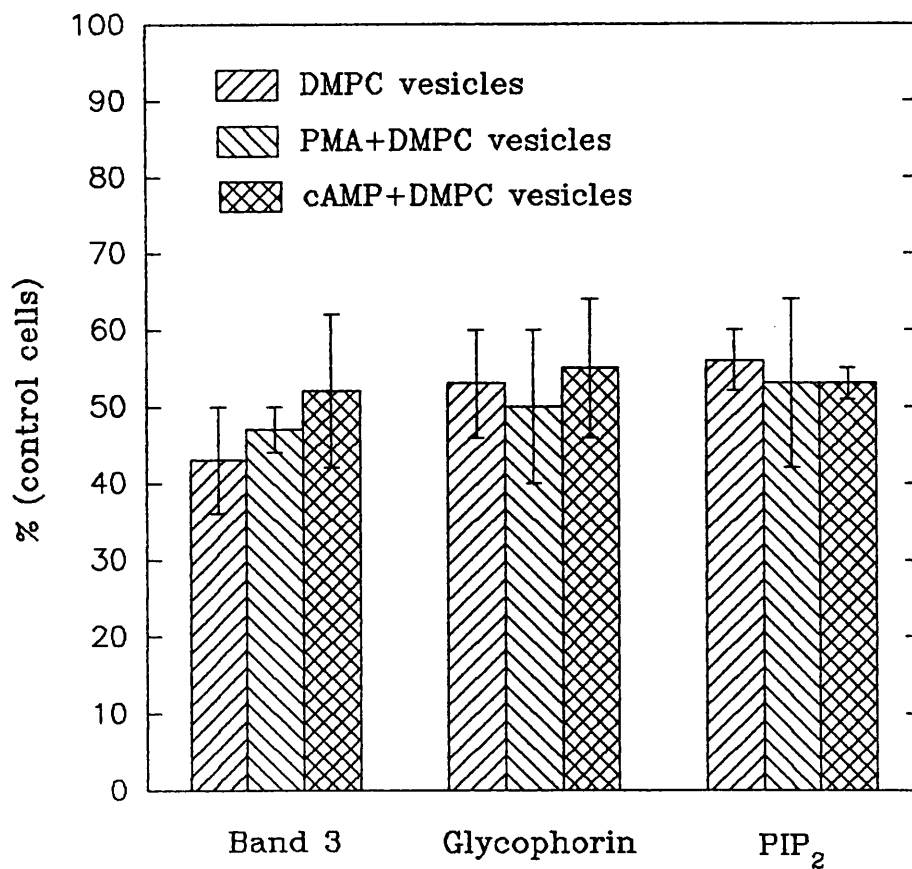
4.3. Segregation of Band 7 into Microvesicles

4.3.1. Introduction

In the previous chapter the segregation of the integral membrane proteins band 3 and glycophorin A into microvesicles from human erythrocytes was discussed. In these experiments it was found that these proteins were depleted in the microvesicles.

Figure 4.4 Band 3, Glycophorin and Inositol 4,5- Bisphosphate Composition Microvesicles from Cells Treated with PMA or cAMP

Erythrocytes were incubated at 37°C with ^{32}P for 2 hours and subsequently with PMA or cAMP for 2 hours as described under Methods. After this pre-incubation, the cells were sedimented and incubated for another 1.5 hours with DMPC and PMA or dbcAMP (to avoid dephosphorylation). The treated cells and microvesicles were separated as described previously. The phospholipids were extracted and analysed by TLC. The proteins were analysed by SDS-PAGE, with Coomassie staining. The results shown are the means \pm S.D. from three experiments for glycophorin and inositol 4,5-bisphosphate and from four experiments for band 3.



From these and other studies a hypothesis was formed suggesting that the isolation and analysis of vesicles represents a way of sampling the freely mobile components of the lipid bilayer, i.e. those components which do not associate with the cytoskeleton. The amount of each protein in the microvesicles and the treated cells was measured by scanning the SDS-PAGE gels. This worked well for Band 3 and glycophorin A because these proteins are present in relatively large amounts and could be measured fairly accurately. This method could not be used for other minor integral proteins such as Band 7. This protein was difficult to measure in the microvesicles as the cytosolic proteins of similar kDa (about 30kDa) often interfered with the relevant SDS- PAGE band.

Band 7 is of interest because it may be involved in the regulation of phospholipid asymmetry [Connor & Schroit 1990]. It was also found to be absent in hereditary stomatocytosis where the cells have an altered morphology and are unusually permeable to K^+ and Na^+ [Stewart 1992]. Band 7 protein, may therefore also be important in the regulation of erythrocyte shape and permeability.

In the erythrocyte several membrane proteins, including Band 7 are phosphorylated when erythrocytes are incubated in the presence of cAMP (see previous section). If ^{32}P is included in the incubation, the extent of phosphorylation can be measured by means of SDS-PAGE and

radioautography. This provides a method by which phosphorylated membrane proteins can be detected in the microvesicles. First the cells are incubated with ^{32}P and then with cAMP. Subsequently microvesicles are produced and the amount of radioactivity in each phosphorylated protein is quantified. If it is assumed that the amount of radioactivity incorporated reflects the amount of protein present, this could give an indication of any segregation between the cell and the microvesicles, and provide information about the interaction of band 7 with the cytoskeleton.

4.3.2. Content of Band 7 in Microvesicles

The results obtained from the SDS-PAGE radioautographs of labelled and cAMP-treated erythrocytes and DMPC microvesicles from these cells showed that approximately 80% of the Band 7 relative to total phospholipid was free to partition into the microvesicles (Table 4.1). This was confirmed by experiments by Stewart & Dash (personal communication) who used a monoclonal antibody to establish the distribution of Band 7 between cells and microvesicles [Stewart 1992].

As mentioned previously, scanning the Coomassie-stained gel was usually unsatisfactory because the cytosolic bands interfered with the Band 7.

Table 4.1 **Content of Band 7 in Control Ghosts, Ghosts from cAMP and DMPC Treated Cells, and Microvesicles from DMPC or cAMP plus DMPC Treated Cells**

Erythrocytes were labelled with ^{32}P . A fraction of these labelled cells were treated with dbcAMP (100 μM) at 37°C for two hours, and both the treated and untreated cells were incubated subsequently with DMPC for two hours. The cells were sedimented, and the microvesicles separated from the supernatant by high speed centrifugation. The proteins were analysed by SDS-PAGE on 15% gels. Quantification of Band 7 was done by scanning both the Coomassie-stained gels and the radioautographs. Results show the content of Band 7, relative to phospholipid, for (a) control ghosts, (b) ghosts from cAMP-treated cells, (c) microvesicles from DMPC-treated cells, and (d) microvesicles from cAMP/DMPC-treated cells.

	(a)	(b)	(c)	(d)
Radioautograph	100	99	--	78.4
Coomassie	100	106	78.9	78.2

However, in one experiment the separation between Band 7 and the cytosolic protein was sufficient to give an estimate of 80% for the relative amount of Band 7 in the microvesicles from both DMPC and cAMP plus DMPC treated cells (Table 4.1). Thus the amount of Band 7 in the microvesicles was not affected by the phosphorylation state of the membrane proteins.

4.4. DISCUSSION

4.4.1. Effect of cAMP-Activated Phosphorylation on the Ca²⁺-Dependent K⁺-Channel Activity of the Erythrocyte

As shown here, and in previous studies, incubation of erythrocytes with cAMP leads to the phosphorylation of several membrane proteins. The results here present some indication of the effects that this phosphorylation may have on the properties of the cell.

As demonstrated previously in other cell types, cAMP-dependent phosphorylation leads to changes in the properties of the ion-channels [Ewald *et al* 1985; Gray *et al* 1990]. In erythrocytes, the Ca²⁺-dependent K⁺-channel activity may be altered after incubation with cAMP, since an increase in the calcium-activated K⁺ efflux was seen (Figure 4.2). It is unclear from these results whether these changes are caused by the phosphorylation of the K⁺ channel itself, or whether phosphorylation of other membrane proteins modulates the channel activity. However, studies in turkey erythrocytes have shown that a membrane protein called goblin undergoes phosphorylation and this is thought to also be the protein involved in the regulation of Na-K-Cl co-transport [Palfrey *et al* 1980].

4.4.2. Decrease in the Phosphatidylinositol 4,5-Bisphosphate

Levels in cAMP-Treated Cells

Another consequence of the changes in the cAMP-activated phosphorylation of the membrane proteins is an indirect effect on the content of phosphatidylinositol 4,5-bisphosphate (PIP₂). The results here indicate that when erythrocytes are incubated with cAMP for a period of up to two hours, the levels of PIP₂ increase slightly compared to control cells. However, when erythrocytes are incubated with cAMP for a period of eight hours the content of PIP₂ but not phosphatidylinositol 4-phosphate (PIP) is markedly reduced.

The results for the shorter incubation period are in agreement with those of others [Enyedi *et al* 1983; Farkas *et al* 1984]. They incubated the plasma membranes of different blood cells with cAMP for a period of five minutes and observed a stimulation in the formation of the polyphosphoinositides. cAMP is thought to stimulate the phosphorylation of the lipid kinases which in turn leads to their activation. Their experiments were carried out on membranes whereas here whole cells were used, so any changes in protein phosphorylation will be slower because the limiting factor is the increase in intracellular cAMP concentration, which is relatively slow with exogenous cAMP added to whole cells.

With the longer incubation period the situation may be more complicated. It may be possible that cAMP lowers the affinity of phosphatidylinositol 4-phosphate kinases for ATP, and so less PIP₂ is formed. This explanation seems unlikely since cAMP does not lead to a decrease in the content of PIP (i.e. the affinity of the phosphatidylinositol kinase for ATP is not affected). Another explanation is that the phosphorylation of the membrane proteins reduces the interaction of PIP₂ with the membrane, allowing more of this lipid to be degraded. This hypothesis however did not reflect the results obtained in the microvesiculation experiments, although other studies support it. In the experiments described here no difference in the proportion of PIP₂ that was free to segregate into microvesicles was detected after incubation with cAMP (see next section). Horne *et al* (1985 & 1990) remarked that the phosphorylation site on protein 4.1 is in the domain promoting the spectrin-actin complex. If this interaction between protein 4.1 and the cytoskeleton is reduced, it may in turn affect the interaction of PIP₂ with the membrane, since it is believed that PIP₂ and protein 4.1 interact [Anderson & Marchesi 1985].

A plausible explanation for the disparity between the results at two or eight hours incubation could be that with the shorter incubation period of two hours a similar situation exists as that of the experiments of Endeyi *et al*, that is, cAMP leads to the activation of phosphatidylinositol 4-phosphate

kinase, whereas with the longer incubation the changes in lipid-protein interaction become more relevant.

4.4.3. Effect of cAMP-Activated Protein Phosphorylation on Microvesicle Release and Composition

Several studies have suggested that protein phosphorylation affects protein-protein interactions within the membrane [Cohen *et al* 1990]. However these changes, which in theory could lead to morphological alterations, are not detected in the experiments described here in cAMP-treated cells. Similarly, in phorbol-treated cells (where protein kinase C is activated) no changes were detected [Raval & Allan 1985].

A destabilisation of the cytoskeleton may also lead to an increase in microvesicle release, as seen in heat-induced microvesiculation [Wagner *et al* 1986], but again the results here, with amphipath-induced microvesiculation, showed no change in microvesiculation. No difference in DMPC-induced microvesicle release was detected between controls cells and cells which had been treated with cAMP. If a change in the affinity between cytoskeletal proteins due to phosphorylation does occur, this change has no evident effect on the microvesiculation process. Increased phosphorylation does not appear to lead to a reduction or increase in microvesicle release.

Although protein phosphorylation has been postulated to play a role in the regulation of erythrocyte morphology, the results here suggest that changes in phosphorylation are not sufficient or necessary to cause alterations in morphology leading to microvesicle release.

If a reduction in the association between the integral membrane proteins band 3 and glycophorin, or PIP₂ (as discussed previously) and the cytoskeleton does occur after cAMP-activated protein phosphorylation, it is expected that this would be reflected in the composition of the microvesicles. It is assumed that microvesicles contain those proteins and lipids that are free to diffuse in the plane of the membrane. In microvesicles from cAMP-treated cells, the proportion of band 3, glycophorin or PIP₂ is no different to those microvesicles from control cells, so either there is no change in the membrane associations after phosphorylation, or any changes which occur have no effect on the proportion of the integral proteins or PIP₂ that is free to segregate into the microvesicles.

4.4.4. Segregation of Band 7 into Microvesicles

These results suggest that approximately 80% of this protein is free to partition into the microvesicles, with 20% unable to diffuse, possibly because of interactions with the cytoskeleton (Table 4.1). In contrast, Yu *et al* (1973) found that only a small fraction of Band 7 was solubilised by Triton-X100

(therefore not associated with the cytoskeleton), with the rest being part of the Triton shells. Thus Band 7 is anomalous in being a constituent of the bilayer on the basis of its partition into microvesicles but being associated with the cytoskeleton on the basis of Triton solubility. It may be that is a protein with binding affinities both to the lipid bilayer and the skeleton. Band 7 is also unusual in being the only protein which partitions into the microvesicles which also shows an increased phosphorylation with cAMP.

One possibility, that cannot be ruled out, is that the phosphorylation itself causes changes in the way this protein is interacting with the cytoskeleton. In other words, that phosphorylation changes the mobility of Band 7 and therefore the amount of protein free to diffuse into the microvesicles. The results presented here suggest that this is unlikely, since a similar content of Band 7 was seen in the microvesicles from DMPC-treated cells and DMPC-treated cells that had been incubated with cAMP (Table 4.1).

5. CONCLUSIONS

5.1. Changes in Erythrocyte Morphology Leading to Microvesicle Release

The work presented here supports the now widely accepted theory that changes in erythrocyte morphology from discocyte to echinocyte or discocyte to stomatocyte occur when the balance between the two leaflets of the lipid bilayer is disrupted. Intercalation of amphipathic molecules into one side of the membrane, or breakdown of one type of membrane lipid, results in the expansion or contraction of one of the membrane layers relative to the other. The results here demonstrate that expansion (or contraction) by as little as three per cent in the area of one leaflet relative to the other leads to membrane bending, and in the extreme case, causes vesicle release.

A reasonable assumption is that only those membrane components which are free to diffuse laterally are found in the microvesicles, making vesicle release a way of fractionating those membrane components which are laterally mobile in the bilayer. This assumption is supported here in the results showing that the protein composition of the microvesicle membranes is markedly different to that of the original cells. The microvesicles are totally depleted of the cytoskeletal proteins and partially depleted of the intrinsic

membrane proteins (band 3 and glycophorin). Band 7 is also depleted, but to a lesser extent. This assumption is further reinforced by the results that acetylcholinesterase (thought to diffuse freely) was found to be enriched in the microvesicles. The results are also consistent with the results of others, which showed by different methods, that a fraction of the membrane proteins are not free to diffuse laterally.

The lipid composition of microvesicles formed as a consequence of outer membrane layer expansion (treatment with MC540 or DMPC) mirrors that of the original cell. An exception is represented by the polyphosphoinositides which are relatively depleted by approximately 50%. Again, a fraction of these lipids is also believed to interact with the cytoskeleton, thus restricting their mobility.

Depleting the cells of ATP or incubating them with calcium plus ionophore causes a decrease in the inner lipid leaflet relative to the outer, resulting in similar morphological changes to those seen with MC540 and DMPC.

Microvesicles from calcium-treated and ATP-depleted cells are also depleted of a fraction of the polyphosphoinositides, band 3 and glycophorin. In the case of ATP-depletion this occurs to a lesser extent, implying that the interaction between these membrane components and the cytoskeleton is reduced after ATP-depletion.

The proposal that an imbalance in the lipid leaflets is sufficient to cause vesiculation is strengthened by the results demonstrating that after incubation of cells with an amphipathic molecule (MC540) no change in phospholipid asymmetry was detected. This suggests that vesiculation events are not dependent on, or do not result in, a loss of lipid asymmetry.

Unsuccessful attempts were made to isolate endovesicles (produced as a result of sphingomyelin breakdown) after lysing the treated cells. It is possible that, unlike external microvesicles, these internal vesicles stay attached to the membrane, making them difficult to isolate. It would have been of interest to analyse the membrane composition of the endovesicles and to compare them to the external microvesicles.

5.2. Effect of Protein Phosphorylation on the Properties of the Membrane

Phosphorylation of membrane protein components through the activation of kinases by cAMP or phorbol may result in changes in the properties of the erythrocyte. An example of this is the increase of the Ca^{2+} -activated K^+ release from erythrocytes which had been incubated with cAMP. From the experiments here it is difficult to ascertain whether the K^+ channels are regulated directly through a change in their phosphorylation state or

indirectly through a change in the phosphorylation of another protein which in turns regulates the K^+ channel.

A difference in the content of PIP_2 was also apparent between control cells and cells which had been incubated for several hours with cAMP. This may be a consequence of protein phosphorylation causing a decrease in the interaction between PIP_2 and the cytoskeleton resulting in increased breakdown of this lipid. No evidence however, could be found for morphological alterations or changes in amphipath-induced microvesicle release following increased phosphorylation of the membrane proteins.

Two of the intrinsic membrane proteins, band 3 and glycophorin were present in the same proportion in microvesicles from control or cAMP/phorbol-treated cells. Assuming that the microvesicles contain a fraction of the proteins which are free to diffuse because they are not attached to the cytoskeleton, increased phosphorylation did not appear to have an effect on the interaction between band 3 or glycophorin and the cytoskeletal proteins.

Finally, this work shows that preparation of exovesicles using an amphipathic molecule such as MC540 is a useful way of analysing the organisation of mobile erythrocyte membrane constituents, and suggests that this organisation is sensitive to metabolic influences.

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David Allan¹, Carla Hagelberg¹, Karl-Josef Kallen² and Cees W.M. Haest²

¹ Department of Physiology, University College and Middlesex School of Medicine, London (U.K.)

and ² Institut für Physiologie der Medizinischen Fakultät der Rheinisch-Westfälischen Technischen Hochschule Aachen, Aachen (F.R.G.)

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Echinocytosis and release of microvesicles from human erythrocytes treated with the impermeant fluorescent dye merocyanine 540 (MC540) has been correlated with the extent of dye binding to intact cells and ghosts. At 20 °C binding appeared to saturate at about $9.3 \cdot 10^6$ molecules per cell (3.6 mol/100 mol phospholipid), equivalent to an expansion of the outer leaflet lipid area of about 2.7%. Stage 3 echinocytes were formed upon binding of $(3-4) \cdot 10^6$ molecules of MC540/cell (about 1.3 mol/100 mol phospholipid), equivalent to an expansion of the outer leaflet lipid area of about 1.0%. Negligible release of microvesicles was observed with MC540 at 20 °C. Binding of MC540 to permeable ghosts was approximately twice that to cells suggesting that there was no selective binding to the unsaturated (more fluid) phospholipids which are concentrated in the inner lipid leaflet of the membrane. At 37 °C apparent maximal binding of MC540 was about 3.2 mol/100 mol phospholipid and correlated with the maximal release of microvesicles from the cells as measured by release of phospholipid and acetylcholinesterase. These results are discussed in relation to the bilayer couple hypothesis of Sheetz and Singer (Proc. Natl. Acad. Sci. USA 71 (1974) 4457–4461).

Introduction

The bilayer couple hypothesis of Sheetz and Singer [1], which built on earlier work of Deuticke [2] and Evans [3], sought to explain drug-induced changes in erythrocyte morphology in terms of the preferential intercalation of drug molecules into one of the leaflets of the membrane lipid bilayer, leading to differential expansion of that leaflet and a consequent local curvature of the membrane. Support for this attractive and simple idea has grown over the last decade [4–7] and the hypothesis has been generalised to include cases where differential contraction of either of the lipid leaflets by removal of lipid also gives rise to predictable changes in morphology [8–11]. However, estimates of the amount of outer lipid leaflet expansion necessary to induce echinocytosis have ranged from 1.5% to 4% [4–7], generally higher than theoretical estimates of 0.7% [7] or 0.4% [12] based on geometrical considerations. Under some circumstances echinocytosis induced

by outer lipid leaflet expansion [13,14] or inner leaflet contraction [15,16] can proceed to the point where membrane material is lost to the medium in the form of microvesicles, but there appears to be no information regarding the precise relative change of membrane leaflet area necessary to cause microvesiculation.

We were initially interested in the binding of MC540 to erythrocytes because of reports [17–19] that this hydrophobic fluorophore only bound to cells which had undergone a rearrangement of their normal asymmetric distribution of phospholipids resulting in an increased fluidity of the outer lipid leaflet. It was suggested that phosphatidylethanolamine and phosphatidylserine which are rich in unsaturated (fluid) fatty acids and which are normally mainly confined to the inner lipid leaflet of the erythrocyte membrane [20] can under some circumstances migrate to the outer leaflet and there promote the binding of MC540 which was presumed to interact strongly with fluid lipids but not with gel-phase lipids [18]. The results of our experiments do not support this suggestion and indicate that MC540 binds equally well to inner and outer leaflet lipids. However, MC540 proved to be an appropriate tool with which to relate the degree of outer leaflet expansion to

Correspondence: D. Allan, Department of Physiology, University College London, London, WC1E 6JJ, U.K.

echinocytosis and microvesicle release, for the following reasons. Firstly, insertion and orientation of MC540 in the membrane can be accurately analysed. Secondly, MC540 selectively inserts into the outer membrane leaflet because its transbilayer migration is very slow [18]. Thirdly, MC540 is a compact and rigid molecule so that from its molecular volume its membrane expanding effect can be easily calculated.

Methods

MC540 was obtained from Sigma Chemical Co. and was dissolved at a concentration of 1 mM in ethanol or 10 mM in dimethylsulphoxide. Egg phosphatidylcholine and dimyristoylphosphatidylcholine were also obtained from Sigma and were sonicated at a concentration of 1 mM in 150 mM NaCl, 10 mM Hepes-NaOH buffer, 0.1 mM EDTA (pH 7.4) using an MSE sonicator (12 μ peak to peak amplitude).

Emission spectra were measured in a Perkin-Elmer luminescence spectrometer using an excitation wavelength of 540 nm. The spectrum of 1 ml of a 100 nM solution of MC540 was analysed in 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.5) in the presence of (a) washed erythrocyte membranes (100 nmol lipid phosphorus); (b) 100 nmol egg phosphatidylcholine sonicated for 5 s; (c) 100 nmol of dipalmitoylphosphatidylcholine sonicated as for (b) but for 20 s; (d) 0.5 mg of bovine serum albumin. The emission spectrum of 100 nM MC540 was also measured in various organic solvents including a range of homologous *n*-alkanols, chloroform and benzene.

Fresh human blood (20 ml) was collected in 150 mM NaCl containing 1 mM EGTA and erythrocytes were washed three times by centrifugation in 50 ml of 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.4). White ghosts were prepared from 1 ml of packed cells by lysis in 50 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.6) containing 0.1 mM EGTA followed by three washes by centrifugation ($30\,000 \times g$ 10 min) in the same solution.

Various concentrations of MC540 (0–20 μ M) were added to washed human erythrocytes (20 μ l of packed cells) or packed ghosts (20 μ l) suspended in 1-ml aliquots of 150 mM NaCl 10 mM Hepes-NaOH buffer (pH 7.5) in 1.5 ml Eppendorf plastic tubes. Parallel control samples containing MC540 but no cells or ghosts were also prepared to correct for non-specific binding to the tubes. After incubation at 20°C for 5 min the samples were centrifuged in an Eppendorf bench centrifuge at 14000 rpm for 5 min and the supernatant solution was removed. The pellet was resuspended in 1 ml of the original buffer and a 50- μ l sample of the suspension was added to 1 ml of *n*-butanol and vortexed for 10 s. The amount of MC540 in the butanol solution was measured from its fluorescence emission at 580 nm in comparison with a standard 100 nM solution of MC540

in butanol using excitation at 540 nm in a Perkin-Elmer LS-5 fluorimeter. Reextraction of the residual cell pellet with butanol, ethanol or 2:1 methanol/chloroform showed that the initial butanol extract of the cells accounted for > 99% of MC540 originally bound and that the results were not significantly (< 2%) affected by contamination with unbound dye. The remaining resuspended cells and ghosts were extracted with 2:1 methanol/chloroform and their total phospholipid content was assayed as described previously [21].

In some experiments separate samples of cells were incubated with MC540 as above and echinocyte formation at each concentration of dye was assessed by light microscopy of the suspension between two plastic cover slips. The degree of echinocytosis was quantified according to the nomenclature of Bessis [22].

Experiments to measure release of microvesicles were carried out as above but at 37°C in glass tubes and using a total volume of 4 ml. Microvesicle release was quantified in terms of phospholipid and acetylcholinesterase lost from the cells as described previously [13,21]. Cell lysis was measured spectrophotometrically (418 nm) and was expressed as the percentage of total cell haemoglobin which was not sedimentable after high speed centrifugation.

Estimates of the dimensions of the MC540 molecule were made from a computer display using Desktop Molecular Modeller (Oxford Electronic Publishing, Oxford University Press, Oxford, U.K.). The dimensions of the hydrophobic part of the molecule were found to be $18.5 \times 9 \times 4.5$ Å, giving a cross-sectional area of about 40 Å². These values agree with a calculation of the membrane volume of the hydrophobic portion of MC540 based on the work of Kita and Miller [23], which gave a value of 700 Å³.

Results

Binding of MC540 to cells and membranes at 20°C (Fig. 1) increased with the concentration of dye but appeared to saturate at about 15 μ M. It was not practicable to investigate the effects of concentrations higher than 20 μ M because the dye became increasingly insoluble. Even at lower concentrations some problems were experienced with non-specific binding of dye to the tubes but this was largely overcome by the use of suitable controls. The maximum amount of dye bound to cells was 3.6 ± 0.4 mol/100 mol lipid phosphorus and the equivalent value for the ghosts (which were freely permeable to small molecules [24]) was 7.4 ± 0.9 . With increasing concentration MC540 induced a progressive change in shape of the cells from discocyte to spherocochinocyte as shown in Fig. 2. Stage 3 echinocytes were produced when binding of MC540 was $(3-4) \cdot 10^6$ molecules per cell.

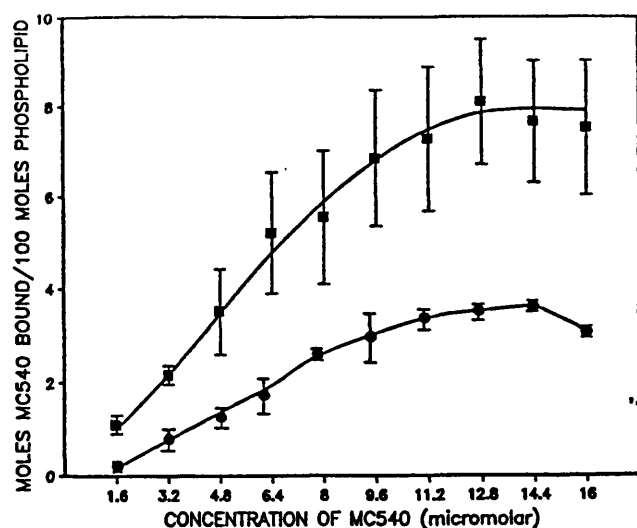


Fig. 1. Binding of MC540 to human erythrocytes and ghosts at 20°C. Various concentrations of MC540 were added to washed human erythrocytes or erythrocyte ghosts and dye binding was measured as described under Methods. Results are shown as means \pm S.D. from four experiments with different samples of cells (●) and ghosts (■).

Cell morphology was assessed at 20°C because it was easier to maintain a constant temperature close to ambient on the microscope slide and the same temperature was used when measuring binding of MC540 to cells and ghosts so that a direct comparison under the same conditions could be made of MC540 binding and shape changes. Microvesicle release was not seen below 20°C but was maximal at 37°C so that the latter temperature was used in those experiments where microvesicles were measured. MC540 was more soluble at

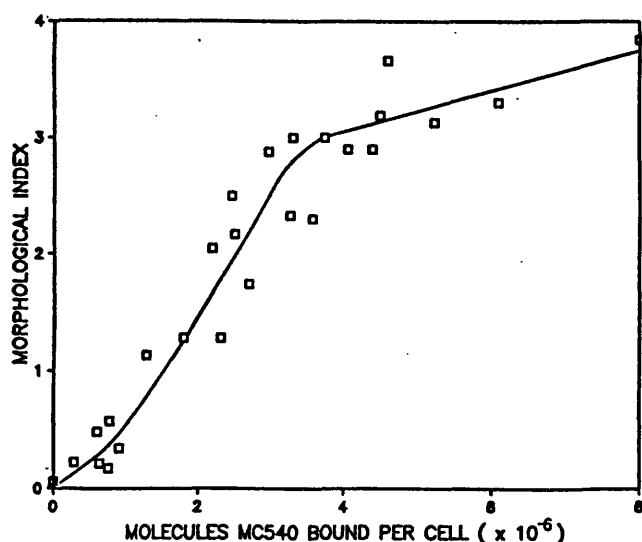


Fig. 2. Morphological index of erythrocytes treated with MC540 at 20°C. Cells were exposed to MC540 in an experiment similar to that shown Fig. 1. Results from four experiments using haematocrits between 0.5% and 2% are combined to illustrate the relationship between MC540 bound per cell and the stage of echinocytosis according to the nomenclature of Bessis [22].

the higher temperature so that it was possible to employ higher concentrations of the dye than at 20°C.

Release of microvesicles from cells incubated at 37°C for 10 min, as judged from phospholipid and acetylcholinesterase activity in the supernatant solution, was maximal at 20 μ M MC540 using a 10-min period of incubation (Figs. 3b and 3c). No microvesicles were released below 5 μ M MC540 and the apparent maximum release of microvesicles coincided with maximal binding of the dye (about 3.2 mol/100 mol phospholipid) (Fig. 3a). Approx. 10% of total cell phospholipid and 20% of acetylcholinesterase were released under these conditions where cell lysis was 2–3% (Fig. 3d) and 90% of the released material could be sedimented after centrifugation for 1 h at 100 000 \times g. Like microvesicles released from erythrocytes by other procedures [13,21,25], these microvesicles contained Band 3 but were largely free of actin and spectrin as judged by electrophoresis on polyacrylamide gels (Fig. 4) although not significantly different from cells in their phospholipid composition (Table I).

In order to obtain an indication of the site at which MC540 bound, the spectrum of the dye was analysed when bound to ghosts and compared with the spectrum of the dye when bound to bovine serum albumin, egg phosphatidylcholine, or dipalmitoylphosphatidylcholine (Fig. 5) and when dissolved in solvents of different polarity (Fig. 6). As shown previously for the absorption spectrum [26], the fluorescence emission maximum of MC540 was red-shifted in solvents of decreasing polarity (Fig. 5) and it was thus possible to assess the effective dielectric constant of the environment of MC540 in a variety of situations. When interacting with erythrocyte membranes the emission maximum of MC540 was shifted from 565 nm in the aqueous medium to 583.3 nm which indicates an environment for the dye which was similar in hydrophobicity to *n*-decanol. An identical change is observed when MC540 interacts with phospholipid vesicles whereas binding to albumin (a protein which has strong binding sites for hydrophobic

TABLE I

Phospholipid composition of (a) untreated red cell ghosts, (b) ghosts from MC540-treated cells, (c) microvesicles

Phospholipids were extracted and analysed as described previously [21]. Results represent mole percent of total phospholipid and are shown as means \pm S.D. from three experiments, each carried out with duplicate samples.

	Phospholipid composition		
	(a)	(b)	(c)
Phosphatidylethanolamine	29.9 \pm 0.3	28.8 \pm 0.9	28.8 \pm 1.9
Phosphatidylserine	15.6 \pm 1.9	14.0 \pm 0.5	12.9 \pm 2.0
Phosphatidylcholine	28.4 \pm 0.7	29.2 \pm 1.3	30.2 \pm 3.3
Sphingomyelin	23.2 \pm 1.4	25.0 \pm 1.8	23.8 \pm 3.4

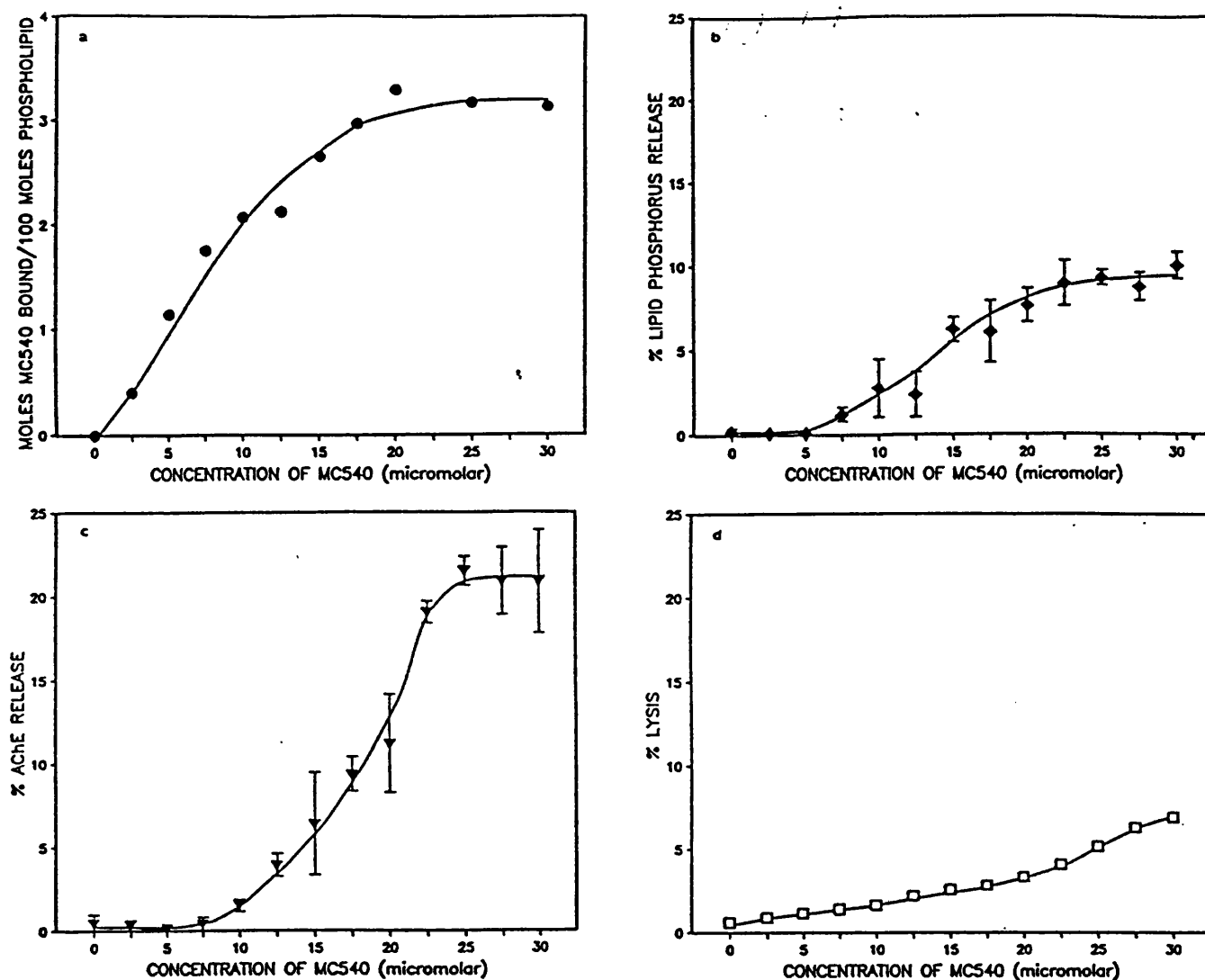


Fig. 3. Binding of dye and release of phospholipid and acetylcholinesterase from erythrocytes treated with MCS40 at 37°C. After incubation with various concentrations of MCS40 for 10 min at 37°C as described under Methods, cells were sedimented and binding of dye (a) was measured as described above. The supernatant solution was assayed for phospholipid (b), acetylcholinesterase activity (c) and haemoglobin release (d). Results shown are the means \pm S.D. from four experiments with different samples of cells. Values for phospholipid and acetylcholinesterase release are corrected for lysis.

molecules) gives an emission maximum at 574 nm, indicative of a relatively hydrophilic environment for the MCS40 (Fig. 5). These data indicate that the major site of MCS40 interaction with membranes is the hydrophobic lipid region and not with membrane protein. The magnitude of the fluorescence maximum of MCS40 when interacting with egg phosphatidylcholine was generally larger than with dipalmitoylphosphatidylcholine but this difference became progressively less when the dipalmitoylphosphatidylcholine was sonicated for longer periods. Similar findings were reported by Schlegel et al. [17] who suggested that this was due to enhanced binding of MCS40 to very small vesicles whose acute curvature allowed the dye to enter the bilayer. However, it was clear in our hands that it required much more sonication to obtain a clear solution from dipalmitoyl-

phosphatidylcholine than from egg phosphatidylcholine and this could have the consequence that the proportion of the phospholipid present as unilamellar vesicles was relatively less with dipalmitoylphosphatidylcholine. Since it is apparent that MCS40 interacts only with phospholipid monolayers which are exposed to the external aqueous medium this factor rather than any intrinsic difference in ability to bind MCS40 between saturated and unsaturated phospholipids could be responsible for the differences observed.

Discussion

Normal human erythrocytes bind significant quantities of MCS40, equivalent to a maximum of 3.6 mol/100 mol phospholipid or to about $9 \cdot 10^6$ molecules per cell

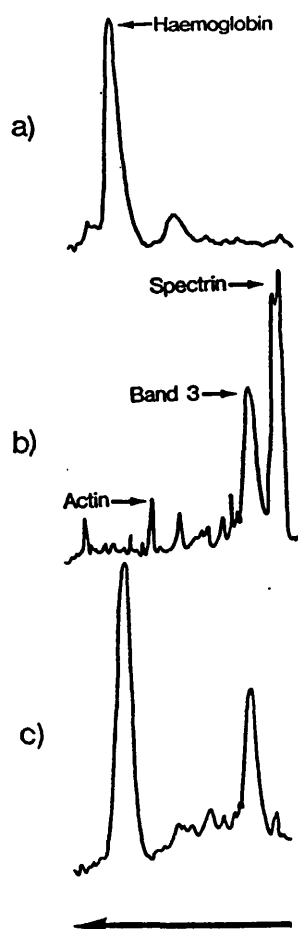


Fig. 4. The polypeptide pattern of vesicles from MC540-treated erythrocytes. The polypeptide patterns of (a) cytosolic proteins, (b) erythrocyte membrane proteins and (c) microvesicle proteins were analysed by SDS-PAGE (11% w/w gel) using the method of Laemmli [42] and a Bio-Rad Minigel apparatus. Bands were visualised with Coomassie-brilliant blue stain and scanned using a Bio-Rad 1650 Scanning Densitometer linked to a Shimadzu C-R3A integrator.

(Fig. 1). This finding appears to conflict with the work of Schlegel and coworkers [17] who claimed that normal erythrocytes do not bind this dye, but their measurements were made in the presence of serum which contains albumin and lipoprotein that would certainly compete with cells for binding MC540. However, in the absence of serum the binding of MC540 to erythrocytes observed by Schlegel et al. was as much as $3 \cdot 10^7$ molecules per cell, more than 3-fold greater than our values. Part of this apparent discrepancy might be due to the use of avian erythrocytes by Schlegel et al. but more significantly, these authors used low ionic strength buffer which could have allowed extra ionic binding of MC540 to the cells.

Erythrocyte ghosts, which in contrast to the intact cells are permeable to MC540, bind twice as much MC540 as cells, for the same amount of lipid phosphorus (Fig. 1) and this suggests that inner leaflet phospholipid has no greater affinity for the dye than

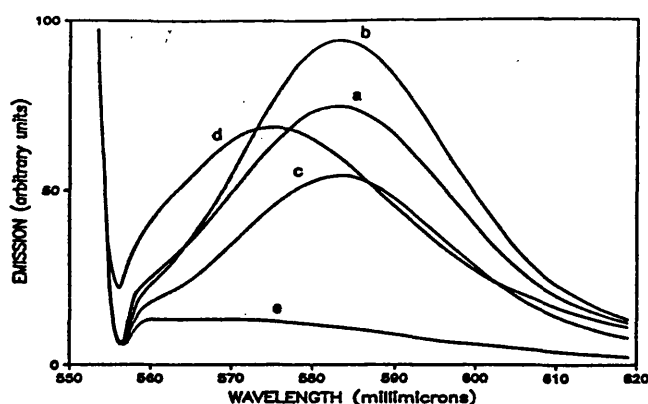


Fig. 5. The spectrum of MC540 in the presence of an excess of erythrocyte plasma membranes, egg phosphatidylcholine, dipalmitoylphosphatidylcholine or bovine serum albumin. The emission spectrum of 1 ml of a 100 nM solution of MC540 was analysed in 150 mM NaCl, 10 mM Hepes-NaOH buffer (pH 7.5) in the presence of (a) washed erythrocyte membranes (100 nmol lipid phosphorus), (b) 100 nmol egg phosphatidylcholine sonicated with a sonic probe for 5 s, (c) 100 nmol dipalmitoylphosphatidylcholine sonicated as for (b) but for 20 s, (d) 0.5 mg of bovine serum albumin, (e) buffer alone.

outer leaflet phospholipids despite the considerable difference in content of unsaturated fatty acids between the two leaflets. Only at the lowest concentration of MC540 ($1.6 \mu\text{M}$) was there a slight indication of a higher affinity of ghosts for the dye and this could suggest preferential binding to inner leaflet lipid. These findings support our earlier conclusions regarding the

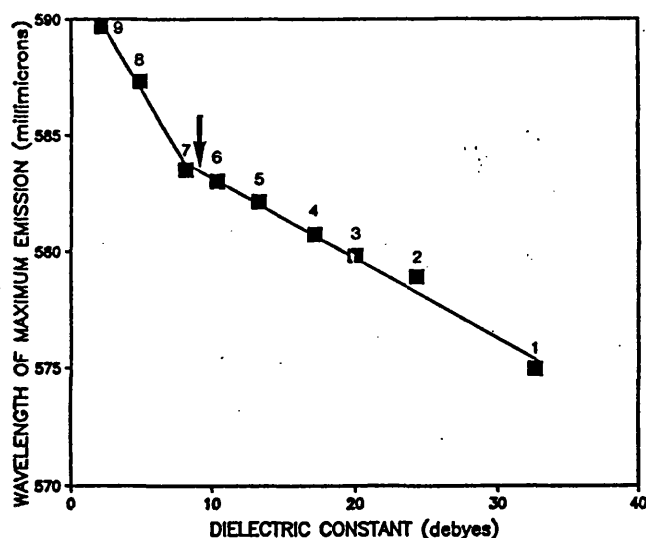


Fig. 6. The dependence of the fluorescence emission peak wavelength of MC540 on the solvent polarity. The spectrum of a 100 nM solution of MC540 was analysed in solvents of different polarity using a Perkin-Elmer LS-5 luminescence spectrometer. Fig. 6 illustrates the relationship between solvent dielectric constant and the wavelength of maximum fluorescence following illumination at 540 nm. 1, methanol; 2, ethanol; 3, *n*-propanol; 4, *n*-butanol; 5, *n*-hexanol; 6, *n*-octanol; 7, *n*-decanol; 8, chloroform; 9, benzene. The arrow marks the wavelength of maximum emission for MC540 in the presence of an excess of erythrocyte membrane.

interaction of MC540 with ghosts and microvesicles from human erythrocytes [27] but do not support the claims of Schlegel et al. [17–19] that inner leaflet but not outer leaflet lipid binds MC540. Our experiments comparing the spectral changes undergone by MC540 after addition of saturated or unsaturated lecithins (Fig. 5) also suggest that any differences in the interaction of the dye with lipids are not due to differences in saturation *per se* but rather to variations in the micellar form of the phospholipid. The observations that ghosts bind about twice as much MC540 as intact cells and that binding to the erythrocytes does not increase upon prolonged incubation are further confirmation that once bound to the surface of intact cells MC540 has little tendency to undergo transbilayer migration. It is noteworthy that binding of MC540 to erythrocytes or to ghosts is a saturable process (Figs. 1 and 3a), perhaps limited by the surface pressure of membrane lipids, by the increase in surface charge due to the introduction of anionic dye into the bilayer [28] or by specific constraints imposed by the underlying membrane skeleton [29]. Thus factors which affect surface charge, pressure or skeletal interactions may in turn affect the binding of dye and the observed changes in fluorescence.

There has been considerable speculation regarding the precise mode of interaction of MC540 with cell membranes, particularly among those workers interested in the small increase in fluorescence which parallels the action potential in stimulated nerves exposed to MC540 [30]. It has been suggested that this change in fluorescence reflects the rotation of the MC540 molecule from a position parallel to the fatty acid chains of membrane lipids to a position at right angles to those molecules [31,32]. It is not clear what are the relative proportions of these two orientations but it would require only a very small proportion of the total bound MC540 to change its orientation in order to explain the small changes observed.

The fluorescence spectrum and hence the environment of MC540 in erythrocyte membranes appears to be almost indistinguishable from that in a sonicate of pure phospholipid (Fig. 5) and this environment seems to be a hydrophobic one which corresponds to a solvent similar in polarity to *n*-decanol, with a dielectric constant of about 9 (Fig. 6). This value is very similar to that measured by Lelkes and Miller from absorption spectra [26] although curiously, these workers concluded that the dye was most likely to be oriented parallel to the surface of the membrane with the chromophore interacting mainly with phospholipid headgroups and apparently accessible to the aqueous medium. On the basis of the spectral evidence we consider it to be more likely that most of the bound dye is oriented so that a large part of the long chromophore is associated with the most hydrophobic region of the membrane, i.e., where the long axis of the molecule lies parallel with the

fatty acid chains of membrane phospholipids. Although there are obvious difficulties in comparing the environment of a dye in an anisotropic membrane environment with the same dye in an isotropic solvent, it seems to us probable that if MC540 really had major interactions with phospholipid headgroups as suggested by Lelkes and Miller, then its spectrum in the membrane would not resemble its spectrum in decanol.

Assuming that intercalation of MC540 between the fatty acid chains of outer leaflet phospholipids accounts for most of the binding of MC540 to intact cells it is possible to calculate the expansion of the outer leaflet corresponding to maximum binding of the dye. Thus taking the cross-sectional area of the MC540 molecule as 40 \AA^2 (based on measurements of molecular models) and assuming that one cell contains $4.3 \cdot 10^{-16}$ mol of phospholipid [33] and has a surface area of $138 \cdot 10^8 \text{ \AA}^2$ [11] then introduction of 3.6 mol MC540/100 mol total phospholipid into the outer lipid leaflet will expand its area by 2.7%. The amount of dye binding necessary to convert all the cells to stage 3 echinocytes is about a third of this value, i.e., a 1.0% expansion. This is less than the lowest estimates previously reported for outer lipid leaflet expansion in echinocytosis [4–7] but greater than estimates obtained theoretically [7,12]. The difference between the experimental values and the theoretical estimates may be due in part to an expansion of the inner lipid leaflet in response to outer leaflet expansion, particularly when the membrane-expanding agent has a long hydrophobic region (e.g., phosphatidylcholine) which can penetrate to the core of the bilayer [10]. Nevertheless, the clear relationship between MC540 binding and shape change is further evidence that the binding that we have measured is not superficial but involves the insertion of specific numbers of dye molecules between the fatty acid chains of the outer leaflet phospholipids as envisaged in the bilayer couple hypothesis [1]. We cannot be certain that all the MC540 is bound in the same region of the membrane; it is possible that some of the dye is bound at superficial sites of hydrophilic character which would not give rise to a visible fluorescence. However, in this case the degree of membrane expansion required to cause the observed morphological changes would be even less than the 1% value calculated above.

At 37°C and at levels of MC540 binding beyond those sufficient to cause echinocytosis the cells respond by shedding microvesicles into the medium (Fig. 3) in a process analogous to that induced by incubation with dimyristoylphosphatidylcholine which is presumed to also act by intercalating between membrane phospholipid fatty acid chains so as to cause unilateral lipid leaflet expansion. However, much less acetylcholinesterase was released by MC540 compared with that seen with dimyristoylphosphatidylcholine [13] (20% versus 70%) and there was no time-lag in the release

with MC540 (results not shown) unlike the latter compound. The faster effect of MC540 on microvesicle release could be due to a more rapid partition of the dye into membranes because the concentration of the dye monomer would be much higher than in the case of dimyristoylphosphatidylcholine, most of which would be in the form of liposomes. The extent of microvesicle release with MC540 and the acetylcholinesterase specific activity of the microvesicles seemed to be much more similar to the equivalent values of microvesicles from cells treated with A23187/Ca²⁺ [21] or with glycocholate [25] than from cells exposed to dimyristoylphosphatidylcholine [13]. Possibly some of the acetylcholinesterase is released in a soluble form in the latter case.

As expected, maximum release of microvesicles correlated with maximum binding of MC540 but cell lysis continued to increase at higher concentrations of dye. If as seems likely, a membrane fusion event is involved in the release of microvesicles then this process only takes place when the expansion of the outer leaflet of the membrane is greater than 1% and is maximal when the expansion is about 2–3%. The marked temperature dependence of microvesicle release, with little release seen below 20°C may be a reflection of a temperature-sensitive decrease in lipid fluidity which affects membrane fusion.

The conclusion that as little as a 1% change in relative area of inner and outer leaflets of the erythrocyte membrane can cause echinocytosis and that a 1–3% change can precipitate vesiculation events has further consequences for erythrocytes and other cells. Thus the echinocytosis and microvesiculation which results from Ca²⁺ entry into human erythrocytes [15,21] or the echinocytosis caused by energy depletion might be explained by a relative diminution of inner lipid leaflet area due to activation of a membrane-bound phospholipase C [34] or phosphatase [35] specific for polyphosphoinositides which account for about 2–3% of inner leaflet lipids. Ferrell and Huestis [35] have estimated that the diminution of the inner monolayer area when phosphatidylinositol 4,5-bisphosphate and phosphatidate are dephosphorylated by energy depletion is about 0.65%. A larger effect would be expected if all the polyphosphoinositides were converted to diacylglycerol by an endogenous phospholipase C especially as diacylglycerol can potentially diffuse into the outer leaflet causing an additional increase in the difference between inner and outer leaflet areas [9]. Phospholipases C similar to that in the red cell are present in the plasma membranes of other cell types where they appear to be involved in the transduction of certain extracellular signals [36], although it is not known if inositide breakdown in these cells has any direct effect on plasma membrane morphology. Certainly there is some evidence in red cell ghosts that the levels of polyphosphoinositides can influence the shape and deformability of

membranes [37]. On the other hand, Ca²⁺-induced decrease in membrane stability has been explained in terms of a decreased association between membrane skeletal proteins mediated either by Ca²⁺-calmodulin [38] or by Ca²⁺-dependent activation of a phospholipase C which attacks polyphosphoinositides [39]. However, it is interesting to speculate that the unilateral insertion of lipids into membranes which appears to occur during lipid biosynthesis in the endoplasmic reticulum [40] and during the process of phospholipid redistribution mediated by cytoplasmic carrier proteins [41], results in predictable changes in membrane curvature which influence subsequent fusion events in those membranes.

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Segregation of proteins and polyphosphoinositides into microvesicles derived from red cells treated with Merocyanine 540

David Allan & Carla Hagelberg

Department of Experimental Pathology, University College London Medical School
University St. London WC1E 6JJ UK.

Release of exovesicles or endovesicles from erythrocytes represent pathological events which occur as part of the natural process of ageing, in certain disease states and also in response to a variety of different chemical treatments. Although the mechanism of red cell vesiculation is poorly understood, the erythrocyte does represent a relatively simple membrane system which may give us useful information regarding the general mechanisms of vesiculation events in cell biology which include such important processes as endocytosis and the release of enveloped viruses.

Red cell vesiculation can be seen as an extreme consequence of a change in normal cell morphology which leads to an locally increased curvature of the plasma membrane. Normal membrane morphology appears to depend on a balance between the lipid bilayer and the spectrin-actin protein skeleton which underpins the bilayer; an imbalance or uncoupling of these two laminar structures allows local membrane curvature which may proceed as far as vesicle release. Suggested mechanisms for red cell vesiculation have generally fallen into two classes; firstly those based on the idea that a defect in the protein skeleton underpinning the lipoprotein bilayer allows herniation and vesiculation of the bilayer and secondly those which suggest that a change in the area of one leaflet of the lipid bilayer relative to the other is the important factor which causes the initial local curvature of the membrane preceding vesiculation. However, both mechanisms can be seen to represent some kind of uncoupling of the membrane skeleton from the bilayer.¹

There is no doubt that defects in the protein skeleton can lead to a destabilisation of the interaction between bilayer and skeleton which allows vesiculation to occur². However, in this article we wish to concentrate on examples of vesiculation which do not appear to involve changes in the membrane skeleton but which seem to be associated with changes in membrane lipid area. The basis of this work goes back to the original suggestions by Deuticke³ and Sheetz & Singer⁴ that localised membrane bending induced by drugs or enzymic attack occurred in a predictable vectorial direction as a result of a change in relative areas of each leaflet of the lipid bilayer. While this 'bilayer couple hypothesis' was only intended to explain the direction of

localised membrane bending it is clear that it could be relevant to the nature of vesiculation events which can be seen as an extreme response of the cell to local membrane curvature.

Although there are good examples of endovesiculation which are induced by decreases in the relative area of the outer leaflet of the lipid bilayer⁵ or by increases in the relative area of the inner leaflet, in general the endovesicles have not been well characterised. For this reason we wish to concentrate our attention here on some well-known cases of exovesicle release from red cells. Perhaps the clearest example of this is obtained by treatment of cells with dimyristoylphosphatidylcholine⁶ which appears to partition selectively into the outer leaflet of the membrane causing an expansion that is accommodated by echinocytosis and eventually results in vesiculation. The vesicles which are released are devoid of spectrin or actin but contain Band 3 protein, glycophorin and acetylcholinesterase. These proteins are presumably free to partition into the vesicles together with the lipids which appear to be the same in the vesicles as in the original cells. Studies with radioactively-labelled and spin-labelled phosphatidylcholines⁷ have indicated that echinocytosis results from an excess of outer leaflet area of only 1–2% over the inner leaflet area, but it is not known in this case what relative expansion is required to obtain exovesiculation.

Recently we have measured the binding to red cells of the fluorescent molecule Merocyanine 540 (MC 540) which inserts specifically into the outer membrane leaflet and which also causes echinocytosis and exovesicle release⁸. We have estimated that echinocytosis requires expansion of the outer leaflet by about 1% and that vesicle release occurs with an expansion of between 1 and 3%. The changes induced by MC540 occur within a few minutes whereas vesicle release by addition of exogenous phosphatidylcholine can take some hours⁶. The effects of MC540 appear to be most analogous to the vesicle release induced by treatment of intact cells with glycocholate⁹ which like MC540 is composed of a large rigid hydrophobic moiety linked to a charged hydrophilic headgroup effectively tethering it in the outer leaflet. Higher concentrations (10–20mM) of glycocholate however, cause endocytosis probably because material is solubilised from the outer leaflet to give a relative decrease in the area of that leaflet.

Exovesicle release from red cells with elevated levels of internal Ca^{2+} may also depend on a change in the relative areas of inner and outer lipid leaflets since there is good evidence that breakdown of polyphosphoinositides occurs under these

conditions and could cause a diminution of the inner leaflet area^{7, 10}. The evidence is ambiguous in this case because raised Ca^{2+} also causes degradation of some elements of the cytoskeleton, particularly ankyrin and polypeptide 4.1, and this might also contribute to the tendency for vesiculation to occur. Such effects of Ca^{2+} may also be important in the exovesicle release which occurs during red cell ageing when intracellular Ca^{2+} appears to be elevated.

In each of the examples of exovesiculation referred to above, the morphology of the vesicles looks very similar and their size is rather consistent at about 100–150nm. This size may be related to the dimensions of the interstices in the spectrin–actin framework underlying the bilayer. Because of this relative homogeneity and to differentiate these vesicles from the larger vesicles (sometimes containing spectrin) which can be released from the cells under other circumstances, we have generally preferred to give them the specific name 'microvesicles'.

The study of protein segregation between cells and microvesicles can give information on protein–protein and lipid–protein interactions in the membrane. There is convincing evidence that most lipids and some of the red cell proteins diffuse rapidly within the plane of the bilayer so that vesicles which become detached from the cell should take with them a representative selection of these diffusible components. Conversely, a lower specific activity of a certain protein component in the microvesicles is an indication that this protein has some interaction with the membrane skeleton. The ratio of Band 3 protein to phospholipid in microvesicles is less than half of that in ghosts (Table 1) and this is consistent with independent evidence¹¹ that about half of the total Band 3 is immobile, probably because it is bound to ankyrin. Considering that there are about ten monomers of band 3 protein for each molecule of ankyrin, the data suggests that band 3 must be associated with ankyrin as aggregates of 4–5 monomers or that some other protein limits its free diffusion. The major glycoprotein, glycophorin A appears to have a similar level of skeletal attachment to Band 3 since about 40% seems free to partition into the microvesicles. Curiously, acetylcholinesterase seems to have a substantially higher specific activity in the microvesicles compared with ghosts, a feature which has been noted previously for microvesicles derived by other treatments^{6, 9, 10}. This is no increase in the total activity of the enzyme since residual cells after treatment with MC540 showed an equivalent decrease in activity. This may suggest that acetylcholinesterase can diffuse more easily into regions of the membrane which contain less of Band 3 and glycophorin so that its relative specific activity increases in proportion to the decrease in amounts of these major proteins. Unlike Band 3 and

glycophorin, there is no evidence that acetylcholinesterase has any tendency to associate with skeletal proteins. No activity of polyphosphoinositide phosphodiesterase could be detected in the microvesicles derived from MC540-treated cells (Table 1) although the dye itself did not inhibit the enzyme. This could suggest that the enzyme is associated with skeletal proteins and consequently does not partition into the microvesicles. Alternatively it could mean that the pool of polyphosphoinositide in the microvesicles is for some unknown reason, not available to the enzyme.

It is true that individual phospholipid species are not generally selected for or excluded from the microvesicles, consistent with the view that there is no barrier to the free diffusion of phospholipids. However one class of phospholipids, the polyphosphoinositides, appear to be depleted in MC540-derived microvesicles compared with the original cells (Table 2). The reduction in polyphosphoinositides in the microvesicles was not due to an enhanced breakdown to phosphatidylinositol since the concentration of this lipid did not rise concomitantly. The data suggest that as much as 50% of these lipids are associated with skeletal proteins or with bilayer proteins which are associated with the skeleton and are therefore not free to diffuse with the other phospholipids into the microvesicles. The decrement of polyphosphoinositide in the microvesicles corresponded to about 1% of the total cellular phospholipid and thus to about 2.4 million molecules per cell which were apparently not free to diffuse. These observations could be consistent with suggestions that polyphosphoinositides mediate interactions between Band 4.1 protein and glycophorin¹². If at least part of the glycophorin is associated with skeletal proteins under normal circumstances, breakdown of polyphosphoinositides by the endogenous Ca^{2+} -dependent phosphodiesterase or by energy depletion of cells may release some of this bound fraction and lead to larger amounts of glycophorin in the microvesicles. This is currently under investigation.

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Table 1 Segregation of red cell membrane proteins into microvesicles

The ratio of protein concentration (measured from gel scans) or enzyme activity to phospholipid was measured for (a) control ghosts (b) ghosts from MC540-treated cells remaining after removal of microvesicles and (c) microvesicles. Results are expressed as means \pm S.D. from three experiments with different samples of cells, taking the values for the control ghosts as 100%. (N.D., none detected).

	<u>MC540 ghosts</u>	<u>Microvesicles</u>
Band 3	102 \pm 5	43 \pm 9
Glycophorin	110 \pm 7	39 \pm 7
Acetylcholinesterase	89 \pm 3	240 \pm 25
PIP/PIP ₂ diesterase	105 \pm 8	N.D.

Table 2. Phospholipid composition of (a) untreated red cell ghosts, (b) ghosts from MC540-treated cells and (c) microvesicles.

Phospholipids were extracted and analysed as described previously. Results represent mole percent of total phospholipid and are shown as mean \pm standard deviation from four experiments, each carried out with duplicate samples.

	(a)	(b)	(c)
phosphatidylethanolamine	29.9 \pm 0.3	28.8 \pm 0.9	28.8 \pm 1.9
phosphatidylserine	15.6 \pm 1.9	14.0 \pm 0.5	12.9 \pm 2.0
phosphatidylcholine	28.4 \pm 0.7	29.2 \pm 1.3	30.2 \pm 3.3
sphingomyelin	23.2 \pm 1.4	25.0 \pm 1.8	23.8 \pm 3.4
phosphatidylinositol	1.0 \pm 0.3	1.1 \pm 0.3	1.0 \pm 0.1
phosphatidylinositol 4 P	1.2 \pm 0.4	1.4 \pm 0.3	0.8 \pm 0.3*
phosphatidylinositol 4,5 bis P	1.6 \pm 0.4	1.6 \pm 0.5	0.8 \pm 0.4**

* Significantly different from controls $p < .05$

** Significantly different from controls $p < .01$

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Restricted diffusion of integral membrane proteins and polyphosphoinositides leads to their depletion in microvesicles released from human erythrocytes

Carla HAGELBERG and David ALLAN*

Department of Physiology, University College and Middlesex School of Medicine, University College London, Rockefeller Building, University Street, London WC1E 6JJ, U.K.

The protein and phospholipid composition of microvesicles released from normal human erythrocytes after ATP depletion, on aging or by treatment with merocyanine 540, dimyristoyl phosphatidylcholine or Ca^{2+} /ionophore A23187 has been compared with the composition of the original cell membrane. It has been shown that these microvesicles are depleted of band 3, glycophorin and phosphatidylinositol 4,5-bisphosphate relative to phospholipid by 40% or more. These data are interpreted to mean that less than half of these membrane components are free to diffuse laterally in the lipid bilayer. Acetylcholinesterase was found to be enriched 2–3-fold in microvesicles, possibly because the removal of non-diffusing proteins from the vesiculating region of the lipid bilayer allows more space for freely diffusing proteins like acetylcholinesterase to enter the microvesicle membrane.

INTRODUCTION

Microvesicle release from echinocytic human erythrocytes occurs naturally during the process of aging [1] and in certain disease states [2,3]. It can also be induced *in vitro* under a variety of different conditions such as calcium loading [4], ATP depletion [5], spectrin oxidation [6] or through the intercalation of certain amphipaths into the outer lipid bilayer [7–9]. In all these cases the composition of the microvesicle membrane resembles that of the original cell, with the notable exception that those membrane proteins which form part of the non-diffusing skeletal complex (e.g. spectrin and actin) are not found in the microvesicles. It has been shown that, in echinocytic blebs, the lateral mobility of antigens is not restricted as it is in the rest of the membrane [10]. This suggests that only those components of the erythrocyte membrane which are free to diffuse in the plane of the bilayer (e.g. lipids and those membrane proteins which are associated with lipid and not with the skeleton) would be expected to be present in the microvesicles. Band 3 does appear among the proteins of the microvesicle membrane, so that at least a fraction of this protein must be diffusible. It is well known that band 3 protein can associate (via ankyrin and possibly band 4.1) with the skeletal complex [11,12], but there appear to be too many molecules of band 3 for them all to be bound to ankyrin and band 4.1 [13], so it might be expected that a fraction of band 3 would be free to diffuse in the lipid bilayer.

We thought that it should be possible to obtain a quantitative measure of the proportion of band 3 molecules which were freely diffusing (i.e. not bound to skeletal proteins) by determining the relative amount of band 3 which partitioned into microvesicles produced under mild conditions. Furthermore, such measurements might reveal differences in the extent of attachment of band 3 under various conditions of microvesicle release. Similar considerations would apply to other components of the bilayer portion of the membrane, such as glycophorin and polyphosphoinositides, which may also have interactions with the membrane skeleton [14].

MATERIALS AND METHODS

Merocyanine 540 (MC540), dimyristoylphosphatidylcholine (DMPC), iodoacetamide, deoxyglucose and Ca^{2+} ionophore A23187 were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Preparation of cells and ghosts

Fresh human blood was collected from healthy volunteers in 150 mM-NaCl containing 2 mM-EDTA (pH 7.4) and used within 1 day of collection. The erythrocytes were sedimented by centrifugation at 500 g for 5 min and were washed twice with 150 mM-NaCl (with the removal of the buffy coat) and once with the incubation buffer [150 mM-NaCl, 10 mM-Hepes/NaOH/0.1 mM-EDTA, pH 7.4 (Hepes-saline)].

Ghosts were prepared by lysing the cells with ice-cold 20 mM-Tris/HCl/2 mM-EDTA, pH 7.4. Membranes were sedimented and washed once with this buffer prior to phospholipid analysis or three times prior to polypeptide analysis.

Protein analysis

Polypeptides were analysed by SDS/PAGE using the Laemmli system [15]. Gels were stained with Coomassie Brilliant Blue or periodate/Schiff reagent [16]. Quantification of the bands was achieved by scanning the gels with a Bio-Rad 1650 scanning densitometer linked to a Shimadzu C-R3A integrator. Acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al.* [17].

Lipid analysis

Lipids were extracted from membranes with chloroform/methanol (1:2, v/v) [18] and were separated by t.l.c. on Merck silica gel 60 plates run in chloroform/methanol/acetic acid/water (75:45:12:2, by vol.) [19]. Polyphosphoinositides were extracted as above, but with the addition of HCl to the extract [20], and were separated on oxalate-impregnated plates [1% in methanol/water (2:3, v/v)] using chloroform/acetic acid/methanol/

Abbreviations used: MC540, merocyanine 540; DMPC, dimyristoyl phosphatidylcholine; AChE, acetylcholinesterase.

* To whom all correspondence should be addressed.

acetone/water (40:12:13:15:7, by vol.) as the solvent system [21]. Phospholipid spots were scraped from the plate and digested with 70% (v/v) HClO_4 at 180 °C for 1 h, and lipid phosphorus was assayed by the method of Bartlett [22].

Radioactive spots were visualized by autoradiography, excised and digested with 70% HClO_4 . After dilution with 10 ml of water the samples were counted for radioactivity in a liquid scintillation analyser. The phosphorus assay for $\text{PtdIns}(4)\text{P}$ was unreliable due to a variable contamination with an unknown lipid; therefore $\text{PtdIns}(4)\text{P}$ was quantified from its radioactivity, assuming that the specific radioactivity is half that of $\text{PtdIns}(4,5)\text{P}_2$ [23].

Labelling of erythrocytes with ^{32}P

Cells at a haematocrit of 20% were incubated for 18 h at 37 °C in Hepes-saline containing MgCl_2 (1 mM), glucose (10 mM), gentamicin (100 $\mu\text{g}/\text{ml}$) and carrier-free [^{32}P]phosphate (5 $\mu\text{Ci}/\text{ml}$) (Amersham International).

T.l.c. lipid analysis of ^{32}P -labelled microvesicles, ghosts and MC540 ghosts confirmed previous results, i.e. the only erythrocyte lipids to be labelled with ^{32}P are those bearing monoester phosphate groups, i.e. phosphatidic acid, $\text{PtdIns}(4)\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ [24].

In some experiments the specific radioactivities of ATP and AMP were measured using h.p.l.c. [25] and compared with the specific radioactivity of $\text{PtdIns}(4,5)\text{P}_2$. No significant differences were found between the specific radioactivity of ATP γ -phosphate group and the specific radioactivities of the labelled phosphates in the polyphosphoinositides.

Incubation of erythrocytes

Unless otherwise stated, labelled cells at a haematocrit of 20% were incubated at 37 °C in Hepes-saline and treated with MC540, DMPC, $\text{Ca}^{2+}/\text{A23187}$ or deoxyglucose/iodoacetamide as detailed below. After incubation the cells were sedimented (500 g, 5 min) and AChE activity, phospholipid release and extent of lysis were measured in the supernatant. Values of 100% AChE activity, phospholipid release and lysis were calculated from a sample taken from the incubation mixture at the beginning of the experiment. Microvesicles were sedimented from the supernatant by centrifugation at 30000 g for 30 min (except for MC540 vesicles, which were sedimented at 100000 g for 1 h). Microvesicles from stored blood were washed by centrifugation several times to remove any contaminating ghosts. SDS/PAGE, t.l.c. and phosphorus analysis were carried out on the vesicles and the ghosts from treated cells.

MC540 treatment. Cells at 5% haematocrit were treated with 62.5 μM -MC540 (added as a 10 mM solution in dimethyl sulphoxide) for 2 min. The incubation was terminated by cooling in ice for 5 min.

DMPC treatment. A suspension of DMPC was prepared by sonicating the dry lipid in the incubation buffer (0.5 mg/ml) using an MSE sonicator (14 μm peak-to-peak amplitude). Cells were incubated for 2.5 h in this suspension as described previously [7]. Because the microvesicle sample was contaminated with DMPC, total microvesicle phospholipid release was calculated by measuring phosphatidylethanolamine and phosphatidylserine after separating the lipids by t.l.c. (assuming that these phospholipids combined represent 40% of total lipid [26]).

Calcium ionophore treatment. Cells were incubated with 1 mM- CaCl_2 and 5 μM -A23187 for 25 min as previously described [27].

ATP depletion. In order to achieve ATP depletion, cells were incubated with 5 mM-iodoacetamide and/or 5 mM-deoxyglucose for 24 h [28].

Aging *in vitro*. Whole blood was stored for 10–15 weeks in citric acid/sodium citrate/dextrose at 4 °C.

RESULTS

When erythrocytes are incubated with MC540 or DMPC, with $\text{Ca}^{2+}/\text{A23187}$, under conditions of energy depletion, or after storage for several weeks, the cells change their morphology from discocyte through echinocyte to spherocyte and release microvesicles into the medium [1,5,7,9,27]. As shown previously [6,9,29], AChE release strongly correlates with phospholipid release.

Membrane protein composition

Based on results from SDS/PAGE, microvesicles released under any of the conditions employed clearly contained only band 3 and glycophorin in addition to cytosolic proteins [5,9,30]. However, the amounts of band 3 and glycophorin relative to phospholipid in microvesicles were substantially less than in either the original cells or the residual treated cells. Thus for MC540, DMPC, $\text{Ca}^{2+}/\text{A23187}$ or storage microvesicles, the amounts of band 3 and glycophorin were between 40 and 50% of the values observed in ghosts from untreated cells (Table 1). Higher values (60–70%) were seen in microvesicles from ATP-depleted cells, and this result was the same whether iodoacetamide or deoxyglucose was used for ATP depletion, although iodoacetamide gave a somewhat higher microvesicle yield. A substantially lower value (20%) for band 3, but not for glycophorin, was observed in the case of microvesicles from stored cells.

In contrast with the relative depletion of band 3 and glycophorin, a 2–3-fold enrichment of AChE relative to phospholipid was observed in the microvesicle preparations, similar to that described previously [29,31]. Total activity of this enzyme was not changed, since there was an equivalent decrease in activity in the residual cells [32].

Membrane lipid composition

The overall phospholipid composition of the microvesicles, the parent erythrocytes and the MC540-treated erythrocytes was very similar, with little variation in the relative amounts of the

Table 1. Segregation of band 3 and glycophorin into microvesicles

The ratio of band 3 and glycophorin concentration (derived from gel scans) to phospholipid was measured for control ghosts from untreated cells, and ghosts and microvesicles from cells treated with MC540, DMPC, $\text{Ca}^{2+}/\text{A23187}$ or iodoacetamide/deoxyglucose (ATP depletion) and from cells aged by storage. Values for the control ghosts are taken as 100%. Results are expressed as means \pm standard error taken from four experiments (seven in the case of MC540) carried out in duplicate. The relative amounts of band 3 and glycophorin in the microvesicles from the different treatments were compared using analysis of variance. * Microvesicles from ATP-depleted cells contained significantly more band 3 and glycophorin than the other microvesicles ($P < 0.01$) and microvesicles from aged cells contained less band 3 than those from the other treatments ($P < 0.01$).

Treatment	Relative amount (%)			
	Band 3		Glycophorin	
	Ghosts	Microvesicles	Ghosts	Microvesicles
MC540	91 \pm 3	36 \pm 6	110 \pm 4	47 \pm 5
DMPC	108 \pm 7	41 \pm 4	99 \pm 8	51 \pm 4
$\text{Ca}^{2+}/\text{A23187}$	94 \pm 8	42 \pm 4	92 \pm 8	43 \pm 6
ATP depletion	124 \pm 9	61 \pm 3*	114 \pm 8	71 \pm 4*
Storage	99 \pm 7	19 \pm 1*	113 \pm 7	41 \pm 4

Table 2. Content of PtdIns(4,5) P_2 and PtdIns(4) P in ghosts from treated cells and microvesicles

Phospholipids were extracted and analysed as described in the text. The amounts of PtdIns(4,5) P_2 and PtdIns(4) P in the ghosts and microvesicles from cells treated with MC540, DMPC, Ca^{2+} /A23187 or iodoacetamide/deoxyglucose (ATP depletion) were measured from the radioactive counts and phosphate analysis, taking the values for the control ghosts as 100%. The results represent mol % of total phospholipid and are shown as means \pm standard error from four experiments (seven for MC540) done in duplicate. Significant differences between treated cells and vesicles, by paired t tests, are indicated as follows: * P < 0.05; ** P < 0.02; *** P < 0.01; **** P < 0.001.

Treatment	Content (mol %)			
	PtdIns(4,5) P_2		PtdIns(4) P	
	Ghosts	Microvesicles	Ghosts	Microvesicles
MC540	100 \pm 4	57 \pm 4****	90 \pm 3	56 \pm 3****
DMPC	114 \pm 9	55 \pm 5***	92 \pm 10	71 \pm 9
Ca^{2+} /A23187	55 \pm 7	35 \pm 5**	53 \pm 8	46 \pm 6
ATP depletion	23 \pm 2	16 \pm 3***	100 \pm 4	67 \pm 10*

major lipids [32]. Similar results were obtained for microvesicles obtained by the other treatments (results not shown).

The amounts of PtdIns(4,5) P_2 and PtdIns(4) P in the ghost samples from untreated cells were found to be approx. 1.6 mol% and 1.2 mol% respectively of total phospholipid (average of seven experiments done in duplicate). This compares well with values of approx. 1.5% and 0.9% respectively given by other workers [33–35]. However, the levels of these lipids, particularly PtdIns(4,5) P_2 , were markedly reduced in the microvesicles from MC540 or DMPC treatment compared with the original cells (Table 2). The level of PtdIns remained constant at 1% in ghosts and microvesicles, so that the reduction in the polyphosphoinositides was not due to their dephosphorylation. Neither was there any evidence for breakdown of these lipids by a phospholipase C, since there was no increase in either diacylglycerol or phosphatidate in the ghosts or microvesicles (except with calcium treatment [27]). A very similar decrease in the content of the polyphosphoinositides was observed in microvesicles from cells subjected to ATP depletion or treatment with Ca^{2+} /A23187, although in these two cases the amount of PtdIns(4,5) P_2 in the treated cells was considerably reduced, due either to dephosphorylation of this lipid as a result of ATP depletion or, in the case of Ca^{2+} /A23187 treatment, to the activation of polyphosphoinositide phosphodiesterase by increased intracellular Ca^{2+} [36].

DISCUSSION

As we have shown recently [9], human erythrocytes undergo echinocytosis and microvesiculation when exposed to MC540. This compound seems to act in a similar way to other amphipathic molecules which partition into the outer membrane leaflet but which do not cross the cell membrane, such as DMPC [7] or glycocholate [8]. Microvesiculation appears to be an extreme consequence of intercalation of these amphipathic molecules selectively into the outer leaflet of the lipid bilayer. This induces localized curvature of the membrane, as predicted by the Sheetz & Singer bilayer couple hypothesis [37]. Very similar echinocytosis and release of microvesicles is seen when cells are subjected to procedures which diminish the amount of the polyphosphoinositides on the inner lipid leaflet of the membrane

bilayer, either as a consequence of phosphomonoesterase activity after ATP depletion [33] or resulting from phosphodiesterase activity stimulated by a rise in the intracellular concentration of Ca^{2+} [27]. Again, these effects can be rationalized in terms of the Sheetz & Singer hypothesis.

The advantage of using MC540 for studies of microvesiculation is that no metabolic changes are involved and the effect is rapid; maximum release of microvesicles is seen in 2 min. This contrasts with the 24 h preincubation with metabolic inhibitors required in order to obtain microvesicles from ATP-depleted cells and with the complex biochemical changes which result from elevation of intracellular Ca^{2+} [27]. Even with DMPC (which does not produce metabolic changes), the lag-time before onset of vesiculation was approx. 1 h [7]. The most likely explanation for the difference in the speed of the effects of MC540 and DMPC is that the entry into the membrane outer leaflet depends on the concentration of free amphipath in the medium and that this in turn is controlled by the critical micellar concentration, which is relatively high for MC540 compared with DMPC (C. Hagelberg & D. Allan, unpublished work). Although Ott *et al.* [7] showed that DMPC intercalates into the bilayer within minutes, it may take an hour for a sufficient concentration to build up in the membrane outer leaflet to allow microvesicle release to occur.

Segregation of proteins into microvesicles

One notable feature of our results is that the content of band 3 relative to that of phospholipid in the microvesicles is only about 40% of that in the original cells or in the residual treated cells. This is consistent with the independent evidence that about 60% of the band 3 protein is bound to the cytoskeletal network via ankyrin or via glycophorin and band 4.1 [38,39], and that accordingly this fraction of band 3 is unable to diffuse into the microvesicles. Glycophorin A is decreased in the microvesicles to a similar extent as band 3, suggesting a comparable degree of skeletal attachment. It is possible that the similar behaviour of glycophorin and band 3 is due to a complex formation between these proteins [40,41].

In contrast with these findings, and as observed previously for microvesicles prepared by other procedures, there was a 2–3-fold increase in AChE specific activity in the microvesicles, although the total activity of this enzyme was not changed. AChE is one of a number of cell surface enzymes which appear to be attached by linkage to a modified phosphatidylinositol [42], so that it might have been expected that such enzymes would share in the rapid diffusion characteristic of lipids. A possible explanation for the markedly increased activity of AChE in the microvesicles is that the removal from the bilayer of some integral proteins which remain attached to the membrane skeleton effectively allows more room in the microvesicle membrane for the accommodation of those proteins like AChE which can diffuse freely. Put another way, removal of half of band 3 and glycophorin leads to a decrease in the surface packing pressure in the microvesicle membrane, which favours the diffusion of AChE out of the more crowded membrane of the residual cells. It seems unlikely that the increased activity of AChE could be due to a greater surface curvature in the microvesicles, since the residual cells are very echinocytic (i.e. locally curved) but show a decrease in enzyme activity [32].

The patterns of protein segregation in the MC540-, DMPC- or Ca^{2+} -induced microvesicles were very similar (Table 2), suggesting that the effects of these agents reflected an underlying organization of the membrane. The only significant difference was with ATP depletion, where it appeared that somewhat more band 3 and glycophorin was free to diffuse into the microvesicles, perhaps suggesting that energy depletion affected the ability of these proteins to bind to the membrane skeleton. This effect does,

however, seem less marked than the results reported by Lutz *et al.* [5], who found essentially no decrease in the amounts of band 3 and glycophorin in vesicles released as a result of energy depletion, indicating no barrier to the free diffusion of these proteins. A substantially lower value for band 3 (20%) but not for glycophorin was observed in the case of microvesicles from stored cells, although here it is possible that degradation of band 3 occurred during the long period of storage.

Partitioning of polyphosphoinositides into microvesicles

The composition of the major phospholipids in the microvesicles prepared by MC540 treatment [32] or any of the other procedures was very similar to that of the original cells. This is to be expected, since phospholipids generally are thought to diffuse freely in the plane of the membrane, and thus the microvesicles should contain a representative sample of these lipids. The only phospholipids to behave differently were the polyphosphoinositides: the microvesicles only contained about half as much of the polyphosphoinositides as the original cells (compared on the basis of total phospholipid content), suggesting that only about half of these lipids are free to diffuse. This inference would be consistent with the idea that the polyphosphoinositides may bind to cytoskeletal proteins and perhaps mediate interactions between band 4.1 and glycophorin [14].

Studies using ^{32}P -n.m.r. have indicated that four molecules of inositol phospholipid are tightly bound to each glycophorin molecule [43]. If it is assumed that the most likely lipid to be associated with glycophorin is $\text{PtdIns}(4,5)\text{P}_2$, these n.m.r. studies could account for a substantial proportion of the non-mobile pool of $\text{PtdIns}(4,5)\text{P}_2$. Thus there are approx. 2.5×10^8 molecules of phospholipid in each cell, and if $\text{PtdIns}(4,5)\text{P}_2$ accounts for 1.5% of this, then there are 3.8×10^6 molecules of $\text{PtdIns}(4,5)\text{P}_2$ per cell. Since 4×10^5 molecules of glycophorin are present in each cell [13], 1.6×10^6 molecules of $\text{PtdIns}(4,5)\text{P}_2$ per cell (only 42% of the total) are calculated to be bound to glycophorin. There is, therefore, too much $\text{PtdIns}(4,5)\text{P}_2$ to explain its distribution between cells and microvesicles solely in terms of binding to glycophorin. However, it may be more than coincidence that such similar proportions of band 3, glycophorin and $\text{PtdIns}(4,5)\text{P}_2$ are able to diffuse into the microvesicles; perhaps this is a reflection of ternary interactions between these proteins, $\text{PtdIns}(4,5)\text{P}_2$ and the membrane skeleton.

This restricted mobility of the polyphosphoinositides in the membrane through the interaction with proteins might be expected to be reflected in metabolic pooling of these lipids. Thus it would not be surprising if accessibility of polyphosphoinositides to phosphatases, kinase and the Ca^{2+} -dependent phosphodiesterase was different for protein-bound inositide compared with freely diffusible inositol lipid. Such considerations could explain the observations of King *et al.* [44], who found that the maximum specific radioactivity of $\text{PtdIns}(4,5)\text{P}_2$ was only 30% of the specific radioactivity of the γ -phosphate of ATP, suggesting that a large part of the polyphosphoinositide pool was not metabolically accessible. However, after the long period of incubation with ^{32}P that we used, the polyphosphoinositides were labelled to equilibrium with ATP (C. Hagelberg & D. Allan, unpublished work) so that we did not find any differences between the specific radioactivities of polyphosphoinositide which partitioned into microvesicles and that which did not.

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