Chromatographic Studies of Solute Interactions with Immobilized Red Blood Cells and Biomembranes

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UPPSALA UNIVERSITY 2002
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ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2002
ABSTRACT


Specific and non-specific interactions of solutes with immobilized biomembranes were studied using chromatographic methods. Liposomes, proteoliposomes and red blood cell (RBC) membrane vesicles were immobilized by a freeze-thawing procedure, whereas whole RBCs were adsorbed in the gel beds using electrostatic interaction, binding to wheat germ agglutinin (WGA) or the streptavidin-biotin interaction.

Superporous agarose gel with coupled WGA was the most promising matrix for RBC adsorption and allowed frontal chromatographic analyses of the cells for about one week. Dissociation constants for the binding of cytochalasin B and glucose to the glucose transporter GLUT1 were determined under equilibrium conditions. The number of cytochalasin B-binding sites per GLUT1 monomer was calculated and compared to corresponding results measured on free and immobilized membrane vesicles and GLUT1 proteoliposomes. This allowed conclusions about the protein’s binding state in vitro and in vivo.

Partitioning of drugs into biomembranes was quantified and the system was suggested as a screening method to test for possible intestinal absorption of drug candidates. We also studied how membrane partitioning of drugs is affected by the presence of integral membrane proteins or of charged phospholipids. An attempt to combine the theory for specific binding and membrane partitioning of solutes in a single equation is briefly presented.

Keywords: Affinity, Binding, Biomembrane, Biotin, Chromatography, Cytochalasin B, Dissociation constant, Drug absorption, Equilibrium, Glucose, GLUT1, Immobilization, Immobilized biomembrane affinity chromatography, Immobilized liposome chromatography, Interaction, Liposome, Membrane protein, Membrane vesicle, Partitioning, Phospholipid bilayer, Proteoliposome, Quantitative, Red blood cell, Solute, Specific, Streptavidin, Wheat germ agglutinin.

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ISSN 1104-232X
ISBN 91-554-5413-5

Printed in Sweden by University Printers, Ekonomikum, Uppsala 2002
Das Herz pumpt schwer
träge blubberndes Blut -
und das ist gut.
Extrabreit, 1980

To the memory of my father
To my mother
PAPERS INCLUDED IN THE THESIS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.


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Other publications

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CB</td>
<td>Cytochalasin B</td>
</tr>
<tr>
<td>CB/mon</td>
<td>Number of CB binding sites per GLUT1 monomer</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Human erythrocyte-type glucose transporter</td>
</tr>
<tr>
<td>IAM</td>
<td>Immobilized artificial membrane</td>
</tr>
<tr>
<td>IBAC</td>
<td>Immobilized biomembrane affinity chromatography</td>
</tr>
<tr>
<td>IBiPaC</td>
<td>Immobilized biomembrane partitioning chromatography</td>
</tr>
<tr>
<td>OG</td>
<td>$n$-Octyl-$\beta$-D-glucopyranoside</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidyl choline</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SPA</td>
<td>Superporous agarose</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
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<tr>
<td>WGA</td>
<td>Wheat germ agglutinin</td>
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1. Biological membranes - life in itself

Living cells and their organelles are usually enclosed by biological membranes that play key roles in their function. Integral membrane proteins in the membranes show an enormous functional diversity and contribute to the fact that biomembranes are more than just envelopes that surround and protect the cells’ life functions. Instead, "life" is focussed very much within the membranes themselves. For example, the respiratory chain in the inner mitochondrial membrane or the photosynthetic membrane assemblies in the chloroplast membrane are the main sources of chemical energy for animals and plants, respectively. Membranes are permeability barriers for larger polar molecules (M_r > approx. 100) and for ions, making control of the critical cytoplasmatic solute balance possible. This means also that mechanisms for transport of essential metabolites or signals across the membrane are required. Transport channels and pores as well as signal receptors at the cell surface are therefore further functional features of the biological membranes that make life possible. In addition, membrane transport of protons is an important feature of the respiratory and photosynthetic machineries. As a consequence, for the chemistry of life it is just as important to study biomembranes and membrane proteins as it is to focus on processes that occur in the inner cell compartments.

2. Solute interactions with biomembranes

The term biomembrane as it appears in the title of this thesis is not well-defined in the literature. Sometimes it may designate only natural membranes of certain cells and organelles, whereas other authors include lipid bilayers in general. In the latter meaning bio- may be understood as composed of biological molecules as opposed to artificial membranes of, e.g., nylon or nitrocellulose. In this thesis, the more general meaning of biomembranes is usually used, although the exact nature of the materials is often specified. For example, the term immobilized biomembrane affinity chromatography (IBAC, cf. Section 10.1 and Paper VI) covers experiments on both cell membranes, membrane vesicles and proteoliposomes, and biomembranes in the title of the thesis is meant to include membrane vesicles and proteoliposomes.

Solute interactions with biomembranes are of major importance in various respects and usually non-specific interactions can be distinguished from specific binding. In the non-specific case, mainly hydrophobic interactions of a solute within the membrane core and ionic and polar interactions with charged and hydrophilic membrane components, respectively, determine the strength of the solute-membrane contact. An equilibrium constant is usually not measurable and the binding is not saturable at high solute concentrations. These non-specific interactions determine the solute partitioning into and diffusion across biomembranes, which were analyzed in Paper I and summarized in Section 6. Specific solute (or ligand) binding to biomembranes, on the other hand, involves a defined contact between the ligand and a membrane protein or lipid and is, in the scope of this thesis,
an important part of protein-mediated solute transport across membranes. The ligand (may it be a transported substrate or a transport inhibitor) binds in a certain steric orientation and with a defined affinity to a definite number of binding sites located on a transport protein in the membrane and the binding is usually saturable at high ligand concentrations.

3. Red blood cells

The human body is composed of an astonishing number (approx. 100 trillion \((10^{14})\) of cells (Guyton 1992) of presumably thousands of different types. As many as one fourth of these smallest living units of our bodies are red blood cells (RBCs), the cell type, which by that measure, is apparently our most abundant one (Guyton 1992; Dixon 1997). Although very flexible, RBCs are biconcave discs with a diameter of 8 µm and a height of 2 µm (Fig. 1A). Provided with this information, how high would a stack of all the RBCs from only one human being be? Yes, that is right, 50 000 km! The reason that there are so many RBCs is their essential task to provide virtually all other of our cells with \(O_2\), a gas that within the RBCs becomes reversibly bound to the protein hemoglobin. Therefore, during RBC-maturation from reticulocytes, all organelles except the cell membrane are reduced to give room for as much hemoglobin as possible. This leaves RBCs with a distinctly simpler intracellular structure in comparison to the highly compartmentalized construction of other typical tissue cells (Fig. 1B). At least 110 other water-soluble proteins and enzymes (Pennell
1964) are present in RBCs, but their amount is small compared to that of hemoglobin, which crowds the cell volume at about 0.34 gram per milliliter (Dixon 1997). The relatively small size and the flexibility of RBCs allow them to enter even the thinnest capillary vessels. Their life span is limited to 120 days, which means that every day 0.8% of our RBCs are replaced by maturing stem cells, which in adults originate mainly from the ribs and the sternum. Considering that RBCs cannot reproduce or synthesize proteins and lack most of the metabolism common to other cells, their energy (ATP) requirements are relatively low and can be covered by anaerobic glycolysis. However, intracellular ATP depletion leads, among other things, to decreased cell membrane deformability, loss of membrane lipids and disturbance of the ion balance (Weed and Lacelle 1969). Therefore, to maintain their function and the essential flexibility of their membrane, RBCs are dependent on a constant supply of energy which originates from glucose.

The only membrane structure of RBCs is their cell membrane, which consists, by weight fraction, of 49% proteins, 43% small amphiphiles (mainly phospholipids), and 8% oligosaccharides (Guidotti 1972). The phospholipids form a bilayer with their hydrophilic parts pointing toward the outside and their hydrophobic parts pointing toward the inside of the membrane, as illustrated below (e.g., Fig. 4). The distribution of phospholipids between the two leaflets of the bilayer is assymmetrical, e.g., phosphatidyl choline (PC) and sphingomyelin are located predominantly within the outer monolayer, whereas phosphatidyl serine (PS) and phosphatidyl ethanolamine are situated mainly within the inner layer (Devaux 1993). Cholesterol is evenly distributed at about 20 weight % of the lipids. At least 30 different integral membrane proteins (Anstee 1990) are embedded in the membrane bilayer, either floating freely or physically coupled to a meshwork of fibrous peripheral membrane proteins called the cytoskeleton. The latter structure is associated with the cytoplasmic face of the membrane and provides the cell with the necessary stability without loss of flexibility. Peripheral proteins can usually be dissociated from the membrane by relatively mild treatments, such as increased ionic strength or pH, whereas extraction of integral proteins usually requires dissolution of the membrane with detergents. The outer membrane face is essentially characterized by the presence of a coat of oligosaccharides linked to the integral membrane proteins or lipids. This increases the hydrophilicity of the cell surface. A high content of sialic acid in the oligosaccharide chains of glycoporin A adds a negative charge, causing the cells to repel each other gently in the blood stream. The sugars also offer lectin-binding sites and sialic acid is known (Adair and Kornfeld 1974) to be the main target for binding of wheat germ agglutinin (WGA).

4. The human glucose transporter GLUT1; a molecular fuel tap

D-glucose is the common major fuel resource for most cells in the human body. The brain alone steadily consumes about 120 g of glucose per day (Stryer 1989). The diffusion of this tremendous number (4 x 10^{23}) of hydrophilic sugar molecules across the blood-brain barrier is
facilitated by the glucose transporter GLUT1, a specific gate for passive diffusion of glucose down a concentration gradient. The richest occurrence of this protein in our bodies is found in the membranes of RBCs, but there is a moderate expression of the transporter in many other tissues. With about $5 \times 10^5$ copies per cell (Mascher and Lundahl 1988; Anstee 1990) GLUT1 is the third most abundant protein of the RBC membrane, just after the anion transporter and glycophorin A (each about $10^6$ copies per cell). Most of the research on GLUT1 has been performed on protein from this source and GLUT1 is frequently called the erythrocyte glucose transporter.

The first functional reconstitution of GLUT1 purified on DEAE-cellulose was done by Kasahara and Hinkle in 1977, who used Triton X-100 for solubilization of the protein. The preparation protocol has been modified many times and the standard procedure employed octylglucoside (OG) (Baldwin et al. 1982; Cairns et al. 1984; Mascher and Lundahl 1988; Baldwin and Lienhard 1989), but octaethylene glycol dodecyl ether (C$_{12}$E$_8$, e.g., Haneskog et al. 1996) can be used instead. A recent protocol involved decylmaltoside detergents (Boulter and Wang, 2001) and on the basis of a direct comparison the authors claim that their preparation procedure yields more stable and monodispersely solubilized GLUT1 than did earlier ones. Maltoside detergents may therefore be promising in future attempts to crystallize GLUT1.

In 1985, Mueckler and coworkers cloned and sequenced a glucose transporter from human HepG2 hepatoma cells which is similar or identical to GLUT1. The transporter turned out to consist of 492 amino acid residues corresponding to a Mr of 54 117 and an $N$-linked heterogeneous oligosaccharide was located at Asn 45. The same paper also presented the 12 $\alpha$-helix topology model for GLUT1 (Fig. 2), which is now generally accepted and supported by additional experimental evidence, including proteolytic digestion (Cairns et al. 1984 and 1987), circular dichroism (Chin et al. 1987), antibody binding (Davies et al. 1990), Fourier transform infrared spectroscopy (Alvarez et al. 1987) as well as glycosylation- and cysteine-scanning mutagenesis (Hresko et al. 1994, Olsowski et al. 1987).

![Fig. 2. The 12 $\alpha$-helix topology model for GLUT1. Reprinted with permission from (Mueckler et al. 1985). Copyright [1985] American Association for the Advancement of Science.](image-url)
al. 1998). Computational 3D-structural models for a glucose channel within GLUT1 (Zeng et al. 1996) or the whole GLUT1 monomer (Zuniga 2001) were published, based mainly on the alignment of polar amino acid residues within the 12 proposed transmembrane α-helices and the supposed α-helix arrangement in the closely related and thoroughly studied E. coli lactose permease (Frillingos et al. 1998; Weinglass et al. 2002). Another model for the arrangement of the α-helices to form a glucose transport channel based on cysteine-scanning mutagenesis had been presented by Hruz and Mueckler in 2000. Although these three models have in common some α-helices which are supposed to be part of the glucose channel (e.g., H7, H8 and H11), a detailed convergence towards the true GLUT1 3D-structure remains to be seen, possibly pending future crystallization of the protein. Alternative GLUT1 topology models containing 16 transmembrane β-sheets (Fischbarg et al. 1993) or a combination of 10 α-helices and 4 β-sheets (Ducarme et al. 1996) have not gained any further support.

GLUT1 is a member of a family of homologous but distinctive facilitative sugar transporters which operate more or less specifically in different organs, and the corresponding gene family is termed solute carriers 2A (SLC2A). GLUT2–GLUT5 were known and cloned by the end of the 80s (Bell et al. 1990, Mueckler et al. 1994) and the recent availability of the human genome data led to a rapid identification of GLUT6–GLUT12 as well as the proton-myoinositol symporter HMIT1, which was included in the GLUT family, all as reviewed by Joost and Thorens (2001).

GLUT1 transport is stereospecific for D-glucose, although some substituted forms (e.g., deoxy-D-glucose) and a collection of other hexoses are transported (cf. Paper III) at different efficiencies. Dehydroascorbic acid, the oxidized form of vitamin C, has also been shown to be a GLUT1 substrate (Vera et al. 1993). One of the most frequently used and potent inhibitors of GLUT1 transport is cytochalasin B (CB), an antibiotic from the fungus Drechslera dematiodeum. CB binds to the cytoplasmic face of GLUT1 close to its C-term (Cairns et al. 1984) by hydrogen bonds and hydrophobic interactions. The CB-binding affinity of GLUT1 is almost 106-fold higher than that for D-glucose and the binding is competitive. CB has been widely used to study the mechanism and kinetics of glucose transport and, in particular, the association state of GLUT1 monomers. Originally, an alternating conformation model was described (Barnett et al. 1975; Baldwin et al. 1982; Lienhard et al. 1992; Barrett et al. 1999) whereby glucose bound to GLUT1 monomers at only one binding site. After a conformational change the glucose was released on the opposite side of the membrane. The number of CB-binding sites per GLUT1 monomer (CB/mon, r in Papers V and VII) is 1.0 in this model. The alternating conformation model is compatible with saturable glucose transport with Michaelis-Menten kinetics as first reported by Widdas in 1952. Already in 1970, GLUT1 tetramers were discussed (Lieb and Stein 1970). During the 80s and 90s CB/mon values of ≈ 0.5 were measured for GLUT1 in cholate solution (Hebert and Carruthers 1992) and maltose- and glucose-binding measurements led to the evolution of the fixed-site carrier model for GLUT1 (Sulzman and Carruthers...
1999). In this model, both \textit{in vitro} and \textit{in vivo} (Cloherty \textit{et al.} 2001), GLUT1 acts as a dimer of dimers, e.g., a tetramer, that exposes two binding sites for CB (CB/mon=0.5) and in the absence of CB simultaneously shows two sugar export and import sites (Fig. 3). Also in the fixed-site carrier model, sugar transport is accomplished by a conformational change that enables substrate to diffuse through a channel within GLUT1 towards another binding site at the opposite face of the protein. The two monomers within a GLUT1 dimer can only change conformation simultaneously (Fig. 3; Paper III, Fig. 3). Alkaline reduction in 10 mM DTT during purification was reported to dissociate the GLUT1 tetramers into two dimers (Hebert and Carruthers, 1992), indicating the presence of disulfide bridges between the dimers. However, such an effect of DTT has not been confirmed by any other research group.

5. Immobilized-cell technologies

The small size of biological cells often makes it desirable to facilitate their handling, e.g., by attaching them to a support of choice. In addition to this technical advantage, immobilization can also protect the cells from unfavourable environmental conditions and high cell densities can be maintained for repeated experiments or for biocatalysis. Therefore, cell adsorption onto surfaces and immobilization of cells in gel matrices are rapidly growing scientific fields. Adsorption or growing of cells on microchips, membranes or other surfaces (Falsey \textit{et al.} 2001; Bisson \textit{et al.} 2002; Prechtel \textit{et al.} 2002) has become a widespread method and enables time- and space-saving analyses. For large-scale use both prokaryotic and eukaryotic cells have been grown in bioreactors (Park and Chang 2000). Some interesting applications are: the degradation of phenol by \textit{Pseudomonas} (Amanda Wu \textit{et al.} 1991), the production of penicillin by \textit{Penicillium chrysogenum} (Mussenden \textit{et al.} 1993), ethanol
fermentation on adsorbed *Saccharomyces cerevisiae* (Holeberg and Margalith 1981; Nagashima *et al.* 1984) and highly efficient production of monoclonal antibodies on hybridoma cells photo-crosslinked to BIX12 gel beads (Kamihira *et al.* 1993). The methods for immobilization of these and other cell types are many, but entrapment of, e.g., yeast cells in calcium-alginate beads (Galazzo and Bailey 1990) is very mild and has received particular attention. In a simple variant of the method a sodium alginate solution is dropped into a yeast culture containing CaCl$_2$. Cell-containing beads are formed which are then incubated in growth medium and finally in the fermentation medium. Within the beads, cells are vital and continue proliferating to a high density and even exhibit ethanol production rates 1.5 times higher than do cells grown in suspension (Galazzo and Bailey 1990). Cells can also be encapsulated in micro suspension droplets enclosed by alginate membranes (Park and Chang 2000) to prevent leakage of cells into the growth medium, while still allowing release of products.

Immobilization of proliferating cells is usually done under optimized conditions regarding temperature, growth medium, etc. Even though the complex structure of these cells may make them vulnerable in many respects, their capability to grow may also be of advantage to keep the number of immobilized cells at a certain saturation level, as compared to immobilized cell organelles or RBCs. Cells that are lost or die can be replaced continuously. Moreover, the viability of many cell types is even dependent on adhesion to a matrix or other cells, for example, mediated by the integrin protein family (Miranti 2002).

In the work of this thesis, RBC adsorption onto agarose gel beads was mediated by the interaction with WGA or streptavidin-biotin, in 10 mM phosphate buffered saline containing 3 mM NaN$_3$, 50 mM mannitol. The effect of adding low concentrations of different preservatives was studied (Paper IV). The RBCs are relatively robust, but do have a limited life-span in buffer suspension at room temperature. Anyhow, the conditions of first choice were those that retained the highest comparability to previous ligand-binding data for GLUT1 in membrane-vesicle- or proteoliposome systems.

6. *In vitro* prediction of drug uptake

Orally administered drugs have to enter the blood circulation and this absorption process usually takes place in the small intestine. The main obstacles to this are biomembranes, e.g., of the mucous epithelial cells or cells of the underlying blood vessels. The different possible routes for the drugs to cross these barriers are simple diffusion (the passive route), transport by a membrane protein (the active route), leakage through the tight junctions in between the cells (the paracellular route) or passage by transcytosis (Artursson *et al.* 1996). In the general case, without involvement of a specific membrane transporter, the passive pathway is the most common. Compounds that are too hydrophilic may therefore never be able to partition into the membranes and pass unabsorbed through the body, whereas very hydrophobic substances instead may accumulate in membranes or fatty tissues.
During the development of new drugs, a large number of different candidates are synthesized and considerable effort and resources can be spared if the compounds with low chances of intestinal absorption may be excluded at an early stage of the process.

One of the first and most frequently used methods to determine the molecules’ ability to partition into lipid phases is to dissolve the analyte in a system of two immiscible phases, e.g., \(n\)-octanol and buffer (Hansch and Fujita 1964). The ratio of the analyte concentrations in the two phases gives the well known partition coefficient \(P\). This method is routinely used in the pharmaceutical industry. A theoretical disadvantage is that the \(n\)-octanol phase lacks structural similarity to biomembranes, which motivated numerous studies of drug partitioning into suspended liposomes (Betageri and Rogers 1987; Ma et al. 1991). Experiments on monolayers of cultured human epithelial cells, such as the Caco-2 cell line, are another commonly used method, which allows valuable determinations of true permeability values rather than just partition data. However, these analyses are quite time-consuming and results from different laboratories are not comparable due to differences in experimental conditions or the cell line itself (Artursson et al. 1996). Within a given laboratory, fairly accurate comparisons between different drug candidates are possible.

At least two chromatographic approaches to prediction of drug absorption have been made. One of them was the system of immobilized artificial membranes (IAMs), which involves monolayers of phospholipid analogues covalently coupled to a silica support (Pidgeon and Vantarekum 1989; Yang et al. 1996). HPLC columns of more than 20 different IAM supports have been prepared and several are commercially available. The bonded phospholipids of an IAM share many of the physical and chemical properties of a biomembrane, despite differences regarding the size and flexibility of phospholipid bilayers. In addition to drug screening, they have been applied for preparative purposes (Pidgeon et al. 1991; Liu et al. 1997) and the immobilization and quantitative chromatographic analyses of integral membrane proteins, such as the nicotinic acetylcholine receptor (Wainer et al. 1999), P-glycoprotein (Zhang et al. 2000) and the estrogen receptor (Moaddel et al. 2002). Apparently, a broad range of detergent solubilized membrane proteins are able to arrange themselves in the phospholipid monolayers in a way that retains specific ligand-binding activity. The other method was originally called immobilized liposome chromatography (ILC, Beigi et al. 1995; Lundahl and Beigi 1997) and was applied to screening of drugs on liposomes immobilized in gel beads. The method is here, as in Paper I, referred to as immobilized biomembrane partitioning chromatography (IBiPaC) to include biomembranes other than liposomes. The preferred way of liposome immobilization was steric entrapment by freeze-thawing (cf. Section 9.2.), but also liposomes absorbed by streptavidin-biotin interaction (Yang et al. 1998) or covalently bound to CNBr activated gel (Yang et al. 1999) have been used for IBiPaC. The latter two immobilization methods allow the immobilization of unilamellar (proteo)liposomes, which may be of advantage for certain experiments. A strength of IBiPaC is that a multitude of different biomembranes can be immobilized (cf. Section 9; Paper I; Lagerquist et al. 2001) and studied repeatedly with the ease and accuracy of a
chromatographic system. In Paper I, various fundamental properties of the system itself were elucidated and a comparison with IAM chromatography was made. The effect on drug partitioning of certain biomembrane components, such as membrane proteins or cholesterol, could also be studied, as reported initially in Paper I and, more extensively, by Lagerquist et al. (2001).

7. Aims of the study

The fundamental chromatographic properties of the newly introduced IBiPaC system for prediction of drug uptake (Beigi et al. 1995, Lundahl and Beigi 1997) were to be elucidated as well as the influence of some physical and chemical properties of the mobile phase (cf. Section 10.5). Also, the consistency of IBiPaC results with those from other systems, such as the related IAM-chromatography, was to be tested and measured absorption values for a number of drugs were to be correlated with IBiPaC capacity factors (Paper I).

Ligand binding to the glucose transporter GLUT1 had been studied in our laboratory in immobilized RBC membrane-vesicles and proteoliposomes of different compositions. For comparison, GLUT1 in a more natural membrane, e.g., in immobilized RBCs, should be studied using the IBAC method (Paper IV).

Using the first immobilized RBC systems developed (Zeng et al. 1997; Paper IV), attempts to improve cell adsorption stability and capacity were made (Papers V and VII), while at the same time monitoring possible effects of the new cell environments on GLUT1. Regarding the importance of RBC research and cytology in general, future analytical applications of immobilized cell systems seem very probable.

8. Membrane preparations

A membrane protein can be studied in membrane systems of different compositions and complexities. Usually, one attempts to avoid possible disturbances from other membrane proteins in the natural biomembrane by purifying the desired protein in detergent solution and reinserting (reconstituting) it into proteoliposomes composed of lipids of choice. Furthermore, attempts to crystallize a protein are only promising with a highly pure preparation. The first IBAC studies of GLUT1 (Yang and Lundahl 1995; Brekkan et al. 1996) were accordingly done on purified protein. However, one generally suspects that detergents involved during purification, reconstitution or crystallization may harm or alter the investigated membrane protein, which potentially complicates conclusions about its in vivo properties. Beyond that, it is understood that various membrane proteins are influenced by changes in their lipid environment (Romsicki and Sharom 1999; Haruna et al. 2000; Hu et al. 2000; Bogdanov et al. 2002). Therefore, examination of the protein at different stages of preparation may reveal crucial information. Figs. 4A–C show sections of the different membrane materials used for IBAC studies of
GLUT1 in Papers IV, V and VII. Materials A and B and, above all, plain liposomes (Fig. 4D) were used for partition chromatography of drugs in Paper I. The preparation of these membrane materials is briefly outlined below.

Fig. 4. Sections of the membrane materials used for this study: A) RBC membrane, B) RBC membrane vesicles, C) GLUT1 proteoliposomes and D) liposomes. Reprinted with modifications from Paper VI, with permission from Elsevier Science.

8.1. **RBCs**

Fresh human blood or RBC concentrate were washed 3 times in phosphate-buffered saline. This removed the buffy coat of the whole blood. The final cell pellet was diluted two-fold used for electrostatic immobilization (Paper I), or five-fold for coupling to WGA-gel beads (Papers IV and VII), or biotinylation (Paper V). Fig. 4A depicts different glycosylated membrane proteins, some of which make contact with the cytoskeleton consisting mainly of spectrin linked to the membrane by peripheral membrane proteins. The phospholipid asymmetry of the membrane double leaflet is also outlined, but it is likely that this feature is lost upon immobilization and long-time analysis of the cells in buffer solution.

8.2. **RBC membrane vesicles**

Membrane vesicles from RBCs were prepared as in Lundahl et al. (1986). Hypotonic lysis of a batch of RBCs (Dodge et al. 1962) yielded membrane ghosts which were separated from soluble proteins by size exclusion chromatography (SEC) at pH 8.2 and pH 10.5. Washing the membranes at pH 12 in 0.2 mM DTE split off the cytoskeleton due to electrostatic repulsion of its protein components and the membranes formed smaller membrane vesicles. The DTE concentration during the pH 12-washing was optionally raised to 10 mM in Paper V to reveal the relevance of disulfide
bridges for GLUT1 oligomerization that had been proposed by others (Hebert and Carruthers, 1992; Hamill et al. 1999). Fig. 4B shows a section of a cytoskeleton-stripped membrane vesicle containing only integral membrane proteins and a certain degree of peripheral membrane proteins. Membrane vesicles were used for IBiPaC studies (Paper I), Hummel and Dreyer chromatography (Papers II and V) and for IBAC analyses (Paper V).

8.3. GLUT1 proteoliposomes

GLUT1 in membrane vesicles was solubilized with OG and purified on DEAE-cellulose at pH 8, making use of the fact that GLUT1 has one of the highest pI values (i.e. 8.0 (Englund et al. 1995)) among the integral RBC-membrane proteins. A substantial fraction of GLUT1 is eluted within the total volume of the column, whereas most other RBC-integral membrane proteins became adsorbed to the ion exchanger. The purity attained by this procedure (Mascher and Lundahl 1988) was estimated to be 90 ± 5% by amino acid analyses of GLUT1, consistent with data in Rampal et al. (1986). A major contaminant is the nucleoside transporter that also has been studied by IBAC (Haneskog et al. 1998). The purified GLUT1 was reconstituted by SEC on Sephadex G-50 medium (Amersham Biosciences) together with co-purified endogenous RBC-membrane lipids or with a 40-fold excess of egg yolk phospholipids. IBAC on GLUT1 proteoliposomes was described in Paper V and compared to identical experiments on proteoliposomes with GLUT1 that had been solubilized in C_{12}E_{8} or that had been reconstituted by dialysis as in Baldwin and Lienhard (1989).

8.4. Liposomes

Protein-free liposomes composed of synthetic PC, PC/PS, egg phospholipids (prepared from hens’ eggs as in Yang and Lundahl (1994)) or endogenous RBC membrane lipids (extracted in 2:1 chloroform:methanol as in Folch et al. (1957)) were used for IBiPaC in Paper I. The relevant lipids were dissolved in organic solvents, which were rotary evaporated and the final lipid film was suspended in buffer to attain large multilamellar liposomes, a leaflet of which is shown in Fig. 4D.

9. Immobilization of membrane materials

9.1. RBC immobilization

Three different methods for RBC immobilization in chromatographic gel beds were used for IBAC or IBiPaC analyses, e.g., adsorption mediated by electrostatic interaction, binding to WGA, or streptavidin-biotin interaction, as illustrated in Fig. 5 and described below.
9.1.1. **Electrostatic RBC immobilization.**

Positively charged gel particles were prepared as described by Zeng et al. (1997) by polymerization of methacrylamide/piperazine diacrylamide in the presence of N-allyldimethylamine. The gel was crushed with a glass rod and the resulting particles of sizes of 40 - 100 µm were incubated with RBCs and packed into plastic columns (1 cm i.d.). Electrostatically immobilized cells were used for IBiPaC in Paper I.

9.1.2. **WGA-mediated RBC immobilization.**

WGA (Goldstein and Hayes 1976) is a lectin dimer of M, 36 000 that binds specifically to N-acetylglucosamine (K$_d$ = 760 µM (Nagata and Burger 1974)) and a number of other sugars, but its affinity for D-glucose is negligible. WGA-agarose gel beads are commercially available (WGA-Sepharose 4B, bead diameter 45–165 µm, [WGA] = 1.96 mg/ml, Amersham Biosciences) with the original purpose to separate cells or cell organelles, rather than to bind them more permanently. Accordingly, when RBCs were incubated together with WGA-agarose beads, almost all of the cells detached from the gel beads upon packing a column. However, pumping the RBCs at 0.15 ml/min into packed gel beds (0.5 – 1.5 ml) of WGA-agarose and incubation for half an hour effected more permanent retention, at least for a few days, during which affinity chromatographic analyses were possible (Papers IV and VII). We assumed that multiple binding of many lectins to one cell was established during the incubation and caused the adsorption of the cells. Five-fold cell application and incubation were usually employed for RBC-coupling to WGA-agarose columns and were sufficient to approach saturation of the gel bed with cells (Fig. 6). Before IBAC analyses (cf. Section 10.1) the
Fig. 6. The following procedure was performed repeatedly: RBCs (5-fold diluted cell pellet) were applied to a column of WGA-SPA (Vt=0.73 ml) at 0.15 ml/min followed by 30 min incubation and 30 min washing at 0.5 ml/min and a frontal run of 2 nM [³H]CB. The elution volumes were supposed to be proportional to the number of adsorbed cells.

columns were washed overnight to remove insufficiently adsorbed cells.

Paper IV describes the use of WGA-Sepharose 4B for IBAC analyses and the effects of different additives in the running buffer, e.g., fructose, pyruvate, adenine, glutamine and inosine, on the stability of the columns were studied. Coating of the immobilized cells with a positively charged polymer (poly-Lys) was also intended initially to improve column stability, but soon we instead focussed on the apparent effects of this treatment on GLUT1. Paper VII describes the use of superporous agarose (SPA) beads (Gustavsson and Larsson 1996) with coupled WGA. These beads had a diameter of approx. 200 µm, and in addition to the diffusion pores of common agarose gel, they possessed 30-µm superpores. The pores were thus large enough to host RBCs in a hopefully more protected environment as compared to cells attached to the surface of WGA-Sepharose 4B beads. A chromatographic buffer flow through the superpores of these gel beads is documented (Gustavsson and Larsson 1996). A SPA bead with a few bound RBCs is shown in Fig. 7., showing a clearly

Fig. 7. A WGA-SPA gel bead. The length of the scale bar is 50 µm.
rougher surface than do Sepharose 4B beads (cf. Fig 1A of Paper IV). WGA was coupled to the SPA beads after *in situ* CNBr-activation (Nandakumar *et al.* 2000; Paper VII). In brief, packed beads were activated by circulating through the gel bed a solution of 0.5 g CNBr dissolved in acetonitrile and diluted in 15 ml of 1 M K$_3$PO$_4$-HCl, pH 11, for 5 min at 10 ml/min to form cyanate esters and cyclic imidocarbonate (Carlsson *et al.* 1989). WGA was then covalently attached to the activated matrix by overnight circulation through the gel in 0.1 M NaHCO$_3$, pH 8.2. Fig. 8A shows a WGA-Sepharose 4B bead covered with some RBCs, panel B a SPA bead filled with immobilized RBCs and Fig. 8C illustrates a column of SPA beads with WGA-immobilized RBCs.

Fig. 8A) A WGA-Sepharose 4B gel bead. Reprinted with modifications from Paper IV, with permission from Elsevier Science; B) A WGA-SPA gel bead; C) A typical WGA-SPA column (1 cm i.d.), all with immobilized RBCs. The length of the scale bars is 50 µm.

9.1.3. Streptavidin-biotin immobilization.

The extraordinarily high affinity ($K_d \approx 10^{-15}\ M$) of the M$_r$ 244 vitamin biotin for binding to the tetrameric proteins avidin or streptavidin (M$_r$ 67 000) is commonly utilized for crosslinking, labelling, purification or immobilization of biomolecules. Paper V describes coupling of the reagent Sulfo-NHS-LC-biotin (Pierce, USA) to primary amines on the RBC surface (Fig. 9) by 45-min incubation in a five-fold diluted RBC-pellet. The N-hydroxysulfosuccinimide was released and the biotin remained bound to the cell via a spacer arm. Possible biotinylation targets on the RBC surface are, e.g., PS, phosphatidyl ethanolamine or amino groups on membrane proteins. The biotinylated cells were washed and pumped into a column of streptavidin-derivatized Sepharose 4B (Amersham Biosciences) at 0.15 ml/min. The process had to be stopped after a few minutes to prevent the column from clogging due to the high binding affinity of the cells. Sulfo-NHS-LC-biotin concentrations of 1-5 mM in the incubation mixture were found to be sufficient to retain the RBCs in the gel bed.
9.2. **Entrapment of membrane vesicles and (proteo)liposomes.**

The observation that very large liposomes can be prepared by freezing and thawing of a sufficiently concentrated liposome suspension (Pick 1981; Mayer et al. 1985) was the basis for a method for sterical entrapment of liposomes and proteoliposomes in gel beads (Yang and Lundahl, 1994). During subsequent years the method was improved several times and extended (Brekkan et al. 1996; Lundqvist et al. 1997). For this thesis, liposomes (Paper I), GLUT1 proteoliposomes (Paper V) or RBC membrane vesicles (Papers I and V) were immobilized. The membranes were mixed with dried Superdex 200 prepgrade gel beads, degassed and incubated for at least 30 min. The beads swelled and 70–80 % of their inner volume became accessible to the membranes (Lundqvist et al. 1998b). The mixtures were frozen in -70°C (EtOH containing CO₂(s)) and thawed in a 25°C water bath, which presumably caused the membranes to fuse and become entrapped in the cavities within the gel beads. After removing non-entrapped membranes by repeated centrifugal washings, the gel beads (0.5–1.0 ml) were packed into glass columns (5 mm i.d.).

9.3. **Attempts on immobilization of cancer cells and yeast.**

Immobilization of cells other than RBCs on agarose gel beads was attempted. Epidermoid carcinoma cells of the human line A-431 that express 10⁶ epidermal growth-factor receptors (EGFR, Carpenter 2000) per cell were grown under sterile conditions and harvested mechanically by scraping off from the culture flasks. Adsorption of these cells onto agarose gel was attempted using the streptavidin-biotin interaction and binding to WGA or concanavalin A. However, IBAC analysis of ¹²⁵I-EGF binding to the receptors never revealed any specific binding. Vinblastine binding to P-glycoprotein in K-562 cells (human chronic myelogenous leukemia) immobilized in similar systems also failed.
Baker’s yeast cells showed no binding affinity for WGA-agarose gel beads. Apparently the chitin (poly-N-acetylglucosamine) moieties in the cell wall of the yeast cells were not accessible for WGA-binding. However, biotinylated yeast cells adsorbed on streptavidin-agarose and freeze thaw immobilization of yeast plasma membrane vesicles enabled preliminary IBiPaC analyses (Suer, Gottschalk and Lundahl, unpublished data).

10. Analytical methods

10.1. Immobilized biomembrane affinity chromatography (IBAC)

Affinity chromatography is well known as a preparative method in bioseparations, but it can also be used for quantitative analysis of ligand binding to biomolecules (first applied by Andrews et al. 1973; Dunn and Chaiken 1974), particularly when frontal sample application is used (Nichol et al. 1974, Kasai and Ishii 1975). In frontal quantitative affinity chromatography a large volume of a relatively dilute ligand sample is applied to the analytical column containing the immobilized acceptor molecules. The experimental set-up is sketched in Fig. 10A. During such an experiment the

Fig 10. A) Experimental set-up for IBAC. B) Frontal elution profiles drawn for a series of samples with $[\text{ligand}] = 0.5–2K_d$ (cf. Paper VI). Both reprinted from Paper VI, with permission from Elsevier Science.

ligand concentration at the top of the column rises until a binding equilibrium is established and the thereby formed ligand boundary migrates through the column. The characteristic shape of chromatograms resulting from frontal runs is drawn in Fig. 10B. The plateau height of the elution pattern corresponds to the ligand concentration of the original sample, which is not diluted during the analysis. The retention volume in a particular run is read at half the plateau height and depends on the ligand concentration $[B]$ in the sample, the constant number of immobilized binding sites $N$ and the dissociation constant $K_d$. The non-specific retention volume under conditions where specific ligand
binding is completely suppressed ($V_{\text{min}}$, cf. Fig. 10B; the determination of $V_{\text{min}}$ is explained below) is also constant at all ligand concentrations. For known $N$, $K_d$ and $V_{\text{min}}$ in a given experiment the retention volume $V$ can be calculated according to Eq. 1 [Kasai et al. 1986]:

$$V = V_{\text{min}} + \frac{N}{[B] + K_d}$$

where the term $N/([B]+K_d)$ is equal to the retention volume, $V_{\text{spec}}$, caused by specific interaction between $B$ and the immobilized analyte. Some inherent advantages of the method are easy handling of the immobilized biomolecules in a chromatographic system and that results can be obtained relatively quickly (compared to dialysis) at equilibrium without separation of free from bound ligand. Both affinity constants and the number of immobilized binding sites can be determined (see below) by use of the relevant theory published, e.g., in Winzor et al. (1985), Kasai et al. (1986), Winzor and Jackson (1993) and Winzor (1998).

The first experiments with frontal quantitative affinity chromatography on membrane proteins were performed in 1996, by Brekkan et al. using GLUT1 in immobilized proteoliposomes and membrane vesicles. The method was later described as immobilized biomembrane affinity chromatography (IBAC, cf. Papers V and VI; Lundqvist and Lundahl 2002). For this thesis, GLUT1 was analyzed in immobilized RBCs, membrane vesicles and proteoliposomes. CB-binding parameters were basically determined from a series of frontal runs of 1 or 2 nM [$^3$H]CB supplemented to total CB concentrations of 2–100 nM (Fig. 11B). Signals were detected on-line in a flow scintillation detector with a six second update-time and the elution fronts were evaluated by curve-fitting of the data to the

![Diagram](image-url)

**Fig. 11.** Frontal elution profiles of 2 nM [$^3$H]CB on RBCs immobilized in a WGA-SPA column. The samples further contained from the right to the left A) 0, 0, 7.5, 12.5, 25 and 50 mM D-glucose and B) 0, 0, 22, 47, 75 and 100 nM cold CB.
four parameter logistic function

\[ f(x) = \frac{a - d}{1 + (c/x)^b} + d \]  

(2),

where \( a \) and \( d \) are the plateau- and background level, respectively; \( b \), the slope coefficient and \( c \), the elution volume of the front at \( f(c) = (a+d)/2 \).

The frontal elution volumes, \( V \), at the different free CB-concentrations, \([CB]\), enabled calculation of the amount \( B \) of bound CB at each \([CB]\),

\[ B = (V - V_{\text{min}}) [CB] \]  

(3).

A plot of \( B \) versus \([CB]\) yielded a binding hyperbola (Fig. 12).

![Figure 12: Binding hyperbola for CB binding to GLUT1 for the data in Fig. 11B. Curve fitting (Eq. 4) resulted in \( N = 527 \pm 11 \) pmol immobilized CB-binding sites and \( K_d = 80 \pm 3 \) nM.](image)

Curve fitting to Eq. 4,

\[ B = \frac{N[CB]}{K_d + [CB]} \]  

(4),

which is analogous to the Michaelis-Menten equation, revealed the number of operative CB binding sites \( N \) and the dissociation constant \( K_d \).

The non-specific retention volume \( V_{\text{min}} \), and the affinity of glucose-binding to GLUT1 was determined for each column by running another series of samples containing 1 or 2 nM \([^3H]CB\) and
varying concentrations of D-glucose (0-50 mM, Fig. 11A) using linear regression (Eqs. 5 and 6; Fig. 13) as in Brekkan et al. (1996).

\[
\frac{1}{V_{\text{max}} - V_i} = \frac{1 + [CB]K_{CB}}{NK_{CB}} + \left(1 + [CB]K_{CB}\right)^2 \frac{1}{NK_{glc}K_{CB}[glc]}
\]  

(5)

\[
\frac{1}{V - V_{\text{min}}} = \frac{1}{NK_{CB}} + \frac{1}{N}[CB]
\]  

(6),

with [glc] being the free concentration of D-glucose; \(K_{glc}\) and \(K_{CB}\), the association constants for glucose- and CB-binding to GLUT1, respectively; \(V_{\text{max}}\), the maximal CB elution volume at \([CB]<<1\) and \([glc]=0\); \(V_i\), the CB-retention volume at \(i\) mM [glc]; \(V\), the CB-retention volume at different [CB] and [glc]=0. \(V_{\text{min}}\) was obtained by extrapolation of the linear plot (Fig. 13A, according to Eq. 5) to infinite glucose concentration. Eq. 6 then gave \(N\) and \(K_{CB}\) and insertion of these parameters into Eq. 5 also allowed calculation of \(K_{glc}\). Hence, although D-glucose itself was not measurably retarded on GLUT1-containing columns due to its low affinity for the protein, inhibition of CB-binding at accordingly higher glucose concentrations revealed the glucose binding constants. It is clear, however, that non-linear regression of binding data, as in Eq. 4, is to be preferred whenever possible, since extrapolation and compression of the data inherent to reciprocal linear plots is thereby avoided (Klotz 1983; Leatherbarrow 1990).

10.2. Hummel and Dreyer SEC

The use of Hummel and Dreyer SEC (Hummel and Dreyer 1962) was described in Papers II and V for the analysis of CB- and glucose binding to GLUT1 in freely suspended membrane vesicles.

Fig. 13. Linear regression analysis of the data in Fig. 11 using A) Eq. 5 and B) Eq. 6.
and proteoliposomes. A gel bed of Superdex 75 (Amersham Biosciences) was equilibrated with 2 nM $[^3\text{H}]\text{CB}$ and 0 – 200 nM cold CB. The biomembranes were incubated with the relevant running buffer and 20- or 50-µl aliquots were applied to the column. In the typical resulting chromatogram (Fig. 9 in Paper II) the baseline signal of $[^3\text{H}]\text{CB}$ first rose to a positive peak corresponding to membranes with bound CB eluting in the void volume and later a negative peak appeared due to depletion of $[^3\text{H}]\text{CB}$ in the mobile phase upon CB-binding to the membranes. The area of the negative peak was used for calculating the amount of bound CB, which increased in runs with increasing concentrations of unlabelled CB included in the mobile phase. The $K_D$ value and the number of binding sites in the aliquots were determined by curve fitting to an analog of Eq. 4.

10.3. *Ultracentrifugal assay*

Ultracentrifugation was used as an alternative method for the measurement of CB binding to free membrane vesicles, as described in Paper V, similarly as by Pinkofsky *et al.* (1985) or Helgersson and Carruthers (1987). GLUT1 proteoliposomes were incubated with CB at different concentrations (0-500 nM) and centrifuged for 60 min at 226 000 g followed by separation of free from bound CB and scintillation counting. The method may be suitable for preliminary screening of the strength of a biointeraction because many samples can be handled at the same time, but this method has the obvious disadvantage that bound ligand is separated from free ligand in a rather time-consuming procedure. The ligand concentration in the emerging pellet almost certainly differs from that in the incubation mixture, which causes ligand to detach or bind during the separation.

10.4. *Transport retention chromatography*

A partial chromatographic separation of D-glucose from L-glucose has been demonstrated on immobilized GLUT1-proteoliposomes (Lu *et al.* 1993) or RBCs (Zeng *et al.* 1997; Paper IV). Mixtures (20 µl) of D-$[^{14}\text{C}]$glucose and L-$[^3\text{H}]$glucose were run through columns containing the immobilized GLUT1 material and the eluent was monitored on-line with a flow scintillation detector using two different energy windows. An elution volume difference, $\Delta V_e$, between the enantiomers was caused by the stereospecificity of GLUT1, which only allowed D-glucose access to the membrane-enclosed volume. The contribution of D-glucose-binding to GLUT1 is negligible according to Eq. 1. Zeng *et al.* (1997) used transport retention chromatography to measure the stability of their immobilized RBC columns, but the very small $\Delta V_e$ involved makes this method less sensitive than measuring the frontal retention volume of an inhibitor such as CB.

10.5. *Immobilized biomembrane partitioning chromatography (IBiPaC)*
A variety of biomembranes were immobilized as described in Section 9 for IBiPaC analysis in Paper I. The retention volumes $V_R$ of drugs (10–50 µl, 0.02–0.5 mg/ml) on the biomembrane-containing columns were measured using a set-up as in Fig. 10A, except that a correspondingly smaller sample loop, UV-detection at 220 nm and paper recording were used. In contrast to the frontal elution mode (cf. Section 10.1.), zonal elution was used in IBiPaC, i.e., the drug samples were eluted as peaks. The capacity factor $K_s$ is a simple normalization of $V_R$ to enable comparison of results obtained on different columns.

$$K_s = \frac{V_R - V_0}{A}$$

(7),

with $V_0$, the elution volume of the reference substance $\text{Cr}_2\text{O}_7^{2-}$ that does not interact with the membranes, and $A$, the molar amount of immobilized phospholipids determined by a phosphorus assay according to Bartlett (1959). Units were chosen to obtain $K_s$ in $M^{-1}$.

Drugs of considerably different nature (β-blockers, phenothiazines and benzodiazepines) were analyzed and compared. $V_R$, the variable in Eq. 7, and accordingly also $K_s$, are dependent on the degree of the drug’s partitioning into the immobilized biomembranes. Eq. 7 is a simplified version of a definition by Lundahl and Beigi (1997) which included the internal volume of the immobilized biomembranes.

In a first set of experiments on entrapped PC liposomes the effect on $K_s$ of varying some chromatographic parameters (cf. Section 11.3.), as well as ionic strength, pH and temperature was investigated. Also the effect of incorporating negatively charged PS into PC liposomes on the retention of positively charged drugs was studied. A set of drugs was run on IBiPaC and IAM columns and the log $K_s$ values were correlated to the corresponding log $k'_{IAM}$ values (Ong et al. 1996). A diagram for prediction of drug uptake was prepared using literature data for absorption of the drugs in humans.

For molecules with specific binding sites within the examined biomembrane, for example CB binding to RBC membranes, IBAC and IBiPaC may be combined by substituting $V_{min}$ in Eq. 3 with $V_R$. Eq. 4 then becomes

$$(V - K_s A - V_0)[\text{CB}] = \frac{N}{[\text{CB}] + K_d}$$

(8).

For Eq. 8 it is assumed that the zonal and frontal non-specific CB retention volumes (inhibiting specific binding to GLUT1 at high glucose concentrations) are approximately equal, which seems likely at least at low drug concentrations. Although Eq. 8 is more complex than Eq. 4 it would
facilitate the experimental work, as the determination of $V_{\text{min}}$ for each column by an experimental series with glucose inhibition (cf. Section 10.1) becomes unnecessary. Instead, a frontal $K_s$ value for CB could be carefully determined once, and measurement of the amount of immobilized lipids, $A$, and the frontal elution volume, $V_0$, of a reference substance would then enable the use of Eq. 8. The zonal log $K_s$ value for CB on egg yolk phospholipid liposomes is 1.99 (unpublished results, Lagerquist Hägglund et al.) indicating a strong partitioning and a good membrane permeability for the molecule (cf. Section 11.3.). Drawbacks of the approximation are that a $K_d$ value for glucose inhibition of CB-binding is not obtained and that changes in $V_{\text{min}}$ caused, for example, by treatment of immobilized cells with poly amino acids might remain undetected.

11. DISCUSSION OF RESULTS

11.1. Can RBCs be adsorbed for chromatographic analyses?

Mainly two methods for RBC immobilization were developed for this thesis; namely, adsorption to agarose beads via immobilized WGA or by means of the streptavidin-biotin interaction (cf. Section 9.1; Papers IV, V and VII). Electrostatic adsorption of RBCs to charged bisacrylamide particles (Zeng et al. 1997) played a minor role in drug partitioning in Paper I. The quality of the immobilization was judged in different ways, e.g., concerning RBC adsorption capacity, stability, relative errors of results or the ratio of specific ($V_{\text{spec}}$) to non-specific ($V_{\text{min}}$) CB binding in the columns (cf. Table 1). The author has chosen to split the data for WGA-Sepharose 4B and WGA-SPA to distinguish plain cells from poly-Lys treated cells and high ($\geq$3.1 mg/ml) WGA-concentrations from lower ones ($\leq$1.2 mg/ml), respectively.

Table 1. Summary of data on immobilized-RBC column capacity, stability and quality of results. Numbers in parentheses are the numbers of used columns; the percentage data given in columns 2, 4 and 5 are relative errors of the respective values.

<table>
<thead>
<tr>
<th>Method</th>
<th>$10^9$ RBCs/ml gel</th>
<th>$T_{90}$[days]</th>
<th>$10^7$ CB sites/RBC</th>
<th>$V_{\text{spec}}/V_{\text{min}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WGA-Sepharose 4B (3)</td>
<td>0.82 ±23%</td>
<td>3–7 (6)</td>
<td>260 ±23%</td>
<td>2.0 ±20%</td>
</tr>
<tr>
<td>WGA-Sepharose 4B + poly-K (6)</td>
<td>0.60 ±17%</td>
<td>4 (1)</td>
<td>500 ±40%</td>
<td>2.9 ±28%</td>
</tr>
<tr>
<td>WGA-SPA, 3.1mg WGA/ml (4)</td>
<td>0.98 ±11%</td>
<td>9 (1)</td>
<td>330 ±12%</td>
<td>4.9 ±14%</td>
</tr>
<tr>
<td>WGA-SPA, 1.2mg WGA/ml (8)</td>
<td>0.58 ±16%</td>
<td>6 (1)</td>
<td>250 ±16%</td>
<td>1.8 ±7%</td>
</tr>
<tr>
<td>Streptavidin-biotin (7)</td>
<td>0.53 ±17%</td>
<td>5 (1); 8 (1)</td>
<td>350 ±14%</td>
<td>1.6 ±13%</td>
</tr>
<tr>
<td>Electrostatic immobilization</td>
<td>1.14 ±11%</td>
<td>10 (1)</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* obtained by automated amino acid analysis after 2 days of IBAC experiments (rows 1–5) or an optical assay directly after immobilization (row 6); * Number of days after RBC immobilization with measured stability $\geq 90%$; * Paper IV; * Papers IV and VII; * SPA with 3.1 (1) or 4.3 (3) mg WGA/ml, Paper VII; * SPA with 0.08 (2) or 1.2 (6) mg WGA/ml; * $[\text{Biotin}]=2$ mM, cf. Paper V; * $[\text{Biotin}]=1$ mM; * Zeng et al. (1997).
The average number of immobilized RBCs per milliliter gel was about equal for poly-Lys treated cells on WGA-Sepharose 4B (0.60 ± 0.10 × 10⁹/ml), WGA-SPA at low WGA concentrations (0.58 ± 0.09 × 10⁹/ml) and biotinylated cells on streptavidin-agarose (0.53 ± 0.09 × 10⁹/ml). It should be noted that the poly-Lys treatment of the cells on WGA-Sepharose 4B, which was originally meant to stabilize the immobilization, lowered the cell density by more than 25%, which probably contributed to the high CB/mon value measured for this material in Paper IV. In general, the immobilized RBC density on WGA-agarose was dependent on [WGA]. The corresponding plot in Fig. 14A described a hyperbola with saturation at 1.3 × 10⁹ bound cells per milliliter gel (Paper VII).

Fig. 14. A) The number of immobilized RBCs per ml gel bed of WGA-SPA (●, Paper VII) or WGA-Sepharose 4B (□, Paper IV) versus the WGA concentration in the gel. B) Dependence of the relative error for the number of CB binding sites per RBC on WGA on the T₉₀ values. Numeral marks refer to rows 1–5 in Table 1. The data gave the best fit to the equation \( f(T_{90}) = (12 + 1200 \times e^{-T_{90}}) \% \).

The high RBC density upon electrostatic adsorption (Table 1, row 6) was obtained directly after immobilization and a different assay was used, which may complicate a direct comparison. In addition, the interpretation of the results in Zeng et al. (1997) suffers from the parallel statements that such a high density of intact cells was immobilized and that a large fraction of the cells became lysed and formed ghosts.

The stability of a series of columns with cells on WGA-Sepharose 4B was assayed by daily frontal runs of CB (Paper IV). The T₉₀ values, e.g., the number of days during which at least 90% stability was observed, were not completely reproducible (i.e., 3–7 days) and were not affected by chromatographic adjustments or cell preservative additives in the mobile phase. For later work on immobilized RBCs, stability measurements were usually done only once per investigated system and the best result (T₉₀ = 9 days) was obtained with WGA-SPA at high lectin concentration. For streptavidin-biotin-immobilized cells (Paper V) the stability decreased for RBCs biotinylated with increasing concentration of Sulfo-NHS-LC-biotin (1–5 mM). Muzykantov et al. (1996) reported that
RBCs biotinylated in 20–700 µM NHS-LC-biotin tended to crosslink and lyse upon addition of streptavidin. Although massive cell lysis was not observed in our chromatographic system it seemed that the cells were unfavourably influenced by biotinylation and/or streptavidin binding (Fig. 1 in Paper V). The stability measurements on the electrostatic immobilization system may not be completely comparable to the present ones, but the reported T90 of 10 days is similar to that for the best WGA-system. It is not surprising that none of the so far investigated RBC immobilization systems had a durability comparable to sterically entrapped GLUT1 proteoliposomes or membrane vesicles (Section 9.2; Paper V). These latter columns could be used for IBAC analyses for several months, apparently without variation of results, retaining 80–90% of the immobilized material (Lundqvist et al. 1998a). The dextran-coated interior of the Superdex 200 beads (cf. Fig. 5 in Paper III) was obviously a very benign environment for the immobilized biomembranes.

The measured number of CB-binding sites per cell (cf. Table 1) was 250–350 × 10^3 for most materials. Poly-Lys-treated WGA-Sepharose 4B (Table 1 and Paper IV) gave a higher value, which was later discarded because of the large error limit (cf. Paper VII). The highest accuracy was obtained for WGA-SPA. The relative errors of 12% or 16% covered values for both plain and poly amino acid treated cells, whereas the individual values for the different materials had even lower error limits (Paper VII).

It is evident from Fig. 14B that the quality of the IBAC results was dependent on the chromatographic stability of the immobilized material. The plot of the relative error for the CB-binding sites per cell (Table 1) decayed exponentially with increasing T90 for the different materials (Fig. 14B). Another requirement for good IBAC results seemed to be a high density of immobilized binding sites giving a high ratio of \( V_{\text{spec}} \) to \( V_{\text{min}} \) (cf. Paper VI). Although no correlation between \( V_{\text{spec}}/V_{\text{min}} \) and the relative errors for CB sites/cell, similar to that above, could be deduced from Table 1, at least the distinctly highest \( V_{\text{spec}}/V_{\text{min}} \) value was obtained for WGA-SPA at high [WGA] which also had the lowest error limit for the number of CB sites/cell. Immediate suggestions for further improvement of the IBAC results are to elaborate lectin coupling conditions that always yield high [WGA] and the use of smaller SPA beads with a larger surface area accessible to RBCs.

Immobilization and/or IBAC analyses of membrane proteins of cancer cells (cf. Section 9.3.) proved to be difficult in the frame of this thesis, although such mild conditions as running in culture medium at 37°C were tried. Although methods for immobilization of animal and plant cells exist (cf. Section 5), these tiny entities may be too sensitive to withstand the non-sterile and non-optimized growth conditions in a chromatographic system. Better results may be obtained upon improvement of the harvesting technique or growing the cells directly in the columns. Also, the observation that EGF binding was retained after fixation of A-434 cells in, e.g., approx. 90% ethanol (unpublished results) could be the key to chromatographic analysis of that interaction.

To answer the title question of this section: Yes! The system can be optimized further but chromatographic analyses on immobilized RBCs with reasonable accuracy are already possible.
Considering the importance of RBCs for our organism and the existence of parasites and major diseases that can affect them, the new technique presented in this thesis might well prove useful for certain specific analytical purposes.

11.2. Implications about GLUT1

The analysis of ligand binding to GLUT1 is an intricate matter. The supply of literature data is abundant and reported results vary considerably, perhaps depending on the preparation status of the protein. A generally accepted picture of the salient attributes of the protein, such as its oligomerization state or transport mechanism, has still not been established (cf. Section 4).

We determined CB/mon values of 0.5 for GLUT1 (designated State 1 in Paper V) in most of our immobilized biomembranes (RBCs, membrane vesicles and GLUT1 proteoliposomes (Papers IV, V and VII)). Suspended membrane vesicles, however, showed a CB/mon close to 1 (State 2, Paper V). The same high value had been obtained with poly-Lys treated RBCs (Paper IV), but an attempt to reproduce these experiments (Paper VII) led to the inconclusive CB/mon values of 0.77 ± 42% or 0.98 ± 40% (corresponding to 500 CB sites per cell, cf. Table 1) without correction of the number of immobilized cells using the standard curve from Paper IV. The CB/mon for streptavidin-biotin immobilized cells (0.68 ± 16%, Paper V) was interpreted to be due to the simultaneous presence of GLUT1 functional dimers and monomers. Considering the results in Paper VII, the author now rather reasons that the loss of hemoglobin from the columns (cf. Muzykantov et al. 1996) caused the elevated CB/mon ratio. The use of WGA-SPA in Paper VII finally solved the somewhat uncertain situation, giving CB/mon values close to 0.5 for both plain cells and those treated with poly-Lys, -Arg and -Glu. The relative errors were 3–18% with a grand average of 0.54 ± 17%. Together with the results for both immobilized and suspended membrane vesicles and GLUT1 proteoliposomes (cf. Paper V), an updated scheme for GLUT1 conversions in the different biomembranes is presented in Fig. 15.

The low CB/mon for free membrane vesicles (0.43 ± 0.03, Paper II) was by mistake obtained on vesicles that retained most of the cytoskeleton due to washing at a maximum pH of 10.5. CB/mon measured on regular membrane vesicles was 0.97 ± 0.04. Zhang and Ismail-Beigi (1998) reported a similar 1.8-fold increase of glucose-sensitive CB binding sites in RBC ghosts upon washing in 0.2 mM EDTA, pH 12.

The number of CB-binding sites per cell (Table 1) was solely based on IBAC results, whereas CB/mon values for RBCs were dependent on the assumption of the presence $5.1 \times 10^5$ GLUT1 copies per cell. The calculation of this value presented in Paper IV resulted in a large relative error of 41%. However, it is likely that the true value is closer to $5 \times 10^5$. The determination of Mascher and Lundahl (1988) of $5.3 \times 10^5$ GLUT1 per RBC by analysis of DEAE purification of the protein and the results of monoclonal-antibody binding by Allard and Lienhard (1985) yielding $5 \times 10^5$ copies/cell (cf. Anstee
1990) gave us the confidence to assume $5.1 \times 10^5$ GLUT1 monomers per cell. The results in Paper V for entrapped GLUT1 proteoliposomes (CB/mon=0.40±0.01) and membrane vesicles (0.50±0.02) were instead dependent on the purity and the fraction of active transporters, but all supported the presence of GLUT1 in State 1. Hence, our CB/mon values support the conclusion that GLUT1 in the RBC membranes works as a functional dimer that can reversibly dissociate into functional monomers during membrane vesicle preparation. It is not clear to which degree a physical dissociation of the dimers takes place. The formation of GLUT1 tetramers, as in the GLUT1 fixed site carrier model (Sulzman and Carruthers 1999; cf. Section 4 and Paper III), is supported by a CB/mon of 0.5, although the formation of larger aggregates (hexamers, octamers, etc) cannot be excluded. Exposure of membrane vesicles to 10 mM DTT during the washing at pH 12 did not effect the CB/mon of the immobilized vesicles themselves nor that of immobilized GLUT1 proteoliposomes prepared from that material (cf. Paper V), in disagreement with the results of Hebert and Carruthers (1992).

The dissociation constants for CB-binding to GLUT1 seem to be independent of the preparation state of the protein. For almost all analyzed GLUT1 materials (Papers IV, V, VII) the $K_d$
values fell within the interval $70 \pm 14 \text{nM}$ determined in Paper VII. Our measured CB-binding affinity is higher than most others reported in the literature, which partly may be due to the use of non-equilibrium methods for the ligand binding assays (e.g., centrifugation or ultrafiltration).

The affinity for D-glucose binding to GLUT1, on the other hand, appeared to be higher in the more natural biomembranes than in proteoliposomes, as determined by displacement of CB. For all immobilized RBCs studied in this thesis work, the $K_d$ was $12 \pm 3 \text{ mM}$, Zeng et al. (1997) reported even 7 mM, whereas for immobilized membrane vesicles and proteoliposomes 18±4 and 41±5 mM, respectively, were measured.

The partial separation of D-glucose and L-glucose in transport retention chromatography experiments succeeded on RBCs immobilized on WGA-Sepharose 4B (Fig. 16).

![Fig. 16. Simultaneous elution of L-[3H]glucose and D-[14C]glucose on RBCs adsorbed on WGA-Sepharose 4B (V_t = 1.3 ml) at 0.1 ml/min. The difference in elution volume ($\Delta V_e = 64 \mu l$) was read at 50% peak height.](image)

An increase in $\Delta V_e$ with the number of immobilized RBCs was described (Paper IV). For unknown reason, the elution volumes of the enantiomers did not differ for columns prepared by any of the other RBC immobilization methods developed in this thesis work. In principle, this could have been caused by glucose leakage from the cells (although they retained hemoglobin), inactive GLUT1 or too slow equilibration of the column with glucose. For the WGA-SPA columns it did not matter whether D-glucose or deoxy-D-glucose, which is transported into but not metabolized in RBCs, were used.

In conclusion, the direct comparison of IBAC results obtained on a multitude of different GLUT1 materials as presented here may yield more insight into the protein’s attributes than do isolated analyses of single preparations. All such knowledge about the properties of GLUT1 may prove helpful in attempts to crystallize the protein.
11.3. IBiPaC for prediction of drug uptake

The typical dimensions of an IBiPaC column containing freeze-thaw immobilized egg yolk phospholipid or PC liposomes were approx. 5 cm × 0.5 cm (i.d.), corresponding to a total volume of ≈1 ml, and a typical gel bed contained about 50 µmol lipids. The retention volumes of different drugs on such columns covered a range of ≈1–500 ml, resulting in log $K_s$ values between ≈0.5 (e.g., for atenolol) and ≈4 (e.g., for chlorpromazine). A remarkable feature of the columns was their extraordinary stability. Once prepared, they could be run (or stored) for months or even more than a year (Österberg et al. 2001), without significant decrease of the drugs’ retention volumes.

The log $K_s$ values were found to be independent of the total column volume (0.1–1.0 ml), the chromatographic flow rate (0.1–1.2 ml/min), the drug concentration and the concentration of immobilized phospholipids (10–55 mM) thereby justifying the use of Eq. 7. However, departure from standard experimental conditions, 150 mM NaCl, pH 7.4 and 23°C, did effect drug partitioning into PC liposomes containing 18.5 mol % PS (cf. Fig. 1 in Paper I). Lowering the salt concentration to 0 increased the log $K_s$ values of the positively charged β-blockers, dramatically for the relatively hydrophilic drugs atenolol and metoprolol, and more moderately for the more lipophilic propranolol. The reason was an increased electrostatic interaction of the drugs with the negatively charged PS. On pure PC liposomes with neutral net charge the effect was only observed with atenolol. Raising the NaCl concentration to 0.6 M resulted at most in a slightly stronger partitioning on the PS-free liposomes due to enhanced hydrophobic interactions. The log $K_s$ values for β-blockers on PS/PC liposomes were essentially constant in the pH interval 6–9, but increased for pH 9–11, probably due to deprotonation of the model drugs. The drugs showed a heterogeneous partitioning behavior upon a temperature increase from 10°C to 60°C, e.g., low log $K_s$ values increased linearly, whereas high values decreased. The analyzed phenothiazines, promethazine and fluphenazine, both showed linearly decreasing membrane partitioning in response to the temperature rise.

Log $K_s$ values of 15 drugs on egg phospholipid liposomes were plotted versus corresponding data obtained on PC liposomes, RBC membrane lipid (ML) liposomes, RBC membrane vesicles and RBCs/ghosts (Fig. 17). All plots were linear with the best correlations ($r^2 = 0.96$) for the membrane lipids (B) and membrane vesicles (C). The slopes of the lines increased through panels A to D in line with the complexity of the immobilized membranes. Low log $K_s$ values increased, whereas high values decreased in the panels to the right. Later work (Lagerquist et al. 2001) showed that the presence of cholesterol in liposomes generally decreased log $K_s$ values, whereas model transmembrane proteins (GLUT1 and bacteriorhodopsin) caused electrostatic attraction or repulsion of the drugs. A combination of these effects may have caused the slope shift in Fig. 17. A complication is that positively charged drugs may get deprotonized, at the surface of the membrane while partitioning into the membrane.
Fig. 17. Comparison of drug interaction with egg phospholipid (EPL) liposomes with results obtained on other biomembranes. ML denotes endogenous RBC membrane lipids. The drugs were a, atenolol; b, acebutolol; c, nadolol; d, metoprolol; e, pindolol; f, oxprenolol; g, fluphenazine; h, pyrilamine; i, flunitrazepam; j, alprenolol; k, propranolol; l, diazepam; m, nitrazepam; n, oxazepam; o, desmethyldiazepam; p, promethazine; q, chlorpromazine. Reprinted from Paper I, with permission from Elsevier Science.

The correlation of log $K_s$ values on PC liposomes and log $k'_{IAM}$ values (Fig. 4 in Paper I) obtained on an IAM.PC.DD column was slightly poorer ($r^2=0.83$) than the above ones, despite the fact that the same type of phospholipid was present in both columns. Sterical hindrance for diffusion of some of the drugs within the IAM lipid monolayers may be among the reasons for the difference.

Fig. 18. Oral absorption of drugs versus log $K_s$ values obtained on RBC membrane vesicles. The different compounds were as listed in Fig. 17 and r, polyethylene glycol (Mw 200); s, mannitol; t, salicylic acid; u, hydrocortisone; v, corticosterone; w, warfarin; x, terbutaline. The absorption data were adopted from Palm et al. (1997) and Fagerholm et al. (1997).

According to plots of drug absorption data in humans versus log $K_s$ values obtained on immobilized membrane vesicles (e.g. Fig. 18), it is likely that drugs with intermediate log $K_s$ ($\approx 1.25–2.25$) are absorbed well. Molecules with lower log $K_s$ values seem to be too hydrophilic for adequate
membrane partitioning. Several drugs in the original plot of Fig. 5 in Paper I have been removed: acetylsalicylic acid is known to be hydrolyzed to salicylic acid upon membrane transport (www.fass.se, search for Aspirin) and sulfasalazine is a substrate for effective efflux systems (e.g., P-glycoprotein) (Österberg et al. 2000). For log $K_s$ values higher than approx. 2.25 the situation appeared to be more intricate. The removal of sulfasalazine from the plot lead to a shallower decrease of absorption for high log $K_s$ values. In Österberg et al. (2000) many drugs with log $K_s$ values > 2.5 on PC liposomes are shown to be almost completely absorbed in humans, making a dramatic absorption decrease for drugs with such high log $K_s$ values more improbable. Obviously, the course of the line in this region is dependent on the choice of model drugs. Features other than the model drugs’ passive diffusion, such as their metabolism, have to be known. A choice of different model compounds representative of different groups of related drug candidates may be considered.

Another important factor for prediction of drug uptake by IBiPaC is the choice of immobilized biomembranes. In Paper I, RBC membrane vesicles of high stability containing membrane proteins and a diversity of lipids were the most reliable and relevant of the available systems. A system of immobilized brush-border membranes from pig mucosa is currently under development (Engvall et al., unpublished).

12. Conclusions

Paper I The simple definition of log $K_s$ presented in the paper worked for normalization of drug partitioning into biomembranes and for prediction of drug uptake in humans. The log $K_s$ values were independent of the chromatographic flow rate, the gel bed volume as well as drug and lipid concentrations, but were affected by changes in ionic strength, pH and temperature. Effects of charge, composition and the complexity of the immobilized membranes on IBiPaC were, for the first time, systematically recorded. Among the drugs tested, those with intermediate log $K_s$ values were absorbed effectively in humans. To make calibration curves that are practically useful for prediction of drug uptake the model compounds have to be chosen carefully. The study of more model drugs is necessary and more work is required for immobilization of the most relevant biomembranes.

Paper II Extension of the size range for chromatographically separable biomembranes in connection with the development of gels with larger pore sizes was made evident. Characteristics of biomembranes, such as flexibility, non-specific binding to gel matrices and light scattering and their impact on chromatography were explained. Preparative and analytical applications, including a previously unpublished method, Hummel and Dreyer analysis of ligand binding to GLUT1, were described. Finally, alternative methods and analyses on immobilized biomembranes were presented briefly.
Paper III  An overview of some of the known features of GLUT1 was given concerning its purification, topology and structure. The alternating conformation model and the fixed-site-carrier model for glucose transport kinetics were described and a possible connection between the two was explained and reconciled with our own results.

Papers IV, V, and VII  Equilibrium constants for ligand binding to membrane proteins in immobilized RBCs can be determined by IBAC. The most promising results were so far obtained upon adsorption of the cells on SPA beads containing more than 3.1 mg/ml coupled WGA (Paper VII). The density of immobilized cells in the gel bed was high \((=10^9\) cells/ml gel) and the cell immobilization stability seemed to be the most critical factor for the quality of the IBAC analyses. Results obtained with RBCs on commercial Sepharose 4B with 1.96 mg/ml coupled WGA showed larger error limits and the column stability was lower, especially for columns treated with polylysine. Biotinylation of RBCs with increasing concentrations of biotin reagent followed by adsorption on streptavidin-agarose seemed to reduce the time available for analysis of the system (Paper V). The method was thus apparently harmful to the cells and was not developed further.

The large amount of data for CB binding to GLUT1 in proteoliposomes, membrane vesicles and cell membranes collected during several years of IBAC analyses was complemented and summarized. A more general picture regarding the association state of the transporter monomers arose, suggesting that the dimeric state of CB binding observed in immobilized proteoliposomes probably also prevailed in the native RBC membrane. The dissociation constant for CB-binding to GLUT1 was approx. 70 nM for most of the analyzed materials. D-Glucose showed a higher affinity for GLUT1 in the more natural the membranes as determined by inhibition of CB binding. The \(K_d\) increased from 12 mM in RBC membranes to 41 mM in proteoliposomes.

In relation to Papers IV–VII, IBAC analyses of human cancer cells attached to agarose gel beads have hitherto not been successful. More favorable conditions for chromatographic analyses of these cells remain to be found.

Paper VI  Methodology and principles of IBAC were presented. The potential of this method to analyze ligand binding to a membrane protein in many different biomembranes was emphasized and exemplified by our studies of GLUT1. Applications of quantitative affinity chromatography on a variety of other immobilized membrane proteins were briefly described, and reference to alternative techniques and the related immobilized-liposome chromatography system was made.
Acknowledgements

Financial support for this work from the University of Uppsala, grants to P. Lundahl et al. from the O.E. and Edla Johanssons Science Foundation, the Swedish Natural Science Research Council and the Swedish Research Council for Engineering Sciences is gratefully acknowledged.

Writing these lines I am about to finish five years of work. It is due to a whole lot of people, colleagues, friends and family that this was a good time of my life, so I think it is about time to thank those who matter most to me.

I owe deep gratitude to my supervisor Per Lundahl for letting me join his research group, trusting me and sharing his vast knowledge and experience in the field of biomembranes. Maybe I will remember our nice and interesting conference trip to Amsterdam the most.

Thank you Janos Hajdu, my examiner, for support and instructions on membrane protein crystallization.

Former members of the biomembrane group who introduced me to the laboratory and with whom I enjoyed to work, discuss and chat are Andreas, Cheng-Ming, Eggert, Eva, Farideh, Lars, Lili, Yanxiao, and Yi-Ming. Thanks Andreas, for support when watching "Saving Private Ryan" and Farideh for being a good party fellow. I appreciated playing darts with Eggert and talking (almost watching) birds with Lars. Today, running into the home stretch, I sense a pleasant climate in the group being created by Caroline (=> glassfika), Christine (=> kaffefika), Juanjo (Mr. Footbag => fotbollsfika a la catalana), Lisa (=> partyfika) and Shu-Sheng (=> sun flowe fika). Neither have I forgotten the undergraduate students in our group: Jan (cool language, idea Fig. 9), Emma (=> torsdagsfika), Chris, Anders and Natalie.

I am especially happy about the doctoral student meetings at Norreda Torp, in 1999 and 2001, which, I think, were first initiated by Ann, Tove and Cynthia. Apart from the good spirits and discussions and nice scenery out there, I got to know and make friends with folks in the other groups of the department. I’d like to name Pelle+Per (håll proggen vid liv!), Nisse (den som klättrar högt behöver inte falla djupt), Peo (YOU introduced me to whitewater-paddling!!), and all regulars at our student pub evenings, such as Sussi, Anna-Karin and Ylva.

Thanks to Christer, who introduced me to teaching at the course lab, and the other nice people with whom I had the pleasure to co-teach and I owe special thanks to Lilian for her kindness and great back-up at the course lab.

I appreciated quick and skilful technical help from Per-Axel and Hasse P. and the organizing skills of Lasse L. Thanks Ulla, Birgitta and Inger for kind help in economical and administrative matters. I am grateful to David, Marie and Karin for amino acid analyses; David and the members of the biomembrane group also for proof reading of manuscripts, in particular, the thesis summary.
Among my co-authors I want to specially thank Per-Erik Gustavsson, who kindly sent me superporous agarose gel without ever having seen me and Bo Ersson for supplying me with WGA.

I would also like to thank my first mentors in biochemistry, Qinghai Zhao and Jörgen Carlsson, as well as Veronika Asplund-Eriksson and Lars Gëdda for help with cell culturing at the Division of Biomedical Radiation Sciences during my thesis work.

My dear friends outside the academic world are important to me. I love(d) to spend time with you around: Sara, Olof, Rebbi, Karl, Benjamin, Maria-José, Fia, Anette, Alex, all former band and percussion group mates, Johan, Ransta Trädgård et al. I was and am always happy to see Britta, Hans, Susan and Morgan in Värmland. My oldest friends live back in Germany, where my roots are. I know times are changing and we do not get to see each other too often these days, but it is important to me to keep contact and I am first thinking of Helge & family, Ralf & family, Arndt, Katrin, Antje, Kai, Stephan, Silke, Henning and many others.

I am glad to have found my way to Uppsala Paddlarklubb where I found the exercise, recreation, and nature experience that helped me even (especially) through periods of concentrated work. Thanks, Hasse, Malin, Peo, Gunnar, Jakob, David, Dan and others for instructions and good fellowship.

This is also where I met Ebba, you are a great support, a wise and humorous person in all matters of life. We have such a wonderful time together.

I wish that my dad Heinrich could be around now. After all, it was you and my mother Helga who enabled my studies and my stay in Sweden. In 1997, after a short but intense struggle against a vicious cancer you passed away in Helgas, my brother Carstens and my arms. I will always keep feeling sorry for your suffering and our inability to help you. Natürlich will ich auch euch danken, Helga und Carsten, für eure Unterstützung und daß ihr in Deutschland nach dem Rechten seht, sowie für Hilfe, zB. sobald es mir einfällt, mal wieder irgendwas aus der Heimat besorgen zu müssen.

Peace,

Ingo
References


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