GLUCOSE TRANSPORT AND ITS' REGULATION IN RODENT MAMMARY GLAND

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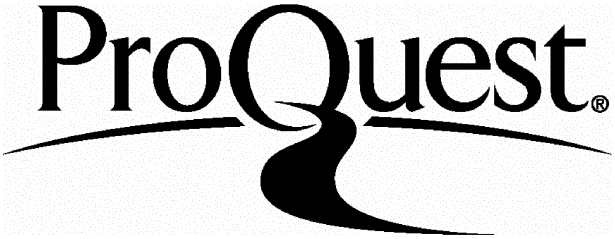

ABSTRACT

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Specific, anti-peptide antibodies against the five known mammalian facilitative D-glucose transporter isoforms (GLUT1-5) were used to investigate the transporter content of lactating rat mammary gland. Western blots of gland homogenate showed the apparent presence of GLUT1 and GLUT4. However, in isolated epithelial cells only GLUT1 was present, a result which indicated the mammary GLUT4 is present within adipocytes. The putative GLUT1 glucose transporter was recognised by antibodies against several hydrophilic regions of human erythrocyte GLUT1 and endoglycosidase F digestion decreased its apparent M_r from 50,000 to 42,000, a value essentially identical to that of the deglycosylated human protein. Its identity as a glucose transporter was confirmed by the ability of anti-GLUT1 antibodies to immunoprecipitate a mammary protein of apparent M_r 50,000, which could be photolabelled in a D-glucose-sensitive fashion by the transport inhibitor [4-^{3}H]cytochalasin B.

Immunocytochemical staining of sections confirmed that the primary location of GLUT1 in the mammary gland is the epithelial cell. Sub-cellular fractionation experiments showed that the transporter is not only located at the cell surface, but also within the Golgi. However, quantitative Western blotting indicated that GLUT1 could only account for about half of the D-glucose-sensitive cytochalasin B binding sites in Golgi vesicles, suggesting the presence of a second, so far unidentified, glucose transporter isoform.

Developmental changes in transporter expression in the gland were investigated both at the level of protein and mRNA. GLUT1 protein levels became detectable in the epithelial cells during the final 24-48hr of pregnancy, rose to a peak immediately following parturition, then fell slightly at the time of peak lactation. No major changes in GLUT1 mRNA were observed, implying a post-transcriptional control of GLUT1 expression. Removal of the litter for 24hr at peak lactation resulted in a total loss of GLUT1, suggesting feed-back inhibition influencing GLUT1 expression. The hormonal basis of these changes was investigated using explant culture of alveoli from mid-pregnant mammary gland. These experiments indicated that GLUT1 expression was dependent upon a lactogenic hormone, prolactin. Insulin and cortisol were also required for explant viability, but neither alone could stimulate synthesis of GLUT1.

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ABBREVIATIONS

ACC	Acetyl Co-enzyme A carboxylase
BSA	Bovine serum albumin
cDNA	Complementary DNA
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
E-64	*Trans*-epoxysuccinyl-L-leucylamido(4-guanino)butane
EDT	Ethanedithiol
EDTA	Ethylenediaminetetra-acetic acid, disodium salt
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
GTA	Galactosyltransferase
HEPES	N-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
i.p.	Intra-peritoneally
KLH	Keyhole limpet haemocyanin
MOPS	3-(N-morpholino)pyropane sulphonic acid
mRNA	Messenger RNA
NaAc	Sodium acetate
N-AG	N-acetylglucosamine
NEM	N-ethylmaleimide
5'-NT	5'-Nucleotidase
PBS	Phosphate-buffered saline (10mM sodium phosphate, 148mM NaCl, pH7.2, unless stated otherwise)
PBSA-T	136mM NaCl, 2.7mM KCl, 1.6mM Na_2HPO_4, 1.2mM KH_2PO_4, pH7.2 containing 0.05% (v/v) Tween-20 and 0.02% (w/v) sodium azide
PBS-T	Phosphate-buffered saline containing 0.05% (v/v) Tween-20
PMA	Phorbol ester 12-myristate 13-acetate
PMSF	Phenylmethylsulphonyl fluoride
PVP	Polyvinylpyrrolidone
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline (20mM-Tris-HCl/500mM NaCl, pH 7.5)
TPA	12-O-Tetradecanoyl phorbol ester
TTBS	Tris-buffered saline containing 0.05% (v/v) Tween-20
TEMED	N,N,N',N'-tetramethylenediamine
TES	N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
Tris	Tris(hydroxymethyl)aminoethane
UDP	Uridine diphosphate

CHAPTER 1

INTRODUCTION

1.1 Introduction

Glucose is the main source of metabolic energy in most animal cells, and some tissues, for example the brain, derive energy almost exclusively from glucose. The uptake of glucose by the lactating mammary gland plays a central role in milk production, as glucose is not only an oxidisable metabolic substrate but also forms the precursors of lactose (milk sugar) and milk fat synthesis. In the lactating rat mammary gland the arteriovenous difference in plasma glucose transversing the gland is between 25-63%, depending upon the conditions under which the measurement is made (Page & Kuhn, 1986). The blood-flow through the mammary gland increases markedly during lactation (Neville & Daniel, 1987), and the increased flow indicates a very high glucose uptake. A higher metabolic clearance rate of glucose during lactation is directly due to its extraction by the mammary gland (Jones *et al.*, 1984). Glucose transport into mammary epithelial cells is rate-limiting for glucose utilisation (Threadgold *et al.*, 1982) and for lactose synthesis, although in rat mammary epithelial cells the latter only comprises 10% of the intracellular glucose utilisation. As glucose is a relatively hydrophilic molecule which does not readily penetrate hydrophobic lipid bilayers, the mechanism of its transport across the cell membrane is fundamental to the survival and function of mammary cells.

The aim of the following investigation was to identify and characterise the glucose transport system(s) that operate in the secretory epithelial cells of the lactating rat mammary gland and, assuming the proteins could be identified, to

study their expression during normal mammary gland development in pregnancy, lactation and involution and their regulation.

1.2 The rat mammary gland

The mammary gland is unique among mammalian exocrine glands in the transience of its existence in a fully functional state, and its subsequent dissolution during involution. Consequently it is a unique non-embryonic system for the study of tissue development and differentiation in mammals. In many species, including the rat, the development of the mammary gland is arrested in the mature virgin female, and the tissue remains dormant until the animal becomes pregnant. Pregnancy triggers a rapid cellular proliferation leading to terminal differentiation associated with the formation of a fully functional milk-synthesizing and secretory system immediately preceding parturition. Milk synthesis and secretion continues as long as required by the litter, until weaning triggers the rapid involution of the mammary gland, with a partial reversion the mature virgin tissue.

The mammary gland contains a number of specialised cell types, the predominant cell type varying with the developmental stage of the tissue. The development of the rat mammary gland through cell proliferation and differentiation is summarised in the following sections and has been reviewed extensively elsewhere (Topper & Freeman, 1980; Mepham, 1987; Neville & Daniel, 1987). Morphologically distinct epithelial stem cells have been identified in mouse mammary gland (Smith & Medina, 1988) from mice at all stages of the reproductive cycle. These epithelial stem cells will undergo mitosis to give rise to daughter cells that will differentiate in the presence of lactogenic hormones, even when isolated from the resting mammary glands of virgin mice, confirming the presence of predestined epithelium

within the mammary tissue. Terminal differentiation of the mammary gland involves both extensive proliferation of the epithelium (during pregnancy) followed by synthesis and secretion of milk-specific elements (during lactation). Proliferation, differentiation and function are controlled by multiple hormone interactions, involving both peptide and steroid hormones, and are also influenced by autocrine, and possibly paracrine, factors. The following synopsis of mammary development and function is divided into the structural (Section 1.3) and biochemical (Section 1.4) aspects, as the mature virgin mammary gland develops through pregnancy and lactation to involution, together with sections on control of cell proliferation and/or function (Section 1.5). Contained within the structural development is a section dealing with the ultrastructure of the secretory epithelial cell, responsible for the synthesis and secretion of milk.

1.3 Structural development of the rat mammary gland

1.3.1 Pre-gestational mammary development

The precursor of the mammary epithelial tissue develops first in the early embryo when mammary buds form by the migration of epidermal cells from the 'mammary crest'. Following this, there is a resting phase until, near the end of term, a rapid proliferation of cells leads to the formation of the mammary cord. These embryonic cells can be induced in explant culture under appropriate hormonal stimuli to synthesize milk-specific proteins (Richards *et al.*, 1982), an indication that they are pre-destined as secretory epithelium as early as the embryo. Further development of the mammary gland does not occur until the young animal becomes sexually mature (about 6 weeks in the rat). This period is identified by a rapid ductal proliferation throughout the mammary fat pad, but there is no formation of

the alveolar structures seen in the differentiated tissue. Ductal cells are surrounded by a continuous layer of myoepithelial cells and basal lamina (Warburton *et al.*, 1982), and are embedded in the mammary adipose tissue. Following the development of this mature tissue a dormant period is entered, which continues in the mature virgin rat until impregnation. In these virgin animals the adipocytes form the predominant cell type within the mammary tissue.

1.3.2 Mammary gland development during pregnancy

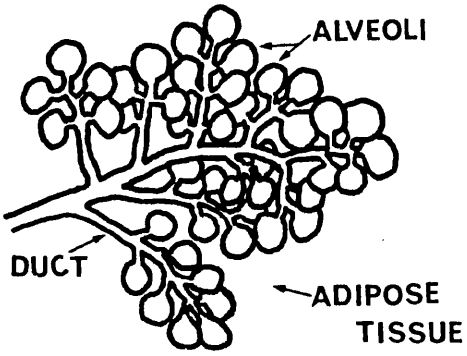
At the onset of pregnancy the mammary gland initiates further cellular proliferation, including ductal growth and the formation of numerous alveoli. The structure of the functional gland developed during pregnancy is schematically depicted in Fig. 1.1(a).

The mammary alveoli are the site of milk production and secretion, and consist of a mixture of epithelial cells, shown in Fig. 1.1(b). The relative numbers of the ductal/alveolar cells to adipocytes increases markedly throughout gestation so that by lactation the predominant cells (>70%) within the gland are the epithelial cells (Kraehenbuhl, 1977). As the alveoli grow and develop the myoepithelial cells become attenuated, and their processes spread out to cover approximately 25% of the surface of the alveoli (Nagato *et al.*, 1980; Warburton *et al.*, 1982).

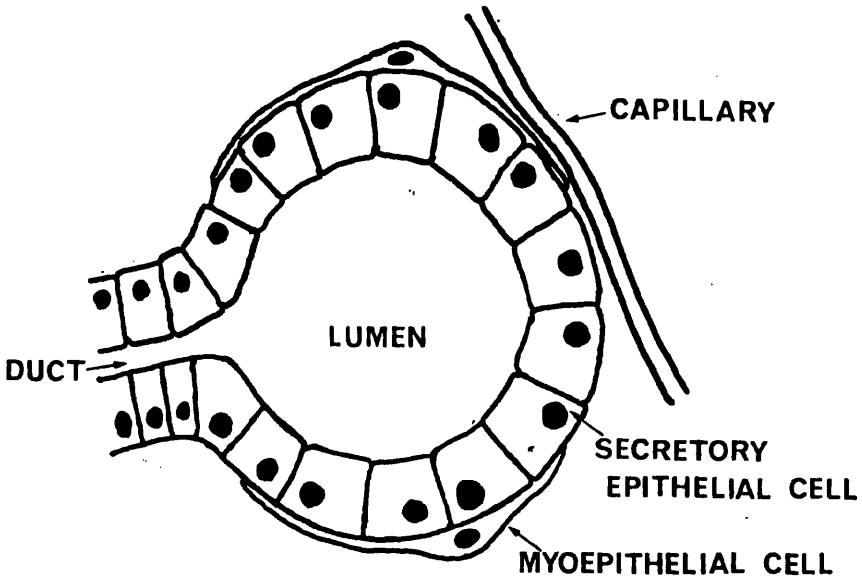
There is a marked increase in the vascularity of the mammary gland during pregnancy; a number of capillaries being associated with each of the alveoli. The vast extent of the mammary blood system reflects the high requirement of the gland for blood-borne metabolites and messengers.

Fig. 1.1 Structure of the rat mammary gland

a) Gross alveolar structure



b) Individual alveolar structure



The arboreal structure of the ducts in the mammary gland of virgin rats expands during pregnancy and develops terminal alveoli, shown in (a). The terminal alveoli are embedded in the mammary fat pad. Each individual alveolus, shown in (b), contains epithelial and myoepithelial cells, surrounded by a continuous basal lamina. Each alveolus is vascularised with numerous capillaries. The alveolar structure is maintained throughout lactation, when milk is secreted into the lumen of the alveoli and expelled by contraction of the myoepithelium, down the ducts. In lactation most of the fat pad is displaced by the epithelial tissue.

1.3.3 Mammary gland development during lactation

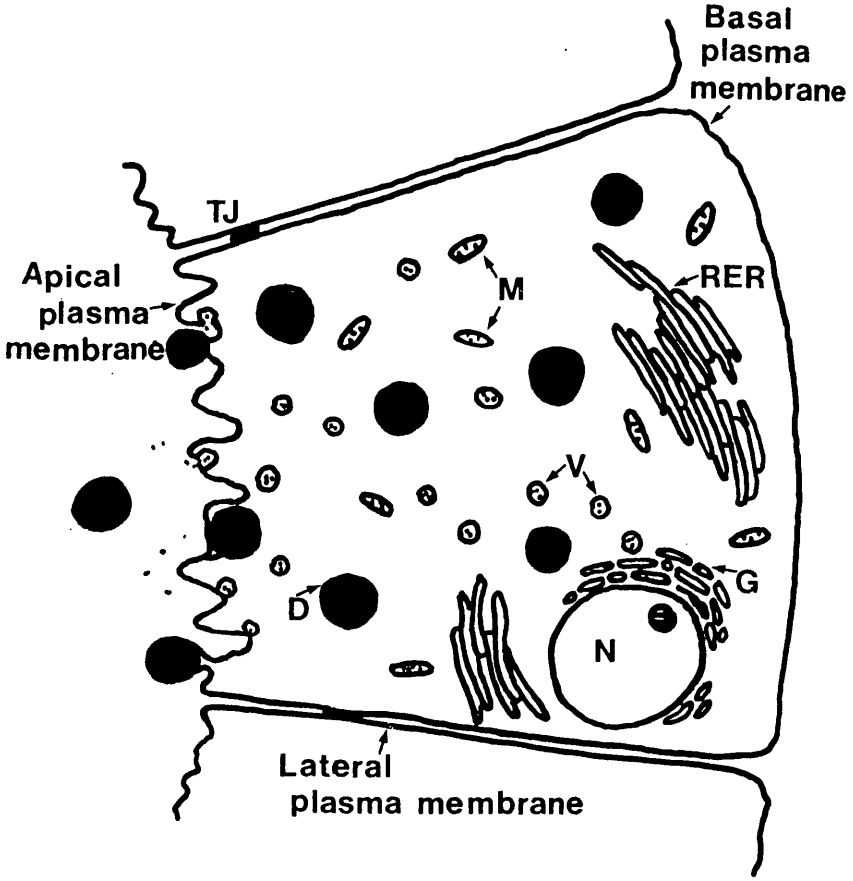
At lactogenesis, occurring during the final hours of pregnancy, the mammary alveoli initiate milk synthesis and secretion. Initially colostrum, and subsequently milk, are stored within the alveoli following secretion. The suckling stimulus generates neural stimulation of the hypothalamus to release oxytocin which then stimulates the myoepithelial cells to contract, expelling milk collected in the mammary lumen and ducts. The structure of the gland developed during pregnancy, shown in Fig. 1.1(a) is maintained throughout lactation. The major changes in structure of the mammary tissue occur at the ultrastructural level, within the secretory epithelial cells. The epithelial cells primed during pregnancy differentiate into fully functional secretory epithelium, described in Section 1.3.4. The synthesis of milk-specific proteins, sugars and lipids is initiated and large numbers of milk fat droplets and secretory vesicles appear in the cells. The differentiated structure is maintained until involution. Terminal differentiation is achieved by both increased synthesis and lower degradation rates of milk specific proteins (Razook-Hasan et al., 1982).

1.3.4 Secretory epithelial cell ultrastructure

The site of milk synthesis and secretion during lactation is the mammary secretory epithelial cell. These cells form the alveoli, become predominant during late pregnancy, and continue increasing in number until around day 5 of lactation (Shipman *et al.*, 1987). The number of secretory epithelial cells then remains constant until involution, when milk production ceases and the alveolar structure breaks down.

Secretory epithelial cells are polar in nature, shown in Fig. 1.2, tight junctions between the cells acting as an impermeable barrier between the alveolar lumen

Fig. 1.2 Ultrastructure of the mammary secretory epithelial cell



The secretory epithelial cell ultrastructure reflects the secretory activity. Cells are polarised, joined by tight junctions (TJ) and have apical, basal and lateral plasma membranes. The cells contain a large rough endoplasmic reticulum (RER), an extensive Golgi apparatus (G) and numerous mitochondria (M). Milk fat droplets (D) and secretory vesicles (V), many containing casein micelles (C) appear in the cells at lactogenesis. The nucleus (N) is present in the basal half of the cell.

containing the milk and the extracellular medium. All milk components must therefore pass through the secretory epithelial cells before they can enter milk, permitting tight control of milk composition. The mammary epithelial cell plasma membrane can be divided into three distinct types:-

i) Apical plasma membrane on the alveolar lumenal surface of the cell, involved in milk secretion;

ii) Lateral plasma membrane, in contact with neighbouring cells;

iii) Basal plasma membrane, responsible for the uptake of substrates from the extracellular medium and attachment to the basal lamina.

Metabolites such as glucose, hormones such as receptor-bound prolactin, and ions are taken into the cell across the basal plasma membrane and the milk constituents secreted out of the cell across the apical membrane. Some membrane proteins have a polarised distribution in the epithelial cells, for example butyrophilin, which is specific to the apical plasma membrane (Franke *et al.*, 1981; Heid *et al.*, 1983).

During gestation epithelial cells develop an extensive rough endoplasmic reticulum (RER) and Golgi apparatus, and also a large number of mitochondria to provide the energy for lactation. Lactogenesis is characterised by the appearance within the epithelial cell of numerous lipid droplets and secretory vesicles, many containing casein micelles.

Milk components are secreted into the lumen of the alveoli by two different mechanisms (Fig. 1.3). Secretory vesicles originate from the trans-Golgi cisternae and translocate, by both constitutive and regulated pathways (Turner *et al.*, 1992), to the apical plasma membrane, where the contents are released into the lumen of

the alveoli by exocytosis. Many components of the aqueous phase of milk are co-localised within the secretory vesicles (Keenan *et al.*, 1979); casein (milk protein) containing secretory vesicles also contain other milk proteins (for example α-lactalbumin and ß-lactoglobulin), certain milk ions (including citrate, potassium and calcium) and, in rats, lactose (Sasaki *et al.*, 1978). This suggests that the constituents of the aqueous phase of milk are compartmentalised by the Golgi cisternae and exocytosed together. Mammary milk fat droplets form near the RER, in the basal part of the cell, and also translocate to the apical plasma membrane. The secretion of milk fat is thought to be apocrine, as the fat globules in the milk are surrounded by a membrane (see Section 1.4.1), which is not observed around fat droplets within the cytoplasm. As a consequence of secretion the apical plasma membrane is in a constant state of transition, with continual vesicle fusion during exocytosis, and membrane shearing away during apocrine secretion.

1.3.5 Mammary gland involution

Following the removal of the suckling stimulus either by natural weaning or premature removal of the litter, the alveolar structures of lactation collapse and the basal lamina breaks up. Macrophages become common, presumably cleaning up the cellular debris and dissolving the basal lamina. Adipose tissue returns to fill the spaces vacated by the epithelial cells and the whole mammary gland reverts to the structure seen in the mature virgin animal. Whilst the morphological changes in the tissue during involution are known the relationship between the removal of the suckling stimulus and retention of milk, and involution of the gland is not yet understood. The initial fall in milk yield seen during involution in goats is thought to result from a decreased number of secretory epithelial cells in the gland, the

remaining cells retaining full activity (Wilde & Knight, 1989), suggesting apoptosis. Plasmin serum protease has recently been implicated in a tissue remodelling system operating in the mammary gland during involution (Turner & Huynh, 1991).

1.4 Biochemical development of the mammary gland

The function of the lactating mammary gland is the provision of milk for maintenance of the litter. Milk is produced exclusively by the secretory epithelial cells of the mammary gland, and contains mammary-specific proteins and sugars. There is considerable variation in milk composition between different species although within a single species milk composition is tightly controlled, for example, platypus milk contains only trace amounts of lactose (Messer *et al.*, 1983), whereas human milk is composed of 7% lactose (Jenness, 1979). The three major calorific components of milk are lipid, protein and carbohydrate, in descending order of calorific contribution in rat, as well as salts, hormones and growth factors, antibodies, etc. Outlines of milk lipid, protein and sugar syntheses are described in the following sections.

1.4.1 Milk lipids and milk fat globules

Milk lipids are synthesized primarily in the form of triglycerides (≈95% in bovine milk) and provide the largest single energy source in milk. Triglycerides are based on a glycerol backbone linked via ester bridges to three long chain fatty acids (usually oleic or palmitic). Free fatty acids, injected intra-venously, are taken up by the lactating mouse mammary gland, esterified and converted into triglycerides within minutes (Stein & Stein, 1967). Fatty acids are also synthesised *de novo* by enzymes present in the RER, using approximately 63% of the intracellular glucose,

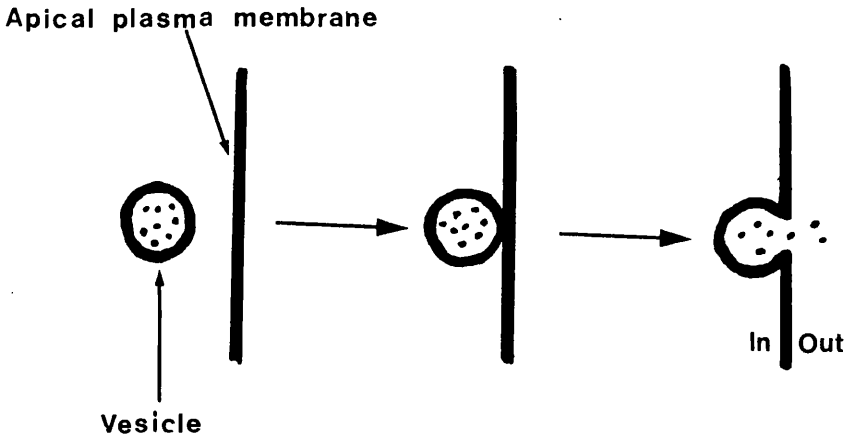
suggesting competition for glucose between lipid and lactose syntheses (Bussmann *et al.*, 1984). These reticular enzymes, including acetyl Co-enzyme A (CoA) carboxylase (ACC) and fatty-acid synthetase, are expressed at high levels during lactation, show pronounced diurnal variations and are hormonally controlled. The activity of lipogenic enzymes in the mammary gland are inversely related to adipose tissue during lactation (Vernon & Flint, 1984; Williamson, 1986; Ros *et al.*, 1990). The milk fat droplets are secreted by a budding mechanism, possibly apocrine, shown in Fig. 1.3(b), and are membrane-bound within the alveolar lumen. This membrane is derived partially from the apical plasma membranes (Basch *et al.*, 1985) and also, in rat milk, from a significant proportion of intra-cellular membranes (Wooding, 1971). High levels of 5'-nucleotidase (a plasma membrane marker enzyme) activity and low levels of galactosyltransferase (a marker of Golgi membranes and secretory vesicles) activity have been detected in bovine milk fat globule membranes. The bovine milk fat globule membrane is asymmetric in character (Mather & Keenan, 1975), with clusters of glycoproteins (Horisberger *et al.*, 1977) indicative of a genuine biological membrane. Insulin receptors have been identified in both rat and goat milk fat globule membranes (Flint & West, 1983), and also some transport systems, for example a $K^+(Rb^+)$ transport protein (Smith *et al.*, 1990).

1.4.2 Milk-specific proteins

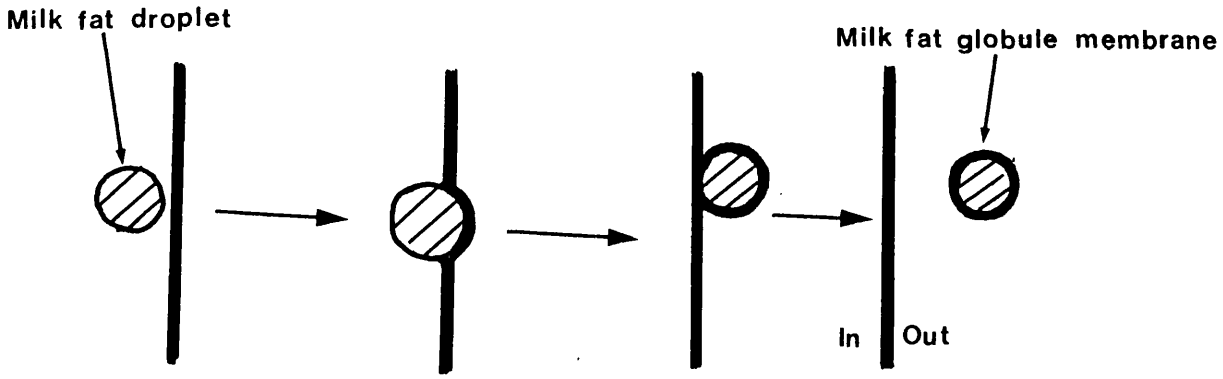
Two groups of proteins are present in milk, one group containing casein, α-lactalbumin and ß-lactoglobulin, and the other containing whey acidic proteins. Casein is a group of proline-rich, acidic phosphoproteins, comprising between 70-80% of rat milk protein. Casein synthesis is initiated at lactogenesis, although a

Fig. 1.3 Mammary epithelial cell secretory mechanisms

a) Exocytosis



b) Budding (apocrine)



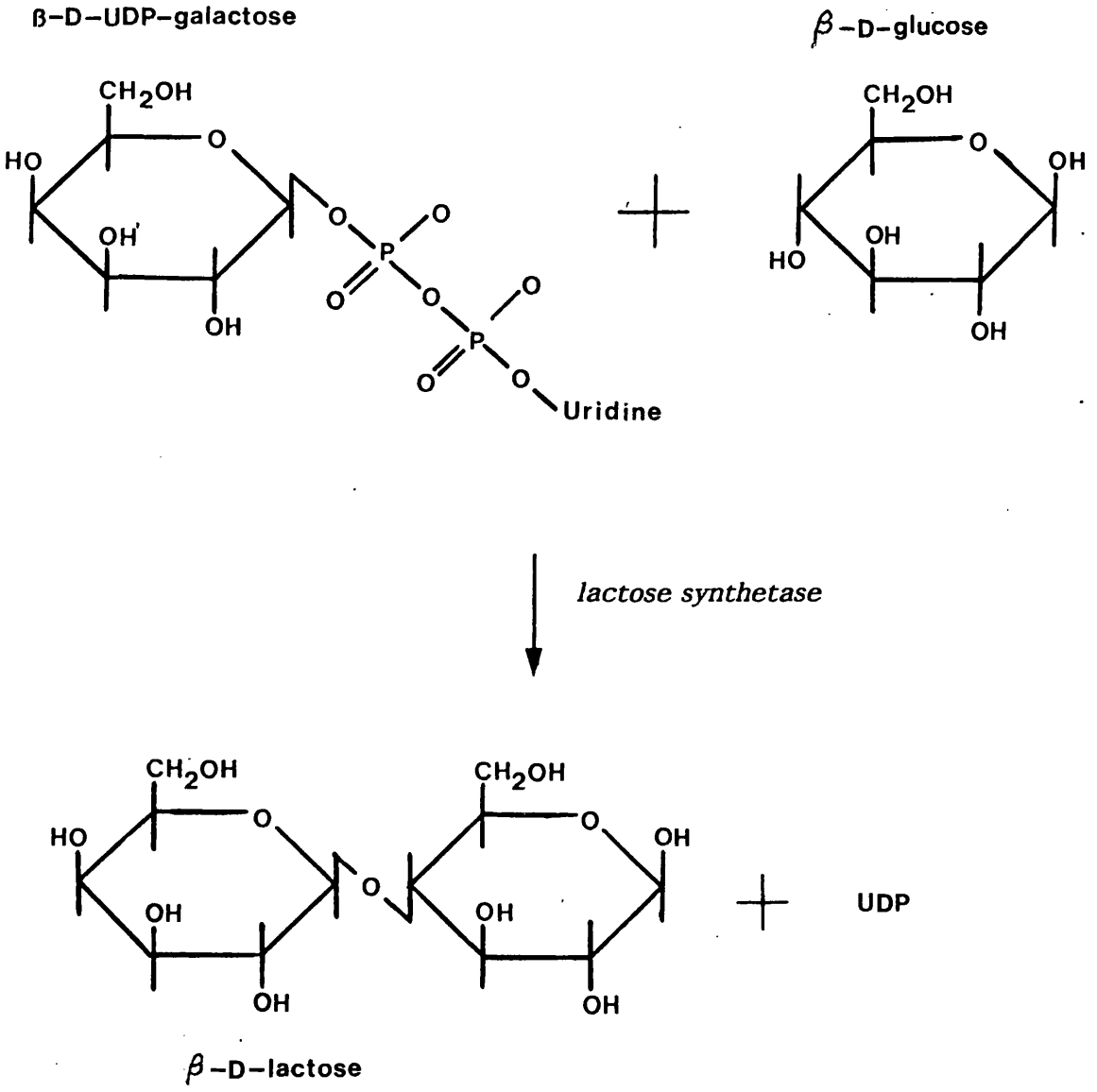
Two mechanisms are employed by the secretory epithelial cells to secrete milk components. Exocytosis (a) involves fusion of membrane-bound vesicles with the apical plasma membrane. The contents of the vesicles are then released into the alveolar lumen and the membrane recycled by the cell. A budding mechanism (b), possibly apocrine, is employed for the secretion of milk-fat. The milk fat droplet becomes surrounded by an apical membrane fraction as it leaves the cell. Occasionally this contains cytoplasmic inclusions. The milk fat droplets are therefore present in the alveolar lumen as milk fat globules surrounded by a continuous milk fat globule membrane.

surprisingly high level of casein messenger RNA (mRNA) is found in total RNA extracts from early and mid-pregnant mammary glands (Rosen *et al.*, 1975) suggesting post-transcriptional regulation of expression. During lactation about 15% of the total mRNA activity in the mammary gland is casein mRNA activity. Caseins are synthesized within the RER then transported to the Golgi apparatus where they are modified for secretion and packaged into secretory vesicles, in the form of micelles, together with the other aqueous milk components. These vesicles translocate to the apical plasma membrane where the contents are released into the lumen of the alveoli by exocytosis (Franke *et al.*, 1976). A smaller proportion of rat milk proteins are the whey acidic proteins, at least one of which is known to be a milk-specific inhibitor of epithelial cell secretory activity (Wilde *et al.*, 1988; see Section 1.5.5).

1.4.3 Milk carbohydrate (lactose) synthesis

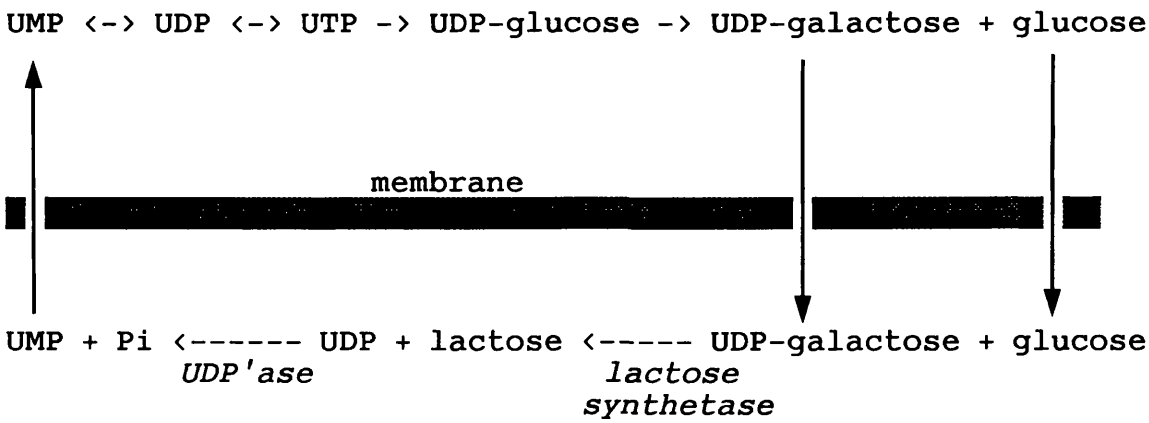
The major carbohydrate in most mammalian milks (including rat and mouse) is lactose. Lactose is synthesized in mammals exclusively by mammary epithelial cells during lactation. Structurally lactose, shown in Fig. 1.4, is a disaccharide, galactose ß(1→4) glucose, formed by a glycosidic bond between the ß-anomer of D-galactose and D-glucose. Lactose synthesis, part of the lactose synthesis cycle shown in Fig. 1.5, is catalysed by a membrane-bound enzyme complex, lactose synthetase, consisting of galactosyltransferase and α-lactalbumin on the lumenal face of the Golgi apparatus (Kuhn & White, 1975; Kuhn *et al.*, 1980). Galactosyltransferase is present in most tissues, catalysing the synthesis of glycoproteins by the attachment of galactose residues to the terminal *N*-acetyl-D-glucosamine of glycoproteins. The modifying sub-unit, α-lactalbumin, present exclusively in the lactating mammary

Fig. 1.4 Structure of lactose



Lactose is formed by a ß(1→4) link between ß-D-UDP-galactose and β-D-glucose, the reaction releasing UDP. The enzyme complex lactose synthetase is found in the lumen of the Golgi apparatus of the mammary secretory epithelial cell.

Fig. 1.5 The mammary uridine nucleotide cycle



A uridine nucleotide cycle was proposed to occur across the membranes of the Golgi apparatus in the lactating rat mammary epithelial cell by Kuhn & White, 1977. Both glucose and UDP-galactose have to enter the lumen in order to be converted to lactose by lactose synthetase, while uridine monophosphate (UMP) has to leave the Golgi lumen, to be converted back to uridine triphosphate (UTP) in the cytoplasm, and keep the cycle going.

gland, changes the specificity of the galactosyltransferase from N-acetylglucosamine to D-glucose. Most of the α-lactalbumin synthesized by the mammary tissue is secreted as a milk protein, only a small proportion being reversibly associated with galactosyltransferase and involved in lactose synthesis. Studies on the expression of both α-lactalbumin and casein (Qasba & Nakhasi, 1988) indicated a post-transcriptional regulation due to either different translation efficiencies or different protein degradation rates.

There is a large increase in lactose synthesis per gram of mammary tissue between parturition and peak lactation (Wilde & Kuhn, 1979), particularly in the final 12hr of pregnancy (Murphy *et al.*, 1973). As α-lactalbumin interacts reversibly with galactosyltransferase, the degree of interaction is a possible controlling factor for lactose synthesis, maximum efficiency occurring around day 4 of lactation. At the initiation of lactogenesis the expression of α-lactalbumin is rate-limiting for lactose synthesis (Murphy *et al.*, 1973) but once adequate α-lactalbumin is available glucose transport is thought to become rate-limiting, as the K_m of 1.5mM for D-glucose of lactose synthetase is above the intracellular concentration of D-glucose, which is between 0.2-0.5mM (Wilde & Kuhn, 1981). The synthesis of lactose rises to a maximum by day 16 of lactation and is unaffected by litter size (Wilde & Kuhn, 1979). Rats show a diurnal variation in lactose synthesis that appears to be related to the food intake (Carrick & Kuhn, 1978), and there is a direct relationship between diet and milk lactose. Starvation inhibits the synthesis of lactose by changing the functional efficiency of galactosyltransferase (Carrick & Kuhn, 1978) before it affects milk fat synthesis, confirming the low metabolic value of carbohydrate. The simultaneous presence of galactosyltransferase and α-lactalbumin in the secretory vesicles (Sasaki *et al.*, 1978) indicates that lactose

synthesis could continue during translocation to the apical plasma membrane.

1.5 Regulation of mammary gland development and differentiation

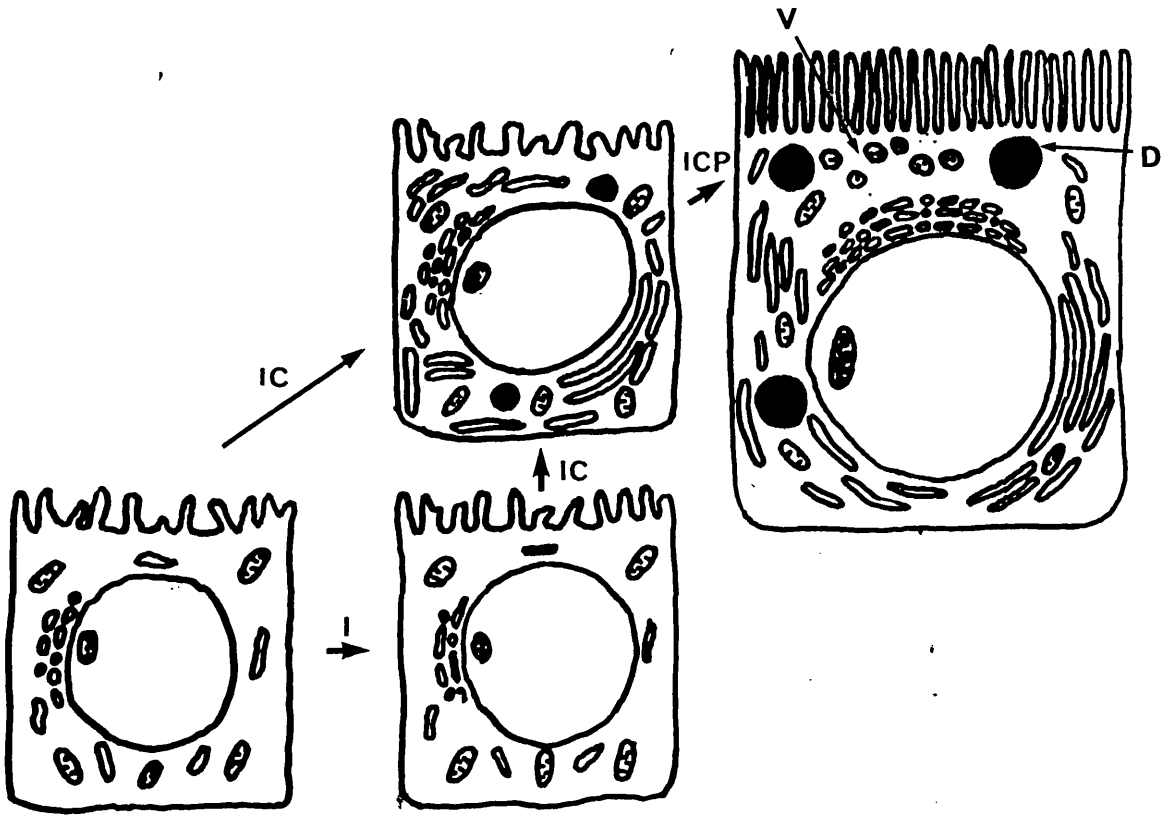
The development of the mammary gland is under the influence of a wide array of hormones and growth factors, as well as other factors such as the extracellular matrix. Hormonal control of the mammary glands' developmental biology is reviewed extensively by Topper & Freeman, (1980) and Mills & Topper, (1970). Hormonal control of lactation has been reviewed by Cowie *et al.* (1980). Hormone interactions have been investigated using a variety of techniques, both *in vivo* and *in vitro*, and the literature is extensive. *In vitro*, both explant and primary epithelial cell cultures have been used. Explant culture has its limitations as explants cannot secrete casein and other milk proteins into the medium, as the cut ends of the ducts seal, producing a closed system (Delouis & Denamur, 1972). The culture of explants is reviewed in detail by Dils & Forsyth, (1981). Primary epithelial cell culture involves plating cells onto collagen gels and allowing the cells to proliferate, often in the presence of serum and hormones, then floating the gels as rafts within the medium. The floating rafts contract as the cells assume the morphology and structure preferred for differentiation. Insulin, at supraphysiological concentrations, and cortisol are required *in vitro* for cell maintenance, but the presence of prolactin, the most potent rat lactogenic hormone, is the minimum requirement for initiating terminal differentiation *in vitro* (Fig. 1.6).

1.5.1 Extracellular matrix

The role of the extracellular matrix in the mammary gland has been studied by

Fig. 1.6 Hormonal effects on mammary epithelial cell development and differentiation

Adapted from Topper & Freeman (1970).



This is a schematic representation of the minimal hormone requirements for structural and functional differentiation of the mammary gland epithelial cell determined *in vitro*. Structural development, characterised by the extensive RER and Golgi apparatus, requires insulin (I) and cortisol (C). Differentiation, characterised by the appearance of milk fat droplets (D) and secretory vesicles (V), and a more highly invaginated apical plasma membrane, requires the additional presence of prolactin (P).

measuring differentiation of primary epithelial cell cultures from mid-pregnant animals (Neville & Daniel, 1987). Both cell shape (Haetuple *et al.*, 1983; Shannon & Pitelka, 1981) and extracellular matrix (Emerman & Pitelka, 1977; Emerman *et al.*, 1977; Yang *et al.*, 1980; Suard *et al.*, 1983) are important for *in vitro* differentiation, indicating that the cells have to attain a certain shape or morphology before milk protein synthesis or secretion can occur (Lee *et al.*, 1985; Rocha *et al.*, 1987). The most commonly used matrix is a collagen gel, but an extracellular matrix prepared from normal mouse mammary gland (Wicha *et al.*, 1982; Wilde *et al.*, 1984), from the human Englebreth-Holm-Swarm (EHS) mammary tumour, or laminin, one of its constituents (Blum *et al.*, 1987), have also been used. The EHS matrix allows cells to polarise giving rise to vectorial secretion of milk protein (Barcellos-Hoff *et al.*, 1989). The flexibility of the growth matrix is essential as mammary cells grown on attached gels or plastic will not differentiate.

1.5.2 Steroid hormones

The ovarian steroids, oestrogen and progesterone, and the glucocorticoids are all involved in the development and differentiation of the mammary gland. High levels of progesterone will inhibit lactation, and progesterone removal (ovariectomy) of pregnant animals will initiate lactogenesis (Murphy *et al.*, 1973). The natural switch from high progesterone to low progesterone at parturition is therefore involved in the initiation of lactation. Oestrogen also affects lactation, stimulating prolactin binding to mammary epithelial cells from virgin rats, while suppressing prolactin binding to mammary epithelial cells from lactating animals (Hayden *et al.*, 1979).

Low affinity, high capacity glucocorticoid receptors are present in the cytosol of mammary gland epithelial cells and the binding of glucocorticoids increases

during pregnancy from a low level in the virgin rat to a maximum during lactation. For α-lactalbumin synthesis there is a biphasic response of mid-pregnant mouse mammary explants to glucocorticoids, with low levels stimulatory and high levels inhibitory. However, this response is not seen in lactating mouse mammary explants, suggesting a suppression of glucocorticoid effects at lactogenesis (Quirk *et al.*, 1988). Glucocorticoids are thought to potentiate the prolactin effects, and enhance cell stability and morphological differentiation *in vitro*.

1.5.3 Peptide hormones

A number of peptide hormones influence the development and function of the mammary gland. These include insulin, prolactin and in some species, growth hormone.

1.5.3.1 Insulin

Serum insulin levels fall during lactation, despite an increased food-intake (Sutter-Dub *et al.*, 1974). The lower insulin levels are thought to be due to either lower glycaemic stimulation of the pancreatic ß-cells (Madon *et al.*, 1990b), as plasma glucose levels are also lower in lactation (Burnol *et al.*, 1983), or a decreased insulin response to secretagogues (Hubinot *et al.*, 1986). Mammary gland glucose transport is not insulin-responsive under normal conditions (Martin & Baldwin, 1971; Williamson & Robinson, 1977; Threadgold & Kuhn, 1984; Prosser & Topper, 1986). Mammary epithelial cells acquire insulin-sensitivity during pregnancy (and transiently during involution) but are not insulin-responsive during lactation (Stockdale & Topper, 1966; Topper *et al.*, 1972; Oka *et al.*, 1974), however, the lactating mammary gland is an insulin-sensitive tissue (Burnol *et al.*, 1983). Insulin

is required for tissue maintenance (reviewed by Forsyth & Jones, 1976) as insulin is not a lactogenic or galactopoietic hormone. There is a reciprocal relationship between the insulin receptors of the mammary epithelial cells and of extra-mammary adipocytes during pregnancy and lactation (O'Keefe & Cuatrecasas, 1974; Flint *et al.*, 1980), consistent with the competitive roles of these two tissues for glucose. Lower insulin stimulation of adipose tissue during lactation may act to increase the amount of glucose available for the mammary gland.

It is thought that insulin may be acting through low affinity-binding to insulin-like growth factor-I (IGF-1) receptors (Imagawa *et al.*, 1988), as supraphysiological concentrations of insulin are required to maintain cells *in vitro*. Insulin can be substituted by physiological concentrations of IGF-1, but not epidermal growth factor, platelet-derived growth factor, multiplication-stimulating factor, fibroblast growth factor or nerve growth factor (Nicholas *et al.*, 1983).

1.5.3.2 Prolactin and growth hormone

Prolactin, the most potent lactogenic hormone in rats, is secreted by the anterior pituitary, its plasma concentration increasing during pregnancy to reach a maximum during lactation. Prolactin stimulates mammary development and milk secretion and is the minimum requirement for differentiation (Cowie, 1969). The lactogenic action of prolactin is inhibited by high concentrations of ovarian steroids, particularly progesterone. The synthesis of milk proteins including casein and α-lactalbumin (Emerman *et al.*, 1977; Suard *et al.*, 1983), and the activity of lipoprotein lipase, acetyl CoA carboxylase (ACC) and other enzymes of the triglyceride synthesis pathway, are regulated by prolactin.

Prolactin binding to mammary gland is low in pregnancy, high in lactation and

declines upon weaning (Hayden *et al.*, 1979; Suard & Kraehenbuhl, 1979). The amount of prolactin bound by mammary epithelial cells varies proportionally to the circulating plasma prolactin concentration (Suard & Kraehenbuhl, 1979), suggesting that it amplifies its own receptor. Internalised prolactin becomes concentrated around the Golgi apparatus, endocytotic vesicles and the nucleus, and is known to stimulate milk protein mRNA synthesis (Chomzynski & Topper, 1974).

Plasma prolactin levels can be decreased by removal of the litter from peak-lactating animals, or *in vivo* treatment with bromocryptine, an inhibitor of prolactin secretion. Both treatments cause a reciprocal increase in plasma glucose and insulin concentrations (Robinson *et al.*, 1978; Agius *et al.*, 1979). Bromocryptine treatment in conjunction with an antiserum raised against rat growth hormone (α-rtGH) inhibits galactopoiesis (Madon *et al.*, 1986).

Prolactin receptors have also been identified in other tissues, notably liver (Hayden *et al.*, 1979), where there is a negative correlation to their expression in mammary gland, and pancreas, however, they are not present in adipocytes (Vernon & Flint, 1984).

Growth hormone is the major galactopoietic hormone in ruminants (Hart *et al.*, 1978). In rats the effect of growth hormone increases as lactation continues (Flint *et al.*, 1992). Although growth hormone receptors themselves have not been identified in mammary epithelial cells, recently mRNAs for growth hormone receptors were identified in bovine (Hauser *et al.*, 1990), rat (Lincoln *et al.*, 1990) and rabbit (Jammes *et al.*, 1990) mammary glands.

1.5.4 Growth factors

IGF-1 has been identified in the milk of rats (Philipps *et al.*, 1991) and goats

(Prosser *et al.*, 1991). In goats, growth hormone-induced increased galactopoiesis is associated with an increase in the level of milk IGF-1 (Prosser *et al.*, 1991). However there is conflicting evidence for the role of IGF-1 in mediating the effects of growth hormone. The ability of exogenous IGF-1 infusion into goats to cause increased blood-flow and milk secretion (Prosser *et al.*, 1990) could not be confirmed using rats (Flint *et al.*, 1992).

1.5.5 Autocrine/paracrine factors

Endocrine control of milk production is modulated by local mechanisms sensitive to the frequency or efficiency of milk removal, both autocrine and paracrine. A 10-30kD milk whey fraction protein has been isolated from goat milk that inhibits functional differentiation of mammary explants (Wilde *et al.*, 1987) and induces a transient dose-dependent reduction in milk yield when injected into the teat canal of lactating goats (Wilde *et al.*, 1988). How this protein inhibits lactation is not known, however one effect of the inhibitory fraction is to increase the degradation of newly synthesised caseins (Stewart *et al.*, 1988; Wilde *et al.*, 1989).

Indirect paracrine effects have been shown in mammary organ culture. A tissue-specific, diffusible growth inhibitor (mammastatin) is secreted by normal human (Ervin *et al.*, 1989), or mouse (Miller *et al.*, 1989), mammary epithelium which inhibits the growth of human, or mouse, mammary sarcomas.

1.6 Mammalian glucose transport

The passage of glucose across cell membranes can occur in one of three ways; simple, non-mediated diffusion, facilitated diffusion and active transport. Simple diffusion through the lipid component of the membrane only becomes significant

when the substrates are lipid soluble. The rate of glucose passage across an artificial lipid bilayer is several orders of magnitude slower than its actual rate of passage across biological membranes, for example those of the human erythrocyte ghost (Jung, 1971). Within biological membranes there are proteins capable of potentiating the passage of glucose, increasing it to physiologically relevant levels. Two functionally distinct carrier-mediated transport systems, active and passive, have been identified.

Active glucose transport systems require energy, usually in the form of an ATP-generated ion gradient, to drive the uptake of glucose against its concentration gradient. These proteins are found in prokaryotic organisms where the extracellular concentration of glucose is highly variable, so there is a need to extract the maximum amount of glucose from the surrounding environment as rapidly as possible. Active glucose transport is also found in eukaryotes where it is restricted to those cells in which the transport of glucose against its concentration gradient is required, for example in the renal proximal tubule brush border membrane (Turner & Moran, 1982) and the apical membrane of the intestinal mucosa (Hopfer, 1987). In mammalian cells active glucose transport is mediated by a Na^+/glucose co-transporter (Maenz & Cheeseman, 1987), which uses metabolic energy in the form of ATP to establish a Na^+ gradient, which is then used to drive the uptake of glucose.

Passive glucose transport can be mediated by proteinaceous pores or facilitative transporter proteins. Pore proteins have very little substrate specificity, allowing most molecules small enough and not restricted by charge to readily penetrate the membrane. Pore proteins are not found in the plasma membranes of mammalian cells, but do exist in intracellular membranes such as the mitochondrial outer

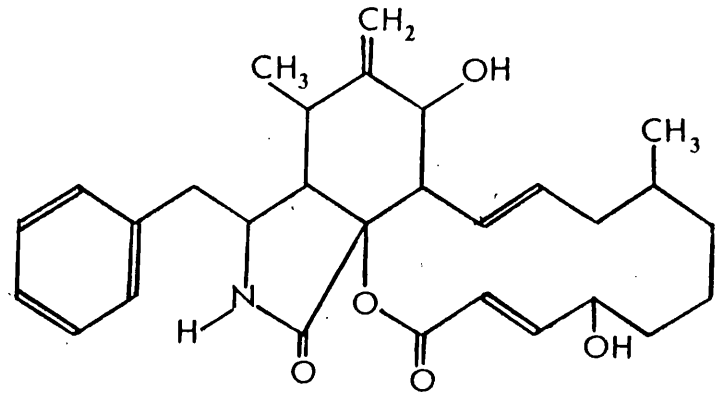
membrane. There is also evidence for the presence of a pore protein in other intracellular membranes of mammary gland (Section 1.10). In contrast passive transport of glucose by facilitative D-glucose transport proteins is found in almost all mammalian cells studied so far. Here the passive transport of glucose down its concentration gradient is facilitated by a family of D-glucose-specific transport proteins. Passive glucose transport is favoured as the extracellular concentration of glucose is maintained between 5-10mM. Different mammalian cell types have functionally distinguishable facilitative D-glucose transporters consistent with the function of the cells, or the tissue (see Table 1.1).

1.6.1 Cytochalasin B

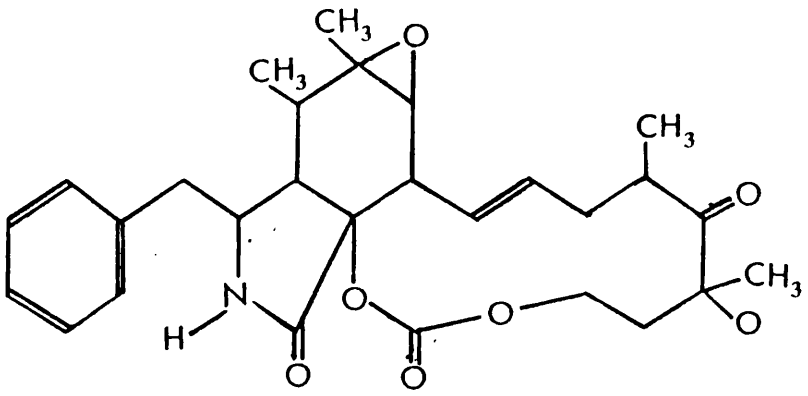
An important feature of all mammalian sugar transporters is the specific, D-glucose-inhibitable, reversible binding of a fungal metabolite, cytochalasin B (Fig. 1.7(a)), which is also a potent inhibitor of glucose transport. Tritiated cytochalasin B has been widely used in the investigation of glucose transporters in mammalian cells. It binds to human erythrocyte glucose transporters with a stoichiometry of approximately 1:1, a K_d of 10^{-7}M (Baldwin *et al.*, 1982) and can be covalently cross-linked to the transport protein by irradiation under U.V. light (Shanahan, 1982; Carter-Su *et al.*, 1982; Shanahan, 1983). The irreversible, covalent photolabelling of glucose transporters by cytochalasin B is also inhibited by D-glucose, but not L-glucose. Photoactivation is thought to proceed via the activation of an aromatic amino-acid within the transporter, rather than by activation of the cytochalasin B itself (Deziel *et al.*, 1984), and as there is only one such residue conserved in the glucose transporters in the relevant area of the protein sequence, the tryptophan residue at amino-acid 412 (Trp^{412}) of the human erythrocyte glucose

Fig. 1.7 Structure of cytochalasin B and cytochalasin E

a) Cytochalasin B



b) Cytochalasin E



The structure of the fungal metabolite, cytochalasin B, a potent, reversible inhibitor of D-glucose transport, is shown in (a). Cytochalasin B can be covalently cross-linked to glucose transporters by irradiation with U.V. light (Carter-Su *et al.*, 1982; Shanahan, 1982). Cytochalasin E, shown in (b), is a related fungal metabolite which will bind to cytoskeletal proteins, thereby inhibiting the binding of cytochalasin B to these proteins. The presence of cytochalasin E during measurement of the number of cytochalasin B binding sites therefore lowers the background signal.

transporter is considered to be the most likely site of cytochalasin B binding. However transporter mutants lacking Trp^{412} can still bind, and be photolabelled with, cytochalasin B, although they have a decreased intrinsic glucose transport activity (Garcia *et al.*, 1992; Katagiri *et al.*, 1991). A method for measuring the equilibrium binding of cytochalasin B to human erythrocyte membranes was published by Zoccoli *et al.*, (1978) to measure the monosaccharide transporter content of the human erythrocyte membrane, and centrifugal binding assays have been widely used to measure the content of glucose transporters in various other membrane types (Cushman & Wardzala, 1980).

One disadvantage of using cytochalasin B is that it will also bind to certain cytoskeletal, peripheral membrane proteins, especially the polymerising ends of F-actin. This binding can be prevented by the simultaneous use of a second fungal metabolite, cytochalasin E, shown in Fig. 1.7(b), which binds to the cytoskeletal membrane proteins but not to the glucose transporters.

1.7 Mammalian facilitative D-glucose transporters

A family of mammalian facilitative D-glucose transport proteins have been discovered in mammalian cells (GLUT1-6, reviewed by Bell *et al.*, 1990 and Gould & Bell, 1990; GLUT7, Waddell *et al.*, 1992), generically called the GLUT family, and numbered in chronological order of their discovery. To date there are six known members of this family (GLUT1-5 and GLUT7), and one pseudo-gene (GLUT6). The characteristics of the individual glucose transporter isoforms are summarised in Table 1.1 and detailed more fully in the following sections. The functional identity of most of the isoforms has been determined by the expression of complementary DNA (cDNA) or mRNA in heterologous cell types.

Table 1.1 Mammalian facilitativeD-glucose transport proteins

Isoform	Amino-acids	K_m (mM)	Major sites of expression	Ref.
GLUT1	492	2-10	Brain, placenta, human erythrocyte	1,2
GLUT2 human rat	524 522	40-60	Liver, kidney, small intestine, pancreatic ß-cell	3 4
GLUT3	496	≈1	Brain	5
GLUT4	509	2-10	Adipocytes, skeletal muscle, heart	6,7 8,9, 10
GLUT5	501	-	Small intestine, kidney, skeletal muscle, adipocytes	11
GLUT6	---	-	Pseudogene	11
GLUT7	528	-	Liver microsomes	12

Table 1.1 details the size (in amino-acids), the K_m of glucose transport and the tissue distribution of six isoforms of the mammalian facilitative D-glucose transporters. Included is the glucose transporter pseudogene, GLUT6. The original publications, containing the amino-acid sequences, are listed below.

References

1. Mueckler *et al.* (1985); 2. Birnbaum *et al.* (1986); 3. Fukumoto *et al.* (1988); 4. Thorens *et al.* (1988); 5. Kayano *et al.* (1988); 6. Fukumoto *et al.* (1989); 7. James *et al.* (1989); 8. Birnbaum (1989); 9. Charron *et al.* (1989); 10. Kaestner *et al.*, (1989); 11. Kayano *et al.* (1990); 12. Waddell *et al.* (1992).

The six sequenced glucose transporters (GLUT1, GLUT2, GLUT3, GLUT4, GLUT5 and GLUT7) have over 25% identity between their aligned amino-acid residues (Bell *et al.*, 1990; Waddell *et al.*, 1992). The highest degree of sequence identity is found within the transmembrane regions, suggesting a functional role for these regions in the mechanism of glucose transport. The variability in the extramembranous domains suggests that these regions may play a role in the differential regulation and kinetics of the transporters. By using sequence alignments an extended family of similar transport proteins were identified in a range of different organisms, both prokaryotic and eukaryotic (Baldwin & Henderson, 1989). While the active H^+/sugar symporters of *Escherichia coli* (*E. coli*), including the D-galactose/H^+ (GalP) symporter, have similar sequences to the facilitative mammalian glucose transporters, the active Na^+/glucose co-transporter of the mammalian small intestine does not. The expression of different glucose transporter isoforms in mammalian cells is tissue specific, and reflects the different needs and functions of specific cells with respect to glucose. Aspects of the regulation of glucose transporter expression, including implications of the different isoforms for glucose utilisation and homeostasis are discussed in the following sections.

1.7.1 HepG2/human erythrocyte-type glucose transporter (GLUT1)

One of the best characterised mammalian, facilitative D-glucose transport proteins is GLUT1, also known as the HepG2/human erythrocyte glucose transporter. Most of the glucose transport proteins are minor components of the membrane, the exception being GLUT1 within the human erythrocyte membrane where it is very abundant, comprising over 5% of erythrocyte ghost protein (Allard & Lienhard, 1985). Human erythrocyte GLUT1 is the only isoform as yet to have

been purified to near homogeneity in a functional form (Baldwin *et al.*, 1982).

GLUT1 is an integral membrane protein which migrates as a broad band on SDS/polyacrylamide gels, with an apparent M_r 45,000-70,000 ($M_r$ave 55,000), due to a single, heterogeneous Asn-linked oligosaccharide (Gorga *et al.*, 1979). Using an antiserum raised against the purified human erythrocyte GLUT1 to screen a HepG2 cDNA library, cDNA encoding a 492 amino-acid glucose transporter protein was identified and sequenced (Mueckler *et al.*, 1985). The amino-acid composition of the HepG2 glucose transporter was comparable with the purified human erythrocyte transporter, suggesting that the two proteins were identical. The calculated M_r 54,117 was close to the estimated M_r 46,000 of the deglycosylated human erythrocyte GLUT1 (Haspel *et al.*, 1985), the difference in apparent M_r probably due to the fact that integral, hydrophobic membrane proteins frequently show increased mobility on denaturing SDS/polyacrylamide gels compared to the soluble protein standards. Following identification of the HepG2/human erythrocyte-type GLUT1, Birnbaum *et al.* (1986) identified and sequenced an equivalent cDNA from an adult rat brain library. This cDNA also coded for a protein of 492 amino-acids, M_r 56,133, with 97.6% identity to the HepG2/human erythrocyte GLUT1. Since then, the 2.9Kb mRNA encoding GLUT1 has been identified in most mammalian tissues. The protein is most abundant in human erythrocytes, foetal tissues and brain, and also in cells associated with a physiological barrier, such as the endothelial cells of the blood-brain barrier (Kasanicki *et al.*, 1987; Kalaria *et al.*, 1988), the blood-nerve barrier (Froehner *et al.*, 1988; Gerhart & Drewes, 1990), the blood-eye barrier of the optic nerve (Harik *et al.*, 1990), the blood-retinal barrier (Takata *et al.*, 1992) and the syncytiotrophoblast of the placenta. The cDNA sequence of GLUT1 has been inserted into the multiple cloning site of pSP65, and

designated pSGT (Mueckler & Lodish, 1986).

GLUT1 is thought to play primarily a house-keeping role in normal cells, supplying their basal glucose requirement and is constitutively expressed, albeit in very low levels in some cells. GLUT1 is over-expressed in most immortal cell lines, for example HepG2, and is one of the two isoforms (the other being GLUT3) for which mRNA is over-expressed in mammalian cancer tissues (Yamamoto *et al.*, 1990).

1.7.2 Liver-type glucose transporter (GLUT2)

The second facilitative D-glucose transporter to be identified was the liver-type, GLUT2. GLUT2 cDNA encodes proteins of 524 or 522 amino-acids in the human (Fukumoto *et al.*, 1988) or rat (Thorens *et al.*, 1988) liver respectively, with 82% homology between the GLUT2 nucleotide sequences, and 55% amino-acid identity to GLUT1. GLUT2 is expressed in liver, kidney and small intestine serosal membranes (Thorens *et al.*, 1990), tissues in which the net efflux of glucose can occur, and in the ß-cells of the pancreas. The glucose transport kinetics and cytochalasin B binding characteristics (Axelrod & Pilch, 1983) of GLUT2 indicated a metabolic role distinct from GLUT1 with a high capacity and a low affinity (K_m 66mM) for glucose (Ciaraldi *et al.*, 1986), the K_m being well above physiological glucose levels. Following a high carbohydrate meal the liver absorbs glucose from the portal vein which it converts to glycogen (Craik & Elliot, 1979). When blood glucose levels are low the liver releases glucose from glycogen, together with glucose formed by *de novo* synthesis, into the vascular system, thus maintaining glucose homeostasis. The high capacity of GLUT2 ensures that the transport of glucose into, and out of, hepatocytes does not become rate-limiting as the intra-

or extra-cellular concentration of glucose changes. The presence of GLUT2 in the basolateral membrane of small intestine ensures that glucose actively imported into these cells from the intestinal lumen by the active Na^+/glucose co-transporter, is rapidly exported into the blood stream. In the pancreatic islet ß-cells, the presence of GLUT2 is thought to permit rapid changes in intracellular glucose concentrations to trigger signalling events leading to insulin release or suppression (Shibasaki *et al.*, 1990; Chen *et al.*, 1990). However the apparent concentration of GLUT2 in the ß-cell membranes in contact with other cells (Orci *et al.*, 1989) and not with the capillaries, indicates a possible involvement in transcellular signalling within the islet. The idea that GLUT2 is involved in the regulation of insulin secretion is supported by the finding that in cultured pancreatic ß-cells expression of GLUT2 is regulated by glucose (Yasuda *et al.*, 1992; Inagaki *et al.*, 1992) and that over-expression increases insulin secretory activity (Sivitz *et al.*, 1989).

1.7.3 Brain-type glucose transporter (GLUT3)

GLUT3 is a 496 amino-acid glucose transporter identified in a human foetal skeletal muscle library (Kayano *et al.*, 1988), with 64% identity to GLUT1 and 52% identity to GLUT2. The mRNA for GLUT3, as for GLUT1, is present in most tissues, predominantly brain, kidney and placenta, however the GLUT3 protein has only been identified in brain, specifically in neurons and microvessels (Gerhart *et al.*, 1992). As with GLUT1 mRNA, elevated levels of GLUT3 mRNA have been identified in mammalian cancer cells (Yamamoto *et al.*, 1990).

1.7.4 Insulin-regulatable glucose transporter (GLUT4)

Of the three facilitative glucose transporters described so far, none are

expressed at high levels in those tissues which dispose of a large part of the blood glucose, muscle and adipose tissue. Both of these tissues express low levels of GLUT1, however in adipocytes insulin stimulation causes an acute 20- to 30-fold increase in the rate of glucose transport, for which the translocation of GLUT1 from an intracellular site to the plasma membrane was found to be insufficient (Simpson & Cushman, 1986). A unique glucose transport protein (GLUT4) was subsequently identified in both rat (James *et al.*, 1989a; Birnbaum, 1989) and human (Fukumoto *et al.*, 1989) adipose tissue, rat heart (James *et al.*, 1989a), both rat (Charron *et al.*, 1989) and human (Fukumoto *et al.*, 1989) muscle and in mouse 3T3-L1 adipocytes (Kaestner *et al.*, 1989). GLUT4 constitutes 90% and GLUT1 10% of the glucose transporters in rat adipocytes, respectively. GLUT4 has between 54-65% similarity to the other glucose transporters. It is expressed uniquely in insulin-sensitive tissues, where its functional expression at the cell surface is controlled by insulin (see Section 1.9.1).

1.7.5 Small intestine-type (GLUT5), liver endoplasmic reticular-type (GLUT7) glucose transporters and the pseudogene (GLUT6)

As well as the relatively well-characterised mammalian facilitative D-glucose transporters described in the preceding four sections, a number of other glucose transporters have also been identified in various mammalian tissues. High levels of an mRNA coding for a second small intestine glucose transporter, GLUT5, were identified in a human small intestine cDNA library (Kayano *et al.*, 1990). This mRNA encodes a protein containing 501 amino-acids, and has between 38-42% sequence identity to the previous four isoforms. Little is known about the transport characteristics or function of GLUT5 but there is some evidence to suggest that

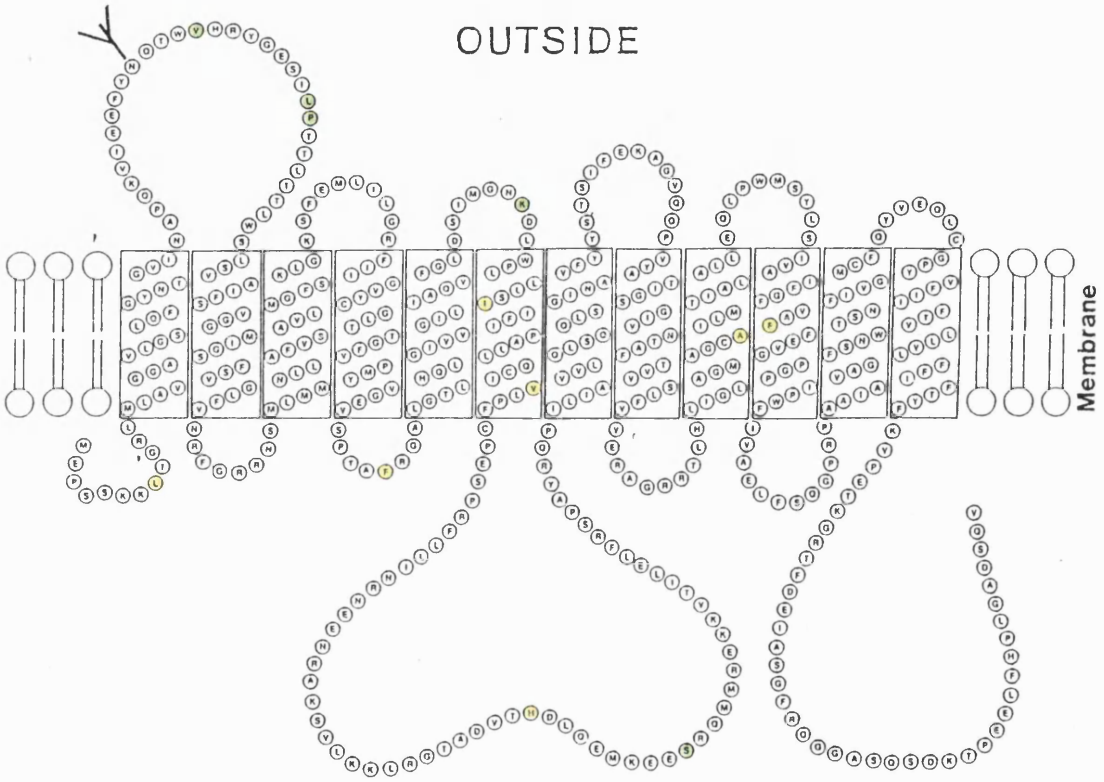
GLUT5 is in fact a fructose transporter (Shepherd *et al.*, 1992). A pseudogene-like glucose transporter sequence (GLUT6) was also isolated from a human small intestine cDNA library and shows substantial sequence identity (76.9%) to GLUT3, however it does not code for a functional glucose transporter protein (Kayano *et al.*, 1990).

A second glucose transporter, GLUT7, was also isolated from rat liver (Waddell *et al.*, 1992). This microsomal glucose transporter has a *C*-terminal endoplasmic reticular retention sequence, and appears to be only expressed in those tissues which also express GLUT2, to which it has 68% sequence similarity.

1.8 Topology and functional organisation of glucose transporters

The best characterised of the glucose transport proteins is GLUT1, its abundance within the human erythrocyte membrane allowing an extensive study to be made of its structure within a lipid bilayer, and the relationship between structure and function. From the amino-acid sequence (Mueckler *et al.*, 1985) a two dimensional structure of GLUT1 was predicted within the membrane, shown in Fig. 1.8. Analysis of the amino-acid sequence using hydropathy plots (Kyte & Doolittle, 1982) predicted the presence of 12 α-helical membrane spanning regions. This secondary structure is found in transporter proteins as diverse as the choline transporters of mammalian nerve terminals (Uhl, 1992) and the galactose/H^+ symporter of *E. coli*, suggesting either conservation of structure or convergent evolution. The membrane spanning domains are joined by predominantly short extramembranous domains, with the exception of a large central cytoplasmic loop between helices 6-7, the *C*-terminus on the intracellular face, and a large loop between helices 1-2 on the extracellular face. The topology of the protein within the membrane has been

Fig. 1.8 Proposed secondary structure of the GLUT1 glucose transporter



This figure is a schematic representation of the proposed secondary structure of the human erythrocyte glucose transporter (GLUT1) within the red blood cell membrane (from Mueckler *et al.*, 1985). The protein contains 12 membrane spanning α-helices usually joined by small connecting sequences. The exceptions are the large extracellular loop which contains a single *N*-linked Asn oligosaccharide, and on the cytoplasmic face, a large, central loop, the large *C*-terminus and the *N*-terminus. Amino-acid differences between the human erythrocyte GLUT1 and the rat brain GLUT1 are denoted by coloured circles. Yellow denotes a conservative amino-acid change, and green denotes a non-conservative change.

determined by using both susceptibility to proteolytic cleavage (Cairns *et al.*, 1987) and site-specific antibodies (Davies *et al.*, 1987, 1990). The large central cytoplasmic loop (213-269) and the *C*-terminus (457-492) are the only parts of the native protein susceptible to proteolytic cleavage, and antibodies raised against synthetic *C*-terminal peptides will bind to the intact protein. The cytochalasin B binding site is in the *C*-terminal half of the protein and the single glycosylation site is extracellular, located in the *N*-terminal half of the protein. The identification of Asn^{45} (Gorga *et al.*, 1979) as the glycosylation site, between helices 1-2, suggested that the *N*-terminal peptide is also cytoplasmic as only a single membrane spanning domain exists between the *N*-terminal and Asn^{45}. The oligosaccharide is not necessary for expression of GLUT1 at the cell surface, however its absence leads to a reduced affinity for glucose (Feugeas *et al.*, 1990; Asano *et al.*, 1991). Each of the glucose transporters subsequently sequenced after GLUT1 were independently predicted to contain 12 hydrophobic membrane spanning α-helices, suggesting both a common ancestry and functional requirement. Neither the *C*- nor the *N*-termini are proteolytically altered after synthesis (Mueckler *et al.*, 1985), therefore there is no cleavable *N*-terminal signal sequence required for insertion into the membrane, which is necessary for some other integral membrane proteins. The three dimensional arrangement of the helices within the membrane is unknown.

Glucose and cytochalasin B are thought to compete for the same binding site. The remainder of GLUT1 left membrane-bound following proteolytic cleavage will bind cytochalasin B in a D-glucose-inhibitable manner, albeit with a reduced affinity, although it no longer transports D-glucose (Cairns *et al.*, 1987). The cytochalasin B binding site has tentatively been identified as Trp^{412} (Deziel *et al.*,

1984), located in the membrane of helix 11, where it is resistant to proteolytic cleavage, suggesting that the glucose binding site is also partially located within the membrane spanning domains. An azidosalicoyl derivative of bis(D-mannose) which binds to the extracellular face of the transporter near the end of helix 9 (Holman *et al.*, 1988) and also inhibits glucose transport, suggests that this helix is also involved. Although the kinetic mechanism of transport remains unknown, it seems likely that a single substrate binding site is alternately exposed to each extramembranous face in turn, by virtue of a conformational change (Walmsley, 1988). Truncation of the *C*-terminus appears to lock the transporter into an inward facing conformation, which is devoid of glucose transport activity (Oka *et al.*, 1990). A number of the helices are predicted to be amphipathic, indicating that formation of a hydrophilic substrate binding cleft or channel might be possible.

Knowledge of the amino-acid sequence of the glucose transporter proteins facilitated the synthesis of synthetic peptide sequences identical to peptides within the transporter proteins. Using synthetic peptides corresponding to the central cytoplasmic loop (Davies *et al.*, 1990) of GLUT1 or the *C*-terminal peptides of GLUT1 (Davies *et al.*, 1987; Haspel *et al.*, 1988), site-specific antibodies were raised, which have proved valuable in both topological and functional studies as outlined above, and in identification of different glucose transporter isoforms in various tissues. As a number of these antibodies will also bind to the intact, native proteins they have proved valuable for immunocytochemistry (Kasanicki *et al.*, 1987) and immunoadsorption (Davies *et al.*, 1987) experiments. Antibodies raised against the small extramembranous loops of the protein do not recognise the intact transporter, probably due to a close association with the polar head groups of the phospholipids, or to different conformations adopted by the synthetic peptide

compared to the native peptide.

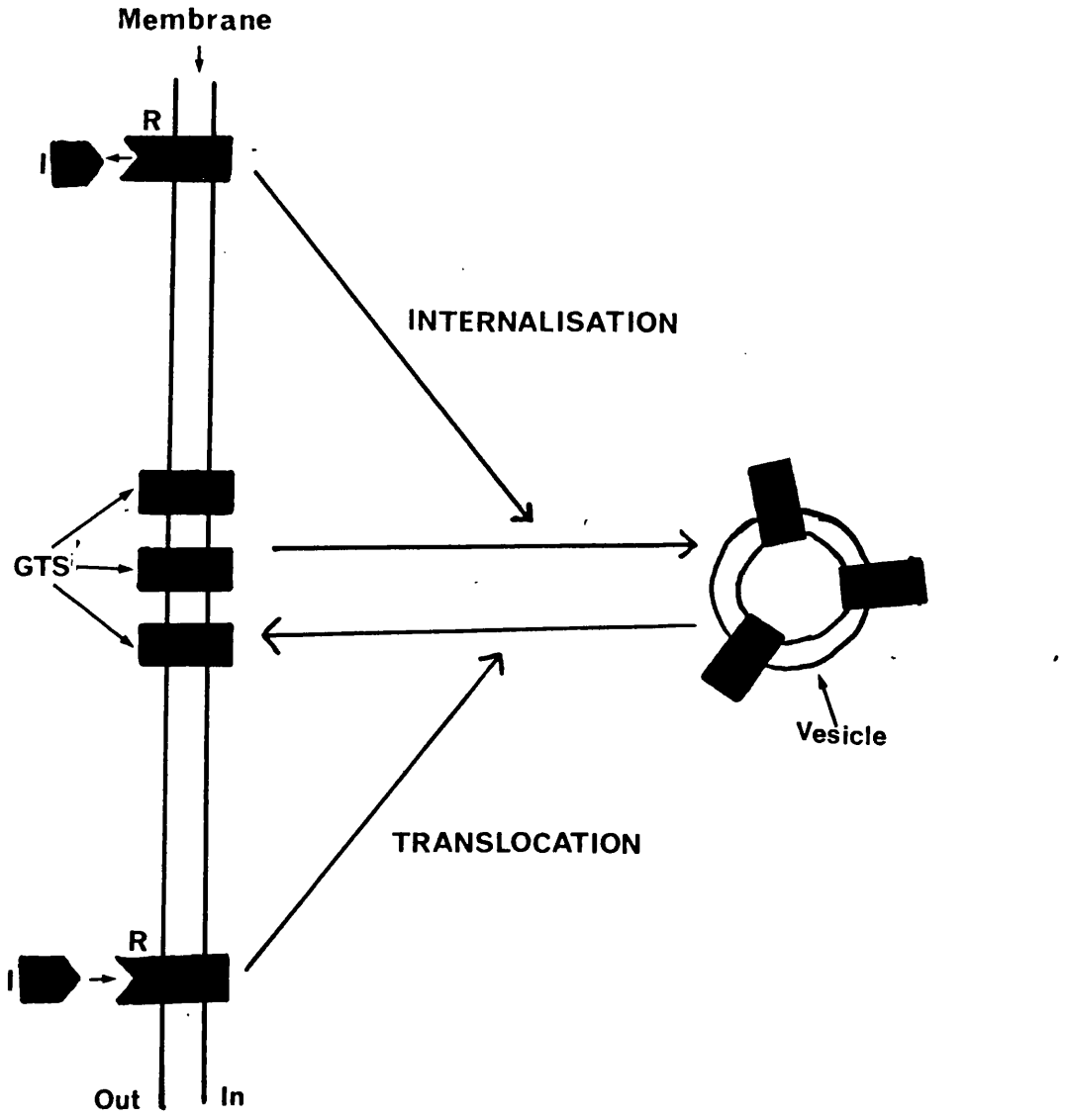
1.9 Regulation of glucose transport

The regulation of glucose transporter expression by mammalian cells is an important aspect of glucose transporter action particularly through its apparent involvement in notable pathological disorders such as non-insulin dependent TypeII diabetes mellitus (review: Mueckler, 1990). Theoretically there are a variety of different mechanisms by which cells can modulate their glucose uptake, including regulation of the synthesis, and expression at the cell surface, of glucose transporter proteins, differential expression of glucose transporter isoforms with different transport characteristics, the translocation of glucose transporters from an intracellular site to the plasma membrane or a change in the intrinsic activity of the transport proteins. The regulation of glucose transport can be acute, occurring within minutes, or slow. Research on the hormonal control of glucose transport in mammalian cells has focused mainly on the insulin effects in adipocytes and muscle, and also in fibroblasts and immortal cell lines.

1.9.1 Glucose transporter translocation

For acute regulatory systems, such as insulin-stimulation of glucose transport in adipocytes (Cushman & Wardzala, 1980; Suzuki & Kono, 1980) and muscle (Klip & Paquet, 1990), the control mechanism is the reversible translocation of glucose transporters from an intracellular site to the plasma membrane, schematically depicted in Fig. 1.9. In basal adipocytes most GLUT4 is internal, and cell surface GLUT1 maintains the basal glucose uptake. Following insulin stimulation GLUT4

Fig. 1.9 Insulin-stimulated translocation of glucose transporters in adipocytes



Adipocyte GLUT4 translocates from an intracellular vesicle pool to the plasma membrane in response to insulin-stimulation increasing the uptake of glucose by up to 30-fold within minutes. Following removal of the insulin-stimulus, GLUT4 is internalised once more into the intracellular site. I=insulin, R=receptor, GTS=glucose transporters.

translocates to the plasma membrane (Joost & Weber, 1989) resulting in an increased number of glucose transporters at the cell surface, and consequently producing a rapid increase in glucose uptake. GLUT1 will also translocate to the cell surface, albeit to a much lesser extent. Translocation of GLUT4 also occurs in 3T3-L1 adipocytes (Blok *et al.*, 1988; Gould *et al.*, 1989; Calderhead & Lienhard, 1988; Shibasaki *et al.*, 1992). A range of compounds including GTP analogues (Baldini *et al.*, 1991), IGF-1 (Bilan *et al.*, 1992; Weiland *et al.*, 1991), acute growth hormone treatment (Tanner *et al.*, 1992; Carter-Su *et al.*, 1988), interleukin-1 (Garcia-Welsh *et al.*, 1990), adenosine (Joost & Steinfelder, 1982), sulphonylureas (Farese *et al.*, 1991) and the phorbol esters, 12-O-tetradecanoylphorbol 13-acetate (TPA) (Muhlbacher *et al.*, 1988; Longo *et al.*, 1992) and 12-myristate 13-acetate (PMA) (Gibbs *et al.*, 1986; Vogt *et al.*, 1991) can all induce translocation of GLUT4 and/or GLUT1 in various cell types.

The ability of a glucose transporter to translocate is related to both cell type and intrinsic activity. GLUT1, which is not insulin-responsive in its native HepG2 cells, translocates following insulin-stimulation in 3T3-L1 adipocytes (Blok *et al.*, 1988; Gould *et al.*, 1989), BHK and COS cells. In BHK cells, GLUT1 translocates not only in response to insulin but also in response to cellular stresses such as hypothermia, arsenite or infection with vesicular stomatitis virus or Semliki Forest virus (Widnell *et al.*, 1990). These findings suggest that in some cells translocation in response to a stimulus is a property conferred onto the glucose transporter by the cell type, under the regulation of tissue specific factors, and not necessarily intrinsic to the transporter itself. Conversely, when GLUT4 is over-expressed in cells for which GLUT1 is the native transporter, GLUT4 remains compartmentalised in an intracellular location, while GLUT1 is expressed at the cell surface as normal

(Haney *et al.*, 1991; Shibasaki *et al.*, 1992), suggesting that the intracellular targeting of GLUT4 is, in this case, intrinsic to the protein itself.

Translocation in response to insulin is inhibited by catecholamines (Green, 1983), dexamethasone (Horner *et al.*, 1987), high cyclic AMP levels (Lawrence Jnr *et al.*, 1992) and high Ca^{2+} levels (Reusch *et al.*, 1991).

1.9.2 Regulation of glucose transporter expression

Regulation of glucose transport involving a change in the total level of the glucose transporter protein appears to be confined to GLUT1, and possibly GLUT3. Long term, or chronic, insulin stimulation increases GLUT1 protein levels (Kozka *et al.*, 1991), as does chronic glucose deprivation (Walker *et al.*, 1988; Tjordman *et al.*, 1990; Kosaki *et al.*, 1991). Growth factors including platelet-derived growth factor (Rollins *et al.*, 1988), epidermal growth factor and fibroblast growth factor (Hiraki *et al.*, 1988) also increase glucose transporter expression. GLUT1 mRNA levels are elevated in cancer tissues (Yamamoto *et al.*, 1990) and GLUT1 expression is increased through the action of oncogenes (Flier *et al.*, 1987; Hiraki *et al.*, 1989), by viral transformation (Salter & Weber, 1979; Birnbaum *et al.*, 1987) and by the action of tumour-promoting phorbol esters, TPA and PMA (Flier *et al.*, 1987). GLUT3 mRNA levels are also increased by treatment with PMA (Longo *et al.*, 1992).

Chronic growth hormone treatment will down-regulate adipocyte basal glucose transport in the long term (Schoenle *et al.*, 1983) through decreased GLUT1 expression (Tai *et al.*, 1990). Cycloheximide does not affect acute stimulation by insulin, suggesting *de novo* synthesis is not required, however after 90min there is a decrease in the absolute number of glucose transporters suggesting that they have

a rapid turnover (Matthaei *et al.*, 1988). Therefore, the expression of GLUT1 seems to be related to maintaining the appropriate basal glucose transport, under conditions of stress or growth. GLUT1 is a member of the glucose-regulated stress-inducible proteins (Wertheimer *et al.*, 1991).

1.9.3 Intrinsic glucose transport activity

A number of agents including adenosine and isoproterenol can change glucose transport activity without apparently affecting transporter distribution, suggesting that the intrinsic activity of the glucose transporters can be modified (Smith *et al.*, 1984; Shanahan *et al.*, 1986; Joost *et al.*, 1986; Kuroda *et al.*, 1987). Isoproterenol, a ß-agonist, will diminish insulin-stimulated glucose transport as long as adenylate cyclase activity is not inhibited (Green, 1983; Smith *et al.*, 1984; Joost *et al.*, 1986). Isoproterenol acts in adipocytes without affecting GLUT4 distribution (Kuroda *et al.*, 1987) possibly by changing the accessibility of the binding site of pre-existing transporters for glucose (Vannucci *et al.*, 1992), and therefore decreasing the intrinsic activity of GLUT4. Adenosine will protect glucose transport from the inhibitory effects of isoproterenol (Green, 1983), and partly augment the stimulation by insulin (Joost & Steinfelder, 1982; Kuroda *et al.*, 1987).

Glucose transporters are phosphorylated in response to cyclic AMP, isoproterenol (James *et al.*, 1989b; Gibbs *et al.*, 1986) and phorbol esters (Gibbs *et al.*, 1986), but not in response to insulin (James *et al.*, 1989b). Phorbol esters also stimulate the phosphorylation of GLUT1 in red blood cells, without affecting their intrinsic glucose transport activity (Witters *et al.*, 1985). Therefore the significance of phosphorylation in the stimulation of glucose transport is not yet understood.

Despite the considerable number of investigations into the intrinsic activity of

glucose transporters, and the involvement of secondary messengers the mechanism controlling translocation and glucose transport regulation are still not fully understood. Most of the findings could result from either the translocation of non-functional transporters, partial fusion of transporter-containing vesicles with the plasma membrane, occlusion of functional transporters within the membrane, or changes in the intrinsic activity, The involvement of microtubule proteins, etc., could all affect the insulin-stimulation.

1.10 Glucose transport in the mammary gland

The lactating mammary gland has been shown in previous sections to have a high requirement for glucose transport, as glucose is required for metabolism, and lactose and fatty-acid synthesis. This requirement is reflected in the 36-fold increase between the glucose transport capacity of acini isolated from virgin compared to mid-lactating rat mammary gland (Prosser & Topper, 1986).

Glucose transport into mammary epithelial cells is rate-limiting for glucose utilisation (Threadgold *et al.*, 1982) and for lactose synthesis. It occurs by a facilitative, temperature-dependent mechanism, specific for D-glucose with apparent saturation kinetics (Wilde & Kuhn, 1981). This suggested the presence of a facilitative D-glucose transport protein within the plasma membrane, probably within the basal plasma membrane. Such a facilitative glucose transport system has been described in both rat (Threadgold *et al.*, 1982) and mouse (Prosser & Topper, 1986) mammary gland. *In vitro* glucose transport is not affected by insulin, corticosterone, dexamethasone, cAMP or adenosine, however it is inhibited by cytochalasin B, $HgCl_2$, N-ethylmaleimide (NEM), phloretin (which will also inhibit lactose synthesis) and structurally related sugars (Threadgold *et al.*, 1982).

The direct relationship between extracellular glucose concentration (Wilde & Kuhn, 1981) and lactose synthesis supports a role of plasma membrane glucose transport in the regulation of lactose synthesis. The low, steady state concentration of intracellular glucose between 0.1-0.5mM (Kuhn *et al.*, 1980) creates a situation where the rate of lactose synthesis is conceivably controlled by changes in the rate of glucose transport and/or glucose phosphorylation/glycolysis. The intra-lumenal location of lactose synthetase suggests that transmembrane Golgi transport pathways must exist for both glucose and UDP-galactose entry. Indirect evidence exists for the specific, facilitative transport of UDP-galactose into the Golgi (Kuhn & White, 1976), that excludes UDP-glucose, a far more abundant cytoplasmic molecule and a potent, competitive inhibitor of UDP-galactose for lactose synthetase. The transport of glucose into the Golgi lumen has proved more difficult to characterise. Golgi vesicles in which lactose synthesis could be inhibited by phloretin were also heavily contaminated with plasma membranes (Kuhn & White, 1975), therefore the influence of the plasma membrane transport protein had to be taken into consideration. Purer Golgi vesicles were found to be permeable to both electrolytes and non-electrolytes under an approximate M_r300, including a range of monosaccharides (White *et al.*, 1981), but impermeable to disaccharides such as lactose. The weight of evidence therefore is for the presence of a non-specific, proteinaceous pore transport system in the mammary Golgi membranes, with a very high capacity, apparent first-order kinetics and resistance to competitive inhibition. The pore properties of the Golgi membranes can be reconstituted into artificial lipid bilayers (Wallace & Kuhn, 1986). However more recent investigations have shown the presence of cytochalasin B binding sites within a Golgi membrane-enriched fraction from lactating mammary gland, a proportion of which appear to

be GLUT1 (Madon *et al.*, 1989, 1990), although this does not necessitate that glucose transport proteins are involved directly in the passage of glucose for lactose synthesis.

Insulin, acutely stimulating glucose transport in other cells has little acute effect on glucose transport in mammary gland (Martin & Baldwin, 1971; Williamson & Robinson, 1977; Threadgold & Kuhn, 1984; Prosser & Topper, 1986), however culture of mid-pregnant mammary explants for 3 days in the presence of insulin, cortisol and prolactin caused a 4-fold increase in the glucose uptake capacity. As the V_{max} increased, but the K_m did not change, the increased uptake was presumed to be due to an increased number of glucose transporters rather than to an increased affinity of the mammary transporter for glucose. In these cultures higher concentrations of IGF-I could be substituted for insulin. Insulin only had a stimulatory effect on glucose transport at concentrations in excess of 8ng/ml (Prosser *et al.* , 1987) which is unlikely to be physiologically relevant, and could correspond to a state of stress.

Elucidation of the mechanism by which the mammary secretory epithelial cells obtain glucose particularly for lactose synthesis was the ultimate aim of this project, by identifying and characterising the glucose transporter isoform(s) expressed by the mammary epithelium in both intracellular and plasma membrane sites, especially in relation to lactation, and glucose transport regulation. Preliminary studies by Madon *et al.* (1989), and results of investigations published during the period of this work, had identified GLUT1 (Madon *et al.*, 1990; Martin *et al.*, 1990; Burnol *et al.*, 1990) in peak-lactating rat mammary gland and there was also evidence for GLUT4 expression in pre-lactating mammary gland (Burnol *et al.*, 1990). The K_m of 7mM for glucose transport into mammary cells (Threadgold *et al.*, 1982) is within the range identified for both of these isoforms (Table 1.1),

although the lack of acute insulin sensitivity of glucose transport would seem to discount the expression of an appreciable proportion of GLUT4 during lactation.

CHAPTER 2

MATERIALS AND GENERAL METHODS

Unless stated otherwise, all materials were obtained from Sigma Chemical Company (Poole, Dorset, U.K.) or BDH Chemicals (Poole, Dorset, U.K.). Nulliparous female Wistar rats (A. Tuck & Son, Rayleigh, Essex) were maintained on Labsure irradiated CRM diet (Labsure, Poole, Dorset) and water ad libitum with a 12hr light:12hr dark cycle. All tissues were removed between 9.00 and 11.00 a.m. to avoid diurnal variations in lactation. Animals were mated (Day 0 of pregnancy) and at parturition (Day 0 of lactation) the litters adjusted to 8 pups.

2.1 Antibody preparations

2.1.1 Conjugation of peptides to ovalbumin

Synthetic peptides were conjugated to ovalbumin using sulpho-m-maleimidobenzoyl-N-hydroxysuccinimide ester (sulpho-MBS, Pierce) following reduction of the terminal cysteine with dithiothreitol (DTT). Peptide (6mg) was dissolved in 1.0ml 50mM Tris-HCl, 1mM ethylenediaminetetra-acetic acid (EDTA), pH8.0 and the pH re-adjusted to 8.0 using 0.1M NaOH. DTT was added to 20mM and the peptide reduced under N_2 for 1hr at room temperature. Reduced peptides were separated from DTT and from any scavengers remaining from the peptide synthesis procedure (e.g. ethanedithiol, EDT) by gel exclusion chromatography on a 1x20cm column of Sephadex G10 (Pharmacia-LKB) equilibrated with 50mM sodium phosphate, pH6.0 to stabilise the reduced form of the peptide. Elution of the peptide was monitored by absorbance at 230nm and of both the peptide and DTT by a thiol assay (Section 2.10) to ensure that they eluted separately, as

contamination of the peptide with DTT will interfere with conjugation. The peak peptide fractions were pooled (3-4ml).

Ovalbumin (17mg) was dissolved in 1ml 10mM sodium phosphate, pH7.4, and dialysed overnight against 2l of this buffer at 4°C. Duplicate 5µl samples were kept for amino acid analysis. Free thiol groups were blocked by incubating 550µl (≈9mg) ovalbumin with 1.4mM NEM at 20°C for 30min, following which the coupling agent, sulpho-MBS (4mg), was added and incubation continued for a further 30min at 25°C. Ovalbumin-MBS was separated from free sulpho-MBS by gel exclusion chromatography using a 1x20cm Sephadex G50 (Pharmacia-LKB) column equilibrated with 50mM sodium phosphate, pH6.0. The eluate was monitored at 280nm, and the peak protein-containing fractions pooled.

Pooled ovalbumin-MBS and peptide fractions were combined and incubated for 4hr at room temperature with constant mixing. The conjugate was dialysed overnight at 4°C against 2x2l 10mM sodium phosphate, 148mM NaCl (Phosphate-buffered saline, PBS), pH 7.2. Protein concentration was determined by the Lowry method (Section 2.9), and aliquots of 100µg protein stored at -20°C.

For determination of the extent of peptide coupling, uncoupled peptide was removed from a 1ml aliquot of the conjugate by gel filtration chromatography on a fresh 1x20cm Sephadex G50 column equilibrated with PBS, pH7.2, as above. Duplicate 80µg samples of the eluted conjugate were taken for amino acid analysis.

2.1.2 Immunisation of rabbits

For the initial immunisation, 250µg conjugate in 300µl PBS, pH 7.2 was emulsified with 900µl Freund's complete adjuvant, and 1ml (≈200µg) injected intramuscularly in two 500µl aliquots into each hind leg of a male New Zealand

White rabbit (from which a pre-immune bleed had previously been taken). Four weeks after the initial injection, the rabbit was boosted with 100μg conjugate emulsified in Freund's incomplete adjuvant, and the first bleed taken after a further 7-10 days. Further bleeds were taken every 4 weeks, and the titre checked using screening enzyme-linked immunosorbent assay (ELISA, see Section 2.1.4). When the titre fell further boosters of 100μg conjugate in Freund's incomplete adjuvant were given, at minimum intervals of 4 weeks.

2.1.3 Antisera preparation

Rabbits were bled from the ear vein (20-40ml) into 10ml glass tubes. Tubes of blood were incubated for 1hr at 37°C, then overnight at 4°C, to complete the clotting retraction, and centrifuged in a bench top centrifuge at 3,000rpm for 10min to compact the clot and pellet free erythrocytes. The serum was transferred to a clean glass tube, and incubated for 30min at 56°C, to inactivate the complement system. Prepared serum was stored frozen at -20 or -70°C.

2.1.4 Screening ELISA

96-well ELISA plates (Nunc maxisorp, Nunc, Kamstrup, Denmark) were coated with either peptide (20ng/well) or purified human erythrocyte glucose transporter (600ng/well) prepared by the method of Cairns *et al.* (1984). Synthetic peptides were dissolved at 1mg/ml in dimethylsulphoxide (DMSO) and stored at -20°C. Stock peptide was diluted to a working concentration of 250ng/ml in 50mM Na_2CO_3, pH 9.6, 80μl loaded into each well and the plates dried down *in vacuo* over sodium hydroxide overnight. Purified human erythrocyte glucose transporter was diluted to 6μg/ml in 50mM Na_2CO_3, pH 9.6 and 100μl loaded into each well and adsorbed

by incubation overnight at room temperature.

Coated plates were washed 5x with 136mM NaCl, 2.7mM KCl, 1.6mM Na_2HPO_4, 1.2mM KH_2PO_4, 0.02% (w/v) sodium azide, 0.05% (v/v) Tween-20 (Bio-Rad Laboratories, Watford, Herts., U.K.), pH7.2 (PBSA-T). The uncoated protein binding sites were blocked by incubating for 2hr at room temperature with 200µl PBSA-T containing 5% (w/v) low fat milk powder (Marvel). The blocking buffer was removed, and the plates washed 5x with PBSA-T. Serial dilutions of antisera (5 or 10-fold) or fractions from the antibody purification procedures (2 or 5-fold) were prepared in PBSA-T containing 1% (w/v) milk powder then 100µl of each dilution loaded into the wells in triplicate, and incubated overnight at room temperature. Plates were washed 5x with PBSA-T, then loaded with 100µl of a 1:3000 dilution in PBSA-T/1% (w/v) milk powder of alkaline phosphatase conjugated anti-rabbit IgG (Bio-Rad). Following a 2hr incubation with this second antibody at room temperature they were again washed 5x with PBSA-T. Immunoreactivity was detected by adding 100µl of the substrate p-nitrophenyl phosphate (Sigma 104 phosphatase substrate) at 1mg/ml in 10mM diethanolamine, 1mM $MgCl_2$, pH9.8. Plates were incubated until a yellow colour developed which was measured at 405nm using a Bio-Rad 2550 EIA plate reader. Readings were taken at various time points until a range of absorbance was obtained for each set of dilutions.

2.1.5 Purification of anti-peptide antibodies

2.1.5.1 Affinity-purification of anti-peptide antibodies by adsorption onto protein-depleted human erythrocyte membranes

The preparation of protein-depleted erythrocyte membranes is described in Section 2.3.5. All procedures were performed at 4°C or on ice. Protein-depleted

erythrocyte membranes (50mg protein) were resuspended in 5ml PBS, pH7.2, in a 50ml Sorvall centrifuge tube, and 5ml antiserum added (this ratio of membrane protein to serum was required for complete removal of specific IgG from antiserum, as shown by small scale ELISA experiments - personal communication, Dr. A. Davies). Membranes and serum were incubated for 2hr with gentle mixing on a rotary mixer. Next, membranes were pelleted at 20,000rpm (Sorvall RC-5B, SS-34 rotor) for 20min and the supernatant stored at -20°C, in order to determine the extent of specific IgG removal. The membranes were resuspended in PBS, pH 7.2 by homogenisation in a glass Dounce homogeniser, then centrifuged as above and the supernatant discarded. Membranes were washed twice more by centrifugation, then bound antibodies eluted by homogenisation in 10ml 0.2M glycine-HCl, pH 2.4, followed by immediate centrifugation as above. The supernatant containing the eluted antibodies was neutralised to ≈pH 7.2 with 2M Tris, and dialysed for 24hr against 2x2l PBS, pH7.2.

The anti-peptide and anti-transporter activities of the antibodies were established by screening ELISA (Section 2.1.4). The purity of the antibody preparation was established by SDS/polyacrylamide gel electrophoresis (method in Section 2.5, results in Section 3.3).

2.1.5.2Affinity-purification of anti-peptide antibodies by affinity-chromatography

This procedure required two steps; first separating serum IgG from other serum proteins (including serum proteases) using a CM Affi-gel blue (Bio-Rad) column, and then isolating specific IgG using an affinity column. Serum proteases were separated from IgG prior to affinity chromatography to try and prevent protease degradation of the peptide column. All procedures were carried out at 4°C unless

stated otherwise.

Affinity columns were prepared by immobilising peptides to an iodoacetyl-activated agarose (SulphoLink, Pierce) matrix. The reactions were done in the dark, to prevent light catalysed oxidation of iodide to iodine ($2I^- \rightarrow I_2$; I^- being released during the coupling reaction) and subsequent reaction of iodine with protein tyrosine residues. To enhance the stability of the matrix SulphoLink was protected from light wherever possible. Peptide (2mg) was dissolved in 1.0ml 50mM Tris, 5mM EDTA, pH8.5 (Tris/EDTA) and the pH readjusted to 8.5 before the peptide was separated from contaminants by gel filtration chromatography on a 1x20cm Sephadex G10 column, equilibrated with Tris/EDTA. Peptide elution was monitored at 230nm and the peak peptide-containing fractions pooled. A 50µl aliquot was removed for later determination of coupling efficiency.

SulphoLink (2ml) was equilibrated with the coupling buffer, Tris/EDTA, by repeated suspension and settling of the gel and removal of the supernatant. Following the final removal of supernatant the peptide, in a volume of 2-3ml, was added, keeping the total volume to < 4ml. Gel was incubated with peptide for 30min at room temperature using a rotary mixer, followed by standing for 30min, following which the supernatant was removed. To determine the extent of peptide coupling a thiol assay (Section 2.10) was performed on duplicate 25µl samples of the supernatant as well as the original decontaminated peptide solution. The extent of removal of peptide from the supernatant was used as a measure of the efficiency of coupling, which was usually around 85%. The gel was washed free of any remaining peptide by repeated settling and suspending in Tris/EDTA and discarding the supernatants, after which any remaining reactive groups on the SulphoLink were blocked using 0.025M cysteine (2ml 0.05M cysteine added to 2ml gel) at room

temperature for 30min with mixing and 30min standing as before.

The coupled gel was packed into a 1x5cm column and washed with 16x volume 1M NaCl. The affinity column was then equilibrated with PBS, pH7.2 containing 0.02% sodium azide and stored at 4°C.

A 1x20cm CM Affi-gel blue column was prepared by washing with 1M acetic acid, 1.4M NaCl, 40% isopropanol until the eluate ran clear. The washed column was equilibrated with 0.01M K_2HPO_4, 0.15M NaCl, 0.02% sodium azide, pH 7.25 (Buffer A), and stored at 4°C. Antiserum (4-5ml) was applied to the CM-Affi gel blue column and eluted at 0.2ml/min monitoring the absorbance at 280nm. The IgG peak, consisting of the first protein fractions to elute, was pooled and a 500µl aliquot stored at -20°C for later analysis. Elution was continued until the absorbance fell to an O.D. <0.01 then the bound serum albumin and proteases were eluted using 0.01M K_2HPO_4, 1.4M NaCl, pH 7.25. The column was regenerated with 2x volume 2M guanidine-HCl, equilibrated with Buffer A and stored at 4°C.

The IgG fraction from the CM-Affi-gel blue column was then loaded onto the peptide-bearing affinity column, which had been equilibrated with Buffer A, and recirculated through the column for 2hr. Alternatively IgG was passed through the column 3x at an elution rate of 0.1ml/min. Using the latter method it was found that no further specific IgG could be removed from the IgG fraction after the second passage, as shown by ELISA. Weakly-bound proteins (non-specific IgG) were washed from the column with Buffer A containing 800mM NaCl, until absorbance of the eluate at 280nm fell to zero. Tightly-bound proteins (specific IgG) were then eluted, first with 10ml 0.2M glycine-HCl, pH2.4, pooling the peak fractions and neutralising with 2M Tris. Following this the column was washed with 10ml Buffer A and eluted a second time with 10ml 50mM diethylamine, pH11.5. The peak

fractions were again pooled, and neutralised using dilute acetic acid. The two eluted peaks were dialysed separately against 2x2l PBS, pH 7.2 overnight, then aliquoted and stored at -20°C. Samples of the antiserum, the albumin from the CM-Affi-gel blue column, total IgG, non-specific IgG and specific IgG were investigated using ELISA and SDS/polyacrylamide gel electrophoresis, and the protein concentration determined.

2.2 Mammary epithelial cell preparation

Female Wistar rats were killed by cervical dislocation, and the mammary tissue gently dissected free of surrounding tissues and placed into Medium A (Medium 199 containing Hank's salts (GIBCO BRL., Uxbridge, U.K.), supplemented with 5mM glucose, 1% bovine serum albumin (BSA, Fraction V, fatty acid-free), and buffered with 13.5mM N-2-hydroxyethylpiperazine-N'2-ethane sulphonic acid (HEPES), pH7.4) at 37°C. Tissue was chopped finely and washed in Medium A, then 4.0g digested in 40ml Medium A containing 4% BSA, 37.5μg/ml soybean trypsin inhibitor (Type I-S), 1.2 mg/ml collagenase (CLSIII, Worthington Biochemical Corporation, New Jersey, U.S.A.) and 150μg/ml hyaluronidase. Digestion was carried out in a 100ml conical screw top flask (Nalgene) in a Gallenkamp orbital incubator at 37°C at 160 rpm, for between 90 and 120min. The rate of digestion was increased by aspirating the cell clumps through pastettes of decreasing bore size, and the stage of digestion monitored under a light microscope. When small clumps (5-10 cells) were seen the digested material was filtered through 150μm nylon mesh (Lockertex, Warrington, U.K.) using a 250ml nalgene filter unit. Filtered cells were transferred to 50ml polycarbonate tubes, and centrifuged at 80g for 4min in a benchtop centrifuge (IEC, Centra). The floating fat layer and supernatant were discarded and

the cells resuspended in 50ml Medium A containing 1μg/ml Deoxyribonuclease I (bovine pancreas DN'aseI, Grade II, Boehringer Mannheim GmbH, Germany) to digest DNA released by cell breakage and help improve cell yield, mixed gently and centrifuged as above. Cells were washed 4x by centrifugation in this buffer before being resuspended in the required volume of appropriate incubation buffer, or pelleted and frozen in liquid N_2.

2.3 Membrane preparations

All membrane preparations were performed on ice or at 4°C unless otherwise stated.

2.3.1 Mammary plasma membranes

This method was adapted from the method of Clegg (1981). Female Wistar rats between days 10 and 14 of lactation were sacrificed by cervical dislocation. The abdominal and inguinal mammary glands were dissected free of surrounding tissues and placed into 0.25M sucrose, 0.1M KCl, 10mM Tris, 1mM EDTA, pH 7.4 (PMHM). Tissue was weighed, and minced with scissors then washed thoroughly with PMHM in a strainer. Minced tissue was resuspended and homogenised in 2.5x volumes PMHM for 3x20 sec at 14,000rpm using a 1cm diameter Ultra-turrax homogeniser. Crude homogenate was filtered through a single layer of damp muslin into a 50ml polycarbonate centrifuge tube and centrifuged for 10min at 1,000rpm using a Sorvall SS-34 rotor.

The floating fat layer was removed with a spatula and the supernatant (designated homogenate) transferred to a clean centrifuge tube. The pellet, consisting of cell debris, was discarded. A 500μl aliquot of homogenate was

removed, frozen in liquid N_2 and stored at -70°C for determination of enzyme activities. The remaining homogenate was centrifuged at 3,000rpm for 10min. The resulting pellet, consisting of nuclei and heavy mitochondria was discarded and the supernatant transferred to a clean centrifuge tube and centrifuged at 6,000rpm for 10min.

The resulting pellet, consisting of light mitochondria, lysosomes and dense microsomes was discarded, and the supernatant divided between two 25ml polycarbonate ultracentrifuge tubes with screw lids, and centrifuged at 40,000rpm for 30min in a Prepspin 50 ultracentrifuge 8x25ml angle rotor. The supernatant from this spin, consisting of cytoplasmic materials, was discarded and the pellets resuspended in 40% sucrose, 20mM Tris-HCl, pH 7.4, (not exceeding 6ml total) and homogenised in a loose glass Dounce homogeniser. Homogenised pellet was placed, in 2ml aliquots, into the bottom of three 25ml cellulose citrate centrifuge tubes, and 6ml each of 36%, 32% and 0.9% sucrose in 20mM Tris-HCl, pH7.4 in turn layered over them. Tubes were centrifuged at 29,500rpm for 2hr using a 3x25ml swing-out rotor (Prepspin 50).

The layer between 32% and 0.9% sucrose was collected (≈10ml) then mixed with an equal volume of 10mM sodium phosphate, pH7.4 and transferred to one 25ml polycarbonate centrifuge tube. The plasma membranes were harvested by centrifugation at 40,000rpm for 30min in the 8x25ml angle rotor. The supernatant was discarded and the pellet was resuspended in 100-200µl of 10mM sodium phosphate, pH 7.4 and homogenised in a glass Dounce homogeniser before aliquoting, freezing in liquid N_2, and storing at -70°C.

2.3.2 Mammary Golgi membranes

This method was an adaptation of that of West (1981). Female rats between days 10-14 of lactation were killed by cervical dislocation and the abdominal and inguinal mammary glands dissected free of surrounding tissues and placed in 37.5mM Tris, 37.5mM maleic acid, 500mM sucrose, 5mM $MgCl_2$, 10mM N-acetyl-L-cysteine, 1% Dextran, pH6.5 containing 1µg/ml bacitracin (GVHM). Glands were weighed, gently cut into small pieces using razor blades, and washed free of blood and milk in a strainer. Tissue pieces were homogenised once in 2.5x volumes of GVHM using a 2cm diameter head Polytron homogeniser at 5000rpm for 30 sec. The crude homogenate was squeezed through a single layer of damp muslin to remove any lumps of tissue into a 50ml polycarbonate centrifuge tube. A 500µl aliquot of the homogenate was removed and stored at -70°C, and the remainder centrifuged for 5min at 1,000rpm, 10°C in the Sorvall SS-34 rotor.

Following this first centrifugation step the speed was increased to 2,500rpm, and the homogenate centrifuged for a further 10min at 10°C, followed by a further 20min at 6,500rpm and 4°C. The first two centrifugation steps were at 10°C to prevent overcooling of the rotor. The floating fat layer was removed using a spatula, and the supernatant, consisting of plasma membranes and lower density microsomes, carefully decanted and discarded. The top, buffy-coloured layer of the pellet, containing the Golgi vesicles, was carefully washed off the more densely packed underlayers, consisting of nuclei and mitochondria, using GVHM and transferred to a loose glass Dounce homogeniser. Following homogenisation, by 3 strokes only, the volume was made up to 21ml with GVHM, and 7ml layered over 10ml 1.25M sucrose, 37.5mM Tris, 37.5mM maleic acid, 5mM $MgCl_2$, 10mM DTT, 1µg/ml bacitracin, pH6.5 (1.25M sucrose medium), in each of three cellulose citrate

centrifuge tubes. The tubes were then filled and balanced with 275mM lactose, 25mM N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES), 10mM DTT, 1μg/ml bacitracin, pH7.0 (LST) and centrifuged at 29,000rpm for 50min in a Prepspin 50 3x25ml swing-out rotor.

The opaque layer between the 1.25M sucrose medium and the GVHM was collected and the Golgi vesicle membranes harvested in one of the two ways, as follows. To concentrate the intact vesicles the Golgi vesicle membranes were diluted with an equal volume of LST, centrifuged at 20,000rpm for 10min in the Sorvall SS-34 rotor and the pellet resuspended in 100-200μl of LST. To obtain a membrane fraction, the Golgi vesicle membranes were diluted into an equal volume of 10mM sodium phosphate, pH7.4 and centrifuged at 40,000rpm for 20min in the Prepspin 8x25ml angle rotor. The pellet was resuspended in 100-200μl 10mM sodium phosphate, pH7.4 and homogenised in a glass Dounce homogeniser, before aliquoting, freezing in liquid N_2 and storing at -70°C.

2.3.3 Rat milk-fat globule membranes

Milk fat globule membranes were prepared by the method of Flint & West, (1983). As an animal licence was required for the procedure of milking rats, I was only able to assist in this, and am grateful to M. Kerr and H.A.C. Fawcett for the milking.

The pups were removed overnight from a day 10 lactating female Wistar rat to permit a build up of milk in the mammary glands. The animal was anaesthetized with 2.7ml/kg Hyponorm:Hyponovel (Janssen:Roche) injected intra-peritoneally (i.p.). Under anaesthesia 0.5U of oxytocin were also injected i.p., to facilitate milk release. The milk was expelled by gently squeezing the glands, collected in glass

capillary tubes and transferred to a sterilin 25ml universal vial on ice. Following milking the rat was killed by cervical dislocation.

The milk was centrifuged twice in a Sorvall bench top centrifuge for 2min at 1,000rpm and the floating cream layers from each centrifugation pooled in a clean Sterilin tube. Cream was washed 4x with 10ml 0.28M sucrose, 1mM $MgCl_2$, 10mM Tris-HCl, pH 7.5, by centrifugation as above, removing the infranatant each time with a needle and syringe. Following the final wash the cream was transferred to a small flat bottomed vial taking care not to disturb the pellet which contained red blood cells and other cell debris. Cream was homogenised on ice for 3x5sec using a 1cm diameter Ultra-turrax, at 14,000rpm. The homogenate was transferred to a 13.5ml polycarbonate ultracentrifuge tube, and centrifuged at 80,000rpm for 2hr at 4°C using an 80Ti rotor in a Beckman L7 ultracentrifuge. The floating fat layer was removed using a spatula, and the infranatant discarded. The pellet of milk fat globule membranes was resuspended by homogenisation in a small glass Dounce homogeniser on ice, with 100-200µl 10mM sodium phosphate, pH7.4, before being aliquoted, frozen in liquid N_2 and stored at -70°C.

2.3.4 Human erythrocyte ghost membranes

Outdated blood was obtained as either whole blood or packed cells from the blood bank of the Royal Free Hospital. Erythrocytes were washed free of plasma components by repeated centrifugation in 4x volumes of PBS (5mM sodium phosphate, 145mM NaCl, pH8.0) at 4,500rpm for 10min using a 6x250ml GSA rotor in a Sorvall RC-5B centrifuge. The supernatants were discarded, and the layer of white blood cells floating on top of the erythrocytes removed by aspiration. Three or four washes were needed depending upon whether packed cells or whole blood

had been used respectively.

Erythrocytes were lysed in 20x volumes of 5mM sodium phosphate, pH8.0 (5P8) containing 1mM EDTA, 0.11mM phenylmethylsulphonyl fluoride (PMSF) and separated from released haemoglobin by tangential flow ultrafiltration through a Pellicon cassette system (Millpore Corp., Bedford, M.A., U.S.A.) using a 0.4μm (coarse screen) durapore cassette filter at a pressure of 400 p.s.i.. Membranes were collected and concentrated by centrifugation at 11,500rpm for 30min using the Sorvall SS-34 rotor, and the hard pellet containing white blood cells removed by aspiration after sliding the sloppy ghost pellet off it. Ghost membranes were assayed for protein and stored at -70°C.

2.3.5 Protein-depleted human erythrocyte membranes

Human erythrocyte membranes were stripped of peripheral proteins by the method of Gorga and Lienhard (1981). Erythrocyte membranes (180mg protein in 45ml of 5P8) were adjusted to pH12 by the addition of 240ml of ice-cold 15.4mM NaOH, 2mM EDTA, 0.2mM DTT, which had been purged with N_2 gas for 20min. After 10min the membranes were separated from the peripheral proteins by centrifugation at 18,000rpm for 20min, in 50ml Sorvall SS-34 centrifuge tubes. The supernatants were discarded and the membranes resuspended in 50mM Tris-HCl, pH6.8 (50T6.8). The membranes were washed 4x in this buffer, by centrifugation as above, before a final resuspension by homogenisation in a glass Dounce homogeniser, determination of protein concentration and storage at -70°C.

2.3.6 Crude liver plasma membranes

Rat liver was obtained from male Sprague-Dawley rats, maintained under the

same regime as the female Wistar-derived rats and killed by cervical dislocation, and plasma membranes prepared by the method of Neville, (1968).

Liver was cut into small pieces using scissors and homogenised using a loose fitting glass Dounce homogeniser in 2.5x volumes 1mM $NaHCO_3$, pH8.0. The homogenate was filtered through a 75μm nylon mesh, and centrifuged at 1,000g for 10min in a bench-top centrifuge. The supernatant was discarded, the pellet resuspended into 1mM $NaHCO_3$ and homogenised in a glass Dounce homogeniser. Following a further centrifugation at 1,200g for 10min, in 10ml glass conical tubes a tripartite pellet was formed, the bottom layer rich in nuclei, the middle layer in plasma membranes and the top layer with mitochondria. The supernatant and mitochondrial layer were discarded. Fresh buffer was added to the plasma membrane layer which was gently resuspended and transferred to a Dounce homogeniser. Following a further homogenisation the membranes were centrifuged as before. The plasma membrane layer was harvested, frozen in liquid N_2, and stored at -70°C.

2.3.7 Rat adipose membranes

Parametrial adipose tissue was dissected free of other tissues and stored at -70°C. Samples of the adipose tissue (500mg) were then thawed into 0.75ml 25mM HEPES, pH7.4 containing 0.2mM PMSF, 4mM EDTA, 1μM Leupeptin, 1μM Pepstatin A, 1μM Aprotinin and 1μM E-64. Tissue was homogenised thoroughly using a Polytron homogeniser at 7000rpm for 3x20sec on ice. Crude homogenate was centrifuged at 3,000rpm for 30sec at 4°C using a Sorvall RT6000B refrigerated benchtop centrifuge and the infranatant (designated homogenate) between the floating fat layer and the pellet removed using a needle and syringe. Aliquots

(0.5ml) of homogenate were solubilised by addition of 1% Triton X-100 and incubation on ice for 1hr (Sinha *et al.*, 1990) and the remaining homogenate frozen at -70°C. The solubilised homogenate was transferred to 10ml Sarstedt ultracentrifuge tubes and centrifuged for 1hr at 239,000g_{av} at 4°C. The supernatant, designated Triton-extract, was stored at -70°C.

2.4 Enzyme assays

2.4.1 5'-nucleotidase

The activity of 5'-nucleotidase was determined by measuring the production of [^{14}C]adenosine from [^{14}C]adenosine-5'-monophosphate ([^{14}C]AMP). Dowex-Cl^- was used to separate the negatively charged AMP from the adenosine by anion exchange. Each membrane sample was assayed in triplicate.

Membranes (12µl of an appropriate dilution, to give ≈10µg of protein) were added to 28µl of an assay mix* to give final concentrations of 50mM Tris-maleate, 0.4mM EDTA, pH7.4, containing 10mM $MgCl_2$, 0.3mM adenosine, 0.4mM AMP, 1.0mM DTT and 0.3µCi/ml [^{14}C]AMP (NEN), in a microfuge tube; (* assay mix was made by mixing together 100µl of a 7x concentration of each with 100µl H_2O). Each reaction was initiated by the addition of the membranes, tubes vortexed briefly, and incubated for 10min at 37°C. The reaction was stopped by the addition of 50µl ethanol and 10µl of a carrier solution of 20mM adenosine, 20mM AMP and 7mM inosine. Samples were vortexed briefly, and stored on ice.

Tubes were centrifuged at top speed in a microfuge for 5min, then 50µl of the supernatant removed and added to 100µl Dowex-Cl^- in a clean tube. The mixture was vortexed for 5sec, and allowed to settle for 10min. This was repeated 3x. Finally the tube was spun for 5min at top speed in a microfuge, and 50µl of the

supernatant added to 10ml Ultima-Gold scintillation fluid. Samples were counted (for ^{14}C) for 10min, using a Beckman scintillation counter. From the specific activity of the [^{14}C]AMP the nmol adenosine formed/mg protein/min was calculated.

2.4.2 Galactosyltransferase

The activity of galactosyltransferase was determined by measuring the transfer of [^{14}C]galactose from uridine diphosphate (UDP)-[^{14}C]galactose to N-acetylglucosamine (N-AG), to form [^{14}C]lactosamine. Lactosamine was separated from the negatively charged UDP-galactose by anion exchange chromatography on a Dowex-formate$^-$ column. Each sample was assayed in duplicate with N-AG, and once with a blank, substituting H_2O for N-AG.

The assay mix was prepared by placing 25µl 80mM TES-NaOH, pH7.4, 30mM $MnCl_2$, 1% Triton X-100 into microfuge tubes. To the tubes were added 10µl 0.1M N-AG or water, followed by 5µl of 5.0mM UDP-galactose containing UDP-[^{14}C]galactose (Amersham, Bucks., England) at ≈2500dpm/5µl. The reaction was started with 10µl membranes (1:20-1:100 dilution), mixed gently, and incubated at 37°C for 10min. The reaction was stopped by boiling for 90sec, after which the samples were cooled immediately on ice and 50µl water added. Condensation was collected by microfuging briefly and the entire sample applied to a 1ml Dowex-formate$^-$ column equilibrated with H_2O. Tubes were washed out with 2x100µl H_2O which was added to the column, and the lactosamine eluted with 1ml H_2O directly into scintillation vials. Scintillant (10ml) was added to each vial, and the samples counted (for ^{14}C) for 10min, using a Beckman scintillation counter, along with duplicate 5µl samples of the UDP-[^{14}C]galactose/UDP-galactose mix, for a total

counts reading.

Dowex-formate$^-$ was formed from Dowex-Cl$^-$ by incubating 50g Dowex-Cl$^-$ with 500ml 1M NaOH for 30min, repeatedly washing with changes of distilled water (dH_2O) until the pH reached neutral, then incubating with 500ml 1M formic acid for 30min, and washing again with dH_2O until the pH reached neutral.

2.5 SDS/polyacrylamide gel electrophoresis

SDS/polyacrylamide gel electrophoresis was performed using 0.75, 1.5 or 3mm thick vertical slab gels containing either 10% or 12% acrylamide, prepared by the method of Laemmli (1970). All reagents were of an electrophoresis grade.

Slab gels were prepared using the Bio-Rad Protean Mk I or the LKB 2050 Midget electrophoresis unit, with a stacking gel of 2 or 1cm respectively, consisting of 3% acrylamide/0.8% bisacrylamide in 125mM Tris-HCl, pH6.8, 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.05% (v/v) tetramethylethylenediamine (TEMED). The separating gels were 10 or 12% acrylamide/0.27 or 0.32% bisacrylamide in 375mM Tris-HCl, pH8.8, 0.1% (w/v) SDS, polymerised with 0.1% (w/v) ammonium persulphate and 0.016% (v/v) TEMED. Protein samples were solubilised in 40mM Tris-HCl, pH6.8, 0.8mM EDTA, 0.8% (w/v) SDS, 4mM DTT, 10% (v/v) glycerol, 15µg/ml Pyronin Y (gel sample buffer), vortexed to dissolve and stored at -20°C. Molecular weight markers from Bio-Rad were routinely used, either as prestained low range markers (M_r 14,000-130,000), to which Coomassie blue stain had been covalently bound, or as low molecular weight range markers (M_r 17,000-97,400) made up in gel sample buffer.

Electrophoresis was carried out using a buffer of 25mM Tris, 190mM glycine, 0.1% (w/v) SDS, pH8.3. Large gels were electrophoresed using the Protean Mk I

electrophoresis cell, and a Pharmacia EPS 5500/400 power supply, at 15mA per 1.5mm thick gel during the migration of the samples through the stacking gel and then at 30mA/gel during resolution in the separating gel. These current values were doubled if 3mm gels were used. Mini-gels (0.75mm thick) were electrophoresed using the Midget mini-gel system at a constant current of 20mA/gel. Gels were electrophoresed until the tracking dye had migrated to the bottom of the gel and a good separation of the prestained markers was seen.

To stain the proteins with Coomassie blue, gels were incubated overnight in 10% acetic acid/25% isopropanol then changed into 10% acetic acid/25% isopropanol/0.025% Coomassie Page blue 83 for 12hr. Gels were destained first in 10% acetic acid/ 25% isopropanol/ 0.0025% Coomassie Page blue 83 for 12hr, then taken through several changes of 10% acetic acid. Stained gels were photographed by the Dept. of Medical Illustration, Royal Free Hospital, and scanned using a Chromoscan 3 densitometer (Joyce-Loebel) at 530nm.

2.6 Western blotting and immunodetection of proteins

Electrotransfer of proteins to nitrocellulose was performed essentially by the method of Towbin *et al.* (1979). Proteins were separated by SDS/polyacrylamide gel electrophoresis as in Section 2.5, and the gels removed from the plates and equilibrated in 38mM glycine, 48mM Tris, 0.0375% (w/v) SDS, 20% methanol (transfer buffer) at room temperature for 20min. Each gel was next placed on top of a sheet of nitrocellulose (0.45μm pore size, Bio-rad or Sartorius) of equal size, pre-wet with transfer buffer, and then sandwiched between two stacks of filter paper pre-soaked with transfer buffer, between the plates of a LKB Novablot electrophoresis transfer system, set up within the LKB Multiphor II electrophoresis

tank. Electrotransfer was at a constant current of 1.5mA/cm^2 of gel, for 1.5hr (1.5mm gels) or 1hr (0.75mm gels) at room temperature. Following transfer the gel was stained with Coomassie blue as in Section 2.5, to check the efficiency of transfer, and the nitrocellulose placed in TBS (Tris-buffered saline, 20mM Tris-HCl, 500mM NaCl, pH7.5).

The migration of the low range M_r standards was determined by staining the appropriate section of nitrocellulose with 0.1% (w/v) amido black, 25% isopropanol, 10% acetic acid until bands were visible, and destained in 10% acetic acid. For immunodetection of proteins the remaining protein binding sites on the nitrocellulose sheets were blocked by incubation, in separate plastic boxes, with TBS containing 0.2% (v/v) Tween-20 (TTBS) and 5% (w/v) low fat milk powder for 1hr. Blocking buffer was then removed, the sheet rinsed once in TTBS and transferred to a straight-sided bottle of an appropriate size, generally a 250ml specimen bottle (Sterilin). The sheets were incubated with primary antibodies in 10ml TTBS, 1% low fat milk powder, at room temperature on a Spiramix 5 roller mixer (Denley) overnight. For this step, antisera were used at a dilution of 1:200 - 1:1500 or affinity-purified antibodies at a concentration of 2-4μg/ml. After removal of the primary antibody solution the nitrocellulose was washed 3x with TTBS for 10min. Bound antibody was then detected by incubation with an iodinated second antibody, [^{125}I] F(ab')$_2$ donkey anti-rabbit IgG (3.7MBq/ml, Amersham), at 9.25 Bq/ml (0.25μCi/ml) in 10ml TTBS, 1% low fat milk powder, for 2hr. Excess second antibody was then removed and the nitrocellulose washed 3x 10min with TTBS, before air drying. Dried nitrocellulose was mounted on filter paper, wrapped in Saran-wrap, and exposed to a sheet of Kodak X-Omat S film, in an autoradiography cassette with a single Hi-speed X intensifying screen at -70^{o}C for

between 12hr and 4 weeks. Films were developed in the X-ray and Accident & Emergency Depts. of the Royal Free Hospital.

2.7 RNA analysis

2.7.1 Plasmid DNA preparation

Complementary DNA encoding the human erythrocyte isoform of the glucose transporter was available in a pSP65 vector, designated pSGT (Mueckler & Lodish, 1986) in glycerol deeps of *Escherichia coli* (*E. coli*) strain JM 109. *E. coli* deeps were thawed slowly, plated out onto 1.5% agar in 2TY (1.6% bactotryptone, 1% bacto-yeast extract, 0.5% NaCl, pH7.0), containing 5μg/ml ampicillin, and cultured overnight at 37°C. Single colonies were used to inoculate 5ml aliquots of 2TY containing 2μg/ml ampicillin, which were again incubated overnight at 37°C, with shaking. Unused colonies on the plate were stored at 4°C for up to one month.

Plasmid DNA was isolated from bacterial DNA and proteins by the following procedure. All steps were performed on ice unless otherwise stated. Cells were pelleted briefly in 1.5ml microfuge tubes, the supernatant discarded and the pellet resuspended in 250μl cold GET (50mM glucose, 10mM EDTA, 25mM Tris-HCl, pH8.0) and incubated for 2min. Bacterial lysis was completed by the addition of 250μl 0.2M NaOH/1% SDS, mixing gently and incubating for a further 10min. To precipitate the dissolved protein and SDS, 200μl 3.0M sodium acetate (NaAc), pH4.8 was added and the tubes incubated for 10-60min. The precipitated materials were pelleted by microcentrifugation at top speed for 10min, and the supernatant carefully transferred to a clean tube. To the supernatant was added 900μl 95% ethanol, and after mixing the plasmid DNA allowed to precipitate at -70°C for 20min. The DNA was pelleted at 14,000g and resuspended in 200μl NE (0.3M NaAc,

1mM EDTA, pH7.0) by vortexing, standing at room temperature for 15min and re-vortexing. To extract any remaining protein, 200µl Tris-saturated phenol, pH8.0:chloroform (1:1) was added. The mixture in the tubes was vortexed and microfuged for 5min at 14,000g. The upper aqueous layer was transferred to a clean tube and the organic layer back-extracted with 200µl NE. The DNA was again precipitated with 800µl 95% ethanol at -70°C, collected by microfuging for 2min and the pellet washed carefully with ethanol. After draining for 5min at room temperature, the DNA was dissolved in 50µl TE (10mM Tris-HCl, 1mM EDTA, pH8.0) and stored at -20°C.

2.7.2 Agarose gel electrophoresis of DNA

All reagents were molecular biology grade. DNA was electrophoresed on both standard agarose and low melting point agarose (LMPA) gels as necessary.

For standard 1.6% agarose gels, 0.8g agarose was dissolved with heating in 50ml TAE (40mM Tris-acetate, 1mM EDTA) and cooled to hand-hot, following which 2.5µl 10mg/ml Ethidium Bromide (EtBr) was added, mixed and the gel poured into an LKB 2013 Miniphor horizontal gel electrophoresis 50ml casting tray. Loading buffer (40% (w/v) sucrose, 0.25% (w/v) bromophenol blue) was added to the DNA samples in a ratio of 1:4. The gel was transferred to the running tank containing TAE, and the whole sample loaded and electrophoresed at 60V for 1-2hr using a GPS 200/400 Gene Power supply (Pharmacia). DNA was visualised by U.V. light using an LKB 2011 Macrovue Transilluminator, and photographed with Polaroid CU-5 land camera and hood, using Kodak 667 film.

For preparative LMPA gels, 0.8% agarose (SeaPlaque) was dissolved with heating in TAE, cooled, and EtBr added to 1µg/ml. The gel was poured and transferred to

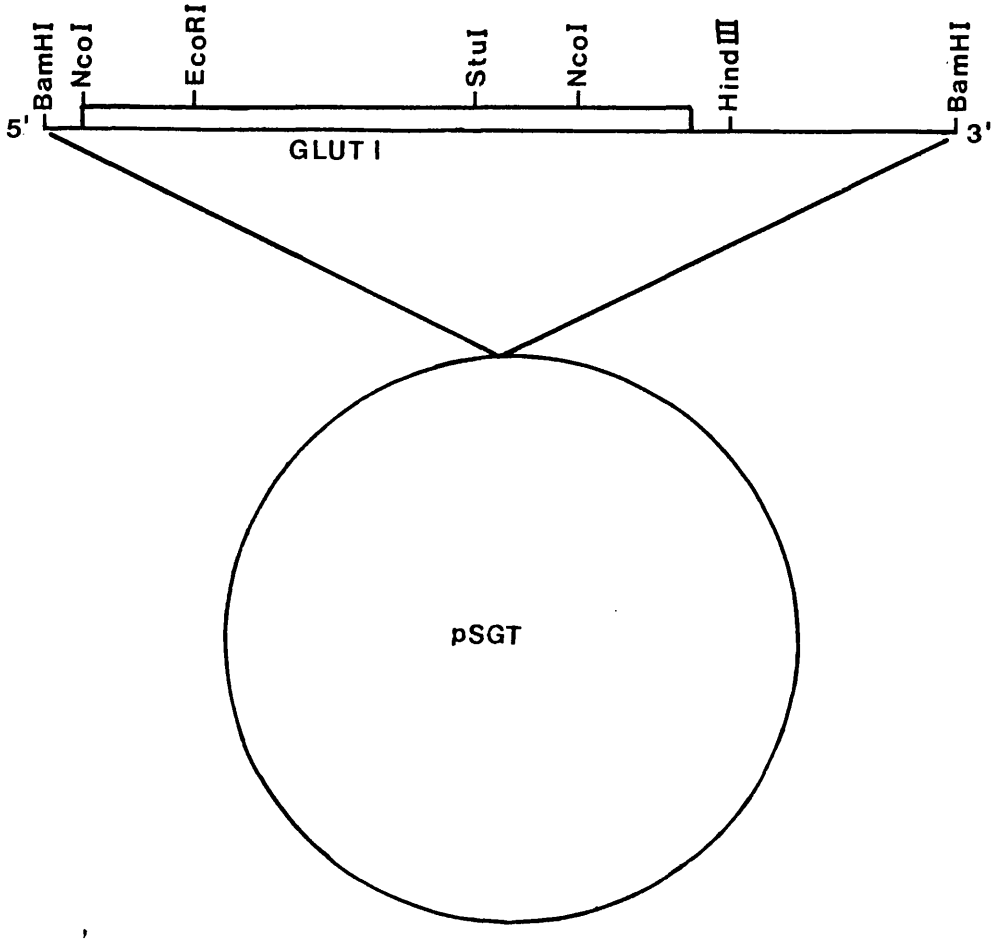
4°C, to prevent cracking upon setting. DNA samples were prepared as above, loaded onto the gel and run at 50V, 4°C. DNA was visualised as above.

2.7.3 Preparation and [^{32}P] labelling of cDNA probes

To prepare a [^{32}P]-labelled cDNA probe to the human erythrocyte/HepG2 glucose transporter the regions of the pSGT insert corresponding to the BamHI fragment and the HindIII-BamHI fragment, shown in Fig. 2.1, were both used, better labelling being obtained with the probe comprising the entire BamHI fragment.

For a double digestion using HindIII and BamHI, plasmid DNA (approximately 100ng) in 180µl TE was digested with HindIII (8µl) and ribonuclease (4µl) in Buffer 4 (Nbl Restriction enzyme kit) which contains 60mM NaCl made up to a total volume of 250µl with water. The digestion was followed by agarose electrophoresis of 10µl aliquots. When digestion appeared to be complete (approx 2hr), 1M NaCl (10µl) was added to the digestion mixture, to give a final concentration of 100mM NaCl (the optimum saline concentration for BamHI activity), together with 8µl BamHI and a further 3µl ribonuclease. The digestion was then continued until complete, followed by agarose electrophoresis as before. The restriction fragments were separated using LMPA electrophoresis, and the fragment of interest (2.0kB fragment) carefully cut from the gel and stored at -20°C. DNA was purified from the LMPA using the Geneclean kit (BIO 101, Inc., La Jolla, CA, U.S.A). Briefly, agarose was melted at 45°C in 3x volumes NaI, then incubated with the GLASSMILK silica beads for 5-10min until the DNA had bound. The beads were pelleted in a microfuge tube, and washed 3x with NEW WASH solution. The DNA was eluted by resuspending the pellet in a small volume of H_2O and heating to 45°C for 5min before pelleting the beads again and removing the DNA-containing

Fig. 2.1 Restriction map of pSGT GLUT1 insert



The restriction map of the intact human erythrocyte GLUT1 cDNA insert within the pSGT plasmid. The GLUT1 sequence is ligated into the polylinker cloning site of pSP65 (Mueckler & Lodish, 1986).

supernatant.

The [^{32}P]-labelled cDNA probe was produced using random hexamer primers, supplied with a kit from U.S.B. (United States Biochem. Corp., Cleveland, U.S.A.). A volume of DNA corresponding to 25ng was made up to 9µl with H_2O and incubated for 1hr at 37°C with 3µl dNTP mix (dGTP:dATP:dTTP (1:1:1)), 2µl reaction buffer, 5µl [α-^{32}P]dCTP (Amersham, 3000µCi/ml) and 1µl Klenow enzyme all provided with the kit except the [α-^{32}P]dCTP. After 1hr, the labelled probe was separated from the free [α-^{32}P]dCTP by exclusion chromatography using a Sephadex G10 Stratagene push column and eluting with STE (0.1M NaCl, 10mM Tris-HCL, pH 8.0, 1mM EDTA). Aliquots of the labelled probe (1µl) were used for scintillation counting to check the efficiency of the labelling reaction and the remainder stored at -20°C.

2.7.4 RNA preparation

Total RNA was prepared by modification of the methods of Glisin *et al.* (1974) and Chirgwin *et al.* (1979). Frozen tissue was ground in liquid N_2 using a pestle and mortar and thawed into 50mM Tris-HCl, pH7.6 containing 3.96M guanidinium isothiocyanate, 10mM EDTA, 2% sodium lauryl sarcosinate and 1% ß-mercaptoethanol (GTC). Alternatively, cell pellets were thawed directly into GTC. Cells or tissue were then homogenised by repeated passage through 19g, followed by 23g, needles to shear the cellular DNA. Homogenate (1.0ml) was layered over 0.8ml 5.7M CsCl, 0.1M EDTA, pH7.5 in 2ml cellulose propionate Beckman ultracentrifuge tubes, and centrifuged for 3hr at 50,000rpm at 20°C using a Beckman TL-100 swing-out rotor. The supernatant was removed, the pellet resuspended in 100µl H_2O and transferred to a microfuge tube. The volume was

made up to 500µl and an equal volume of chloroform:butan-1-ol (1:1) added and vortexed, to extract any remaining protein. The aqueous layer was removed and the organic layer back-extracted with a further 500µl water. RNA was precipitated from the aqueous layers with 0.2x volumes 3M NaAc, pH5.3 and 2x volumes 95% ethanol, overnight at -70°C. The precipitate was collected by microfugation at top speed for 10min. RNA pellets were freeze-dried for 30min, and dissolved in 30-100µl double-distilled, deionised water. The absorbance was measured at 260nm and 280nm. A ratio of absorbance at these wavelengths (260/280) of 1.8-2.0 indicated a low degree of protein contamination. Given that:-

A_{260} 1 = 40mg RNA/ml (for a path length of 1cm)

the concentration of RNA in the samples was estimated. RNA was stored at -70°C.

2.7.5 Formaldehyde/agarose gel electrophoresis and Northern blotting

RNA was electrophoresed on 1.2% agarose/formaldehyde gels. Agarose (0.5g) was dissolved with heating in 38ml 1xMOPS buffer (10xMOPS = 200mM sodium 3-(N-morpholino)propane sulphonic acid (MOPS) with 50mM NaAc, 10mM EDTA, pH7.0) and cooled to hand-hot. Formaldehyde (37% solution) was added to 5.4%, together with 2.6µl 10mg/ml EtBr, the solution mixed and poured into a 50ml LKB horizontal gel casting apparatus, in a fume hood, and left to set.

RNA samples (5-35µg RNA) were made up to 2.5µl with H_2O and 7.5µl of denaturing buffer (50% formamide, 7% formaldehyde (37% solution), in 1xSSC:-20xSSC=3M NaCl, 3.3M sodium citrate) added. The samples were heated to 65°C for 5min to denature the RNA, and 6µl of the dye mix (50% glycerol, 1mM EDTA, 0.2% bromophenol blue, pH8.0) added after cooling. Following a brief microcentrifugation, the samples were loaded into the wells of the formaldehyde

gel and run in 1xMOPS at 60V until the dye front had moved 2/3 of the way down the gel.

The gels were photographed under U.V. light, then washed for 2x 20min in 10xSSC to remove the formaldehyde. The RNA was transferred to Hybond N nylon membrane (Amersham) by capillary action overnight in a 10xSSC buffer system, using the set-up shown in Fig. 2.2. The following day the Northern blot was air-dried and irradiated with U.V. light for 4min, using the transilluminator, to cross-link the RNA to the nylon membrane.

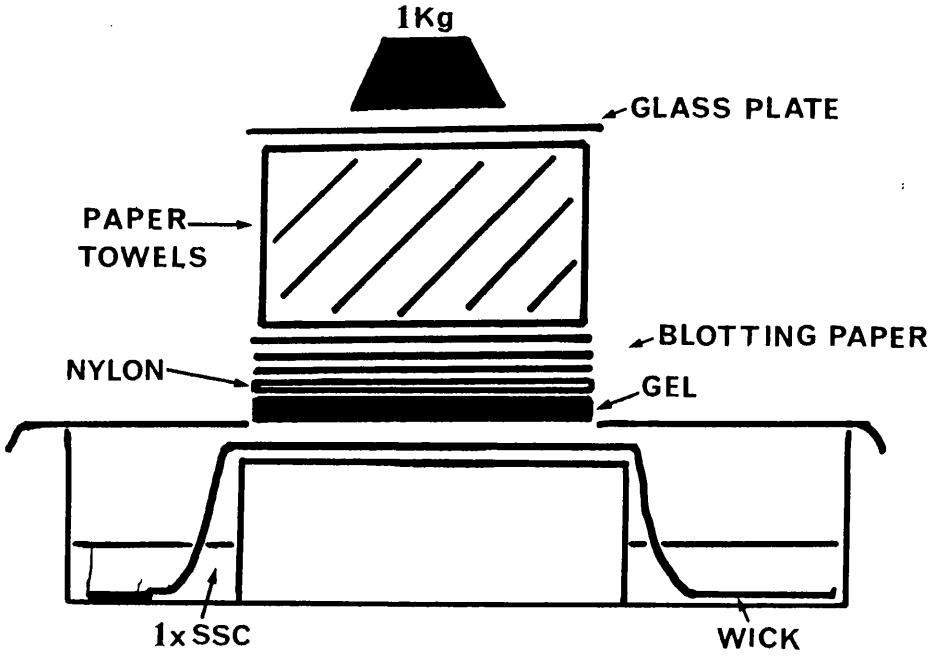
2.7.6 Slot blotting

Aliquots of RNA (1µl = 5µg) were heated to 65°C for 5min with 40µl 50% formamide, 20% formaldehyde (37% solution) in 1xSSC and made up to 500µl with 20XSSC. RNA was then applied to Hybond N using a standard slot blotting apparatus. When all the buffer had been sucked through, the membrane was removed and air-dried, then irradiated under U.V. light for 4min using the transilluminator, to cross-link the RNA to the membrane. Slot blots were stored at room temperature.

2.7.7 Hybridisation

The procedure for hybridisation to both slot and Northern blots was the same. Membranes were washed for 2 x20min with 2xSSPE (20xSSPE=3M NaCl, 177mM NaH_2PO_4, 20mM EDTA) at 42°C, then prehybridised using 5ml 50% formamide, 5xSSPE, 0.2% SDS, 5xDenhardt's (50x Denhardt's = 1% Ficoll 400, 1% polyvinylpyrrolidone (PVP), 1% BSA) and 200µg/ml sheared salmon sperm DNA at 42°C for 2hr with constant rolling in a hybridisation oven (Hybaid). After 2hr, [α-

Fig. 2.2 Northern blotting RNA onto nylon by capillary action



RNA separated on denaturing formaldehyde/agarose gels can be transferred to nylon membranes by capillary action using the apparatus shown above. The transfer stack stands in 1x SSC, and consists of a wick of blotting paper, the gel, the nylon (Hybond N), three sheets of blotting paper and a stack of absorbent paper towels. A glass plate is placed on top of the paper towels and a weight (approximately 1kg) is placed on top of this. The buffer passes through the wick, drawn by capillary action from the dry paper towels, transferring the RNA to the nylon sheet. The stack is left overnight (approximately 16hr) and the following morning the nylon is removed, dried and the RNA cross-linked by irradiation over U.V. light for 4min.

^{32}P] cDNA was added to the prehybridisation buffer to give 10^6dpm/ml, and the incubation continued overnight at 42°C. The following day the hybridisation medium was removed and the blot washed for 2x 20min with 0.2% SSC/0.2%SDS at 50°C. Labelled blots were wrapped in saranwrap and exposed to Kodak X-Omat S autoradiographic film for between 12hr and 6 weeks.

Hybridised blots were stripped of labelled probes by placing into boiling 2% SDS, and incubating with shaking until the SDS reached room temperature. The 2% SDS was then discarded and any excess rinsed away using 0.2% SSC. Stripped blots were drained of excess liquid, autoradiographed to check for stripping efficiency, and stored moist at 4°C.

2.8 DNA assay

DNA concentrations were determined by the method of Labarca & Paigen (1980). A 1mg/ml stock solution of the fluorescent reagent, bisbenzimide (Sigma Hoescht reagent H33258), stored at -20°C, was diluted to 2µg/ml in assay buffer (500mM NaCl, 5mM sodium phosphate, pH7.4) immediately preceding the assay. Samples of cell homogenate or calf thymus DNA standards (1-10µg DNA), in a volume of less than 200µl, were made up to 2ml with assay buffer, then 2ml fluor added and mixed by vortexing. After incubation at room temperature for 15-30 min, sample fluorescence was measured using a Perkin Elmer fluorimeter at an excitation wavelength of 356nm and emission of 445nm, and the concentration of DNA determined by comparison to the fluorescence of a calibration curve prepared using samples containing 0, 1, 2, 4, 6, 8 and 10µg calf thymus DNA.

2.9 Protein assay

Protein concentrations were determined by the method of Lowry *et al.* (1951). The solutions used for the Lowry were; Solution A : 2% (w/v) sodium carbonate in 0.1M sodium hydroxide; Solution B : 1% (w/v) copper sulphate (pentahydrate) in water; Solution B^* : 2% (w/v) sodium/potassium tartrate in water. The working solutions were; Solution C : 40ml solution A containing 0.5% (w/v) SDS, to which 0.4ml of solutions B and B^* had been sequentially added; Solution D : 1:1 dilution of Folin's phenol reagent with water. 1ml of Solution C was added to 200µl of unknown sample or protein standards (0-50µg protein), vortexed and incubated at room temperature for 20min. Solution D (100µl) was added to each sample and immediately vortexed, and incubated for 30min. The absorbances of the resulting solutions were read at 750nm, and the concentration of the unknown protein samples determined by comparison with the absorbances of a calibration curve prepared using 0, 5, 10, 20, 30, 40 and 50µg of BSA.

2.10 Thiol assay

Sulphydryl groups were assayed by an adaptation of the method of Ellman (1959), using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The samples (containing approximately 50nmol thiol groups) or water blanks, were added to 67µl 10mM DTNB and 600µl 100mM sodium phosphate buffer, pH7.5 in glass tubes, and the volumes made up to 1ml with water. The tubes were vortexed and incubated at room temperature for 10min, then the absorbance measured at 412nm. The concentration of free thiol groups could then be calculated, given the molar extinction coefficient of the thionitrobenzoate anion of $14,100 M^{-1} cm^{-1}$.

CHAPTER 3

CHARACTERISATION OF ANTIBODIES TO THREE GLUCOSE TRANSPORTER ISOFORMS

3.1 Introduction

Numerous studies have shown that it is possible to raise antibodies against short, synthetic peptides if these are coupled to large, antigenic carrier proteins such as keyhole limpet haemocyanin (KLH) or ovalbumin before use as antigens. When this approach was applied to the human erythrocyte glucose transporter (GLUT1), antibodies raised against peptides corresponding to several hydrophilic, extramembranous regions of the sequence were found to recognise not only the peptides themselves, but also the intact protein on Western blots (Davies *et al.*, 1987; Haspel *et al.*, 1988). One of the most immunogenic peptides was that corresponding to the *C*-terminal region of the protein. This region of the sequence is one of the least conserved between different mammalian glucose transport isoforms, as illustrated in Fig. 3.1. Antibodies against this region of each of the transporters can therefore be used as specific probes with which to detect the different isoforms on Western blots. Furthermore, antibodies against this region of the sequence have been shown to also recognise the native conformations of the transporters (Davies *et al.*, 1990) and are therefore useful for both immunoprecipitation of transporters and for immunocytochemical investigations. Antibodies raised against synthetic peptides from GLUT1-5 were available in the laboratory, or raised specifically for the purpose of this study, as described below.

Fig. 3.1 *C*-terminal peptides of the glucose transporter isoforms

Species	Isoform	Sequence	Residues	Reference
*Rat	GLUT1	KTPEELFHPLGADSQV	477-492	(1)
Rat	GLUT2	RKATVQMEFLGSSETV	507-524	(2)
		:: : : ::: :::		
Human	GLUT2	PKAAVEMKFLGATETV	505-522	(3)
Human	GLUT3	NSIEPAKETTTNV	484-496	(4)
*Rat	GLUT4	VKPSTELEYLGPDEND	494-509	(5,6)
Human	GLUT5	YPEKEELKELPPVTSEQ	485-501	(7)

* *C*-terminal peptide sequences identical in the rat and human isoforms

The amino-acid sequences of the glucose transporter *C*-terminal peptides used to raise antibodies, obtained from the primary sequences of five mammalian facilitative D-glucose transporter proteins (see references below), are shown. These regions of the transporters are both highly immunogenic and show the greatest sequence divergence between isoforms. Although species variability exists between the human and rat sequences, antibodies raised against the rat GLUT2 peptide will cross-react with the human GLUT2 protein and *vice versa*. Antibodies raised against the human GLUT3 peptide will also cross-react against the rat GLUT3 protein.

References- 1. Birnbaum *et al.* (1986); 2. Thorens *et al.* (1988); 3. Fukumoto *et al.* (1988); 4. Kayano *et al.* (1988); 5. James *et al.* (1989); 6. Charron *et al.* (1989); 7. Kayano *et al.* (1990)

3.2 Peptide synthesis

Synthetic peptides corresponding to the *C*-terminal peptides of GLUT1, GLUT2 and GLUT4 had been synthesized by the N^{α}-fluorenylmethoxy-carbonyl-polyamide solid phase method (Atherton and Sheppard, 1985) using N^{α}-fluorenylmethoxycarbonyl-amino acid pentafluorophenylesters (Fmoc-amino acid OPfp's) or N^{α}-fluorenylmethoxycarbonyl-amino acid oxo-benzotriazine esters (Fmoc-amino acid ODhbt's), coupled to Pepsyn KA resin (Milligen, Bedford, M.A., U.S.A.). An additional cysteine was added to the *N*-terminal of each peptide to facilitate coupling to the carrier protein or to media for affinity-chromatography.

3.3 Preparation of anti-human erythrocyte (GLUT1) glucose transporter antibodies

Site-specific antibodies raised against various peptide sequences within the human erythrocyte/HepG2 and rat brain GLUT1 were provided by Dr. A. Davies. These antisera all recognised a protein of $M_r$ave 55,000 on Western blots of human erythrocyte membranes. The range of antisera provided covered most of the cytoplasmic domain of GLUT1 (shown in Fig. 4.7). As it was known that a number of these site-specific antibodies, raised against the *C*-terminal peptides including the amino-acids 450-467, 460-477 and 477-492, would also bind to the native protein, these antibodies were particularly useful. To ensure specificity antibodies raised against the peptides 450-467, 460-477 and 477-492 were affinity-purified. Antibodies raised against the extreme *C*-terminal residues 477-492 are referred to as anti-*C*-terminal peptide antibodies and were routinely used to immunologically detect GLUT1, unless stated otherwise. The specificity of all of these antisera had been determined by Dr. A. Davies.

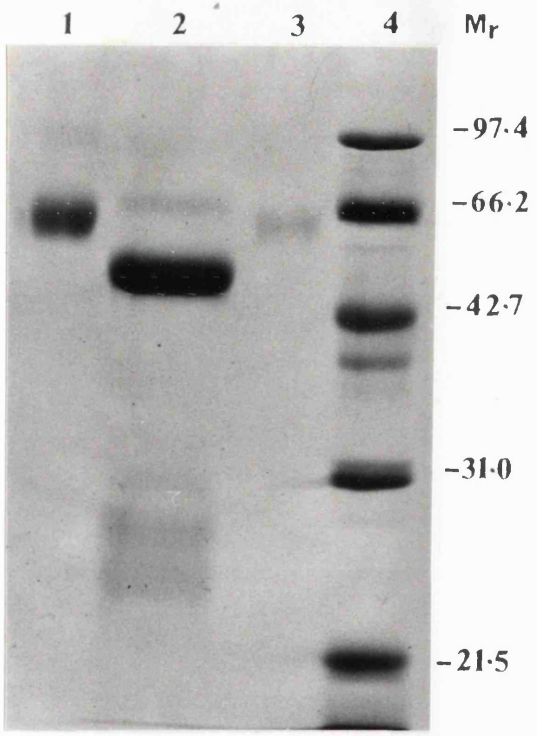
Antibodies raised against the *C*-terminal peptide were affinity-purified as

described in Section 2.1.5.1 by adsorption onto, and elution from, protein-depleted human erythrocyte membranes, and the concentration of the purified antibodies determined using the Lowry assay (Section 2.9). The average yield of affinity-purified antibodies was 500μg protein/ml serum.

To check the degree of contamination of the IgG with other serum proteins, gel samples containing approximately 5μg original serum, eluted IgG and the IgG-depleted serum remaining following the membrane-serum incubation (supernatant), were boiled for 5min then electrophoresed on 12% SDS/polyacrylamide gels. Following electrophoresis proteins were stained using Coomassie blue. It was possible to distinguish the serum IgG from the serum albumin, shown in Fig. 3.2. Contamination of the purified IgG fraction with other proteins was shown to be low, therefore it was assumed that the concentration of protein determined for the IgG fraction was equivalent to the concentration of IgG.

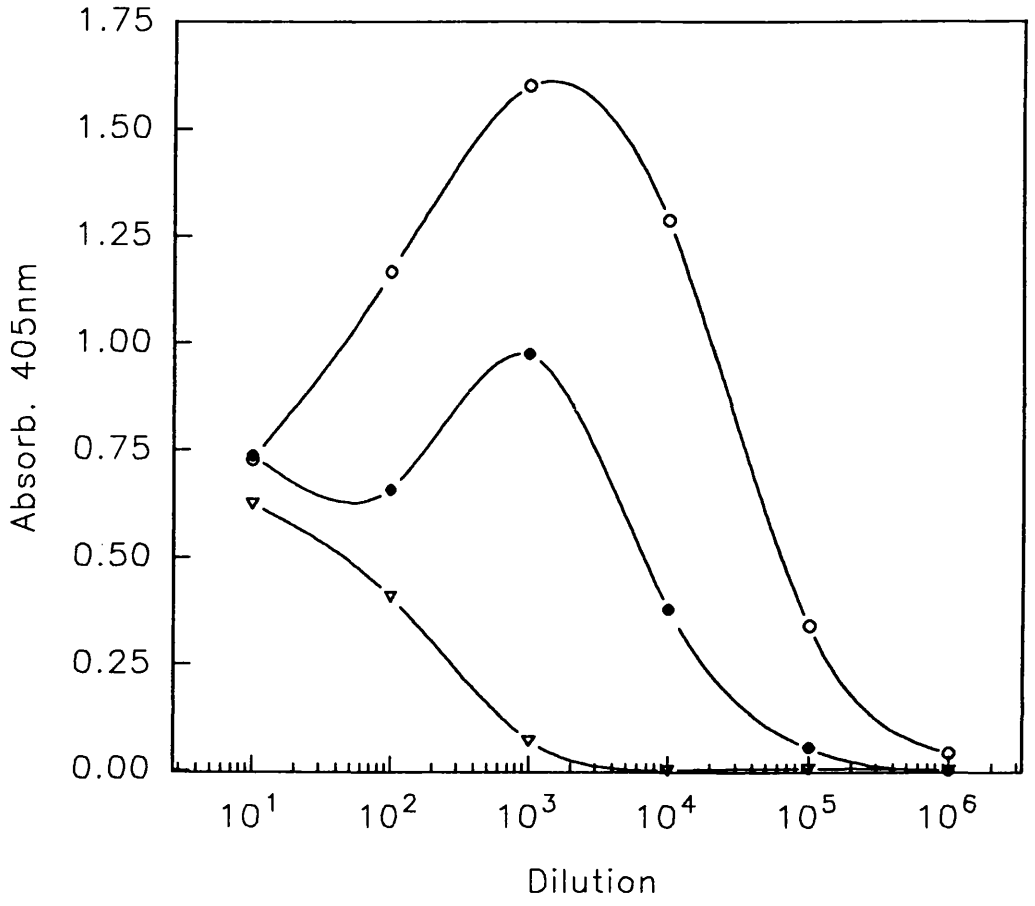
To determine the titre of the affinity-purified antibodies, serial dilutions (usually 10-fold) of the serum, eluted IgG and supernatant were investigated by a standard screening ELISA as described in Section 2.1.4, using both the peptide and the intact purified GLUT1 protein (provided by Dr. A. F. Davies) as antigens. By plotting the absorbance at 405nm against the dilution factor, as shown in Fig. 3.3, the titre (arbitrarily described as dilution at half maximal absorbance) was determined. The results in Fig. 3.3, were obtained from a standard purification of antibodies raised against the extreme *C*-terminal peptide (residues 477-492) of GLUT1, using purified GLUT1 to coat the ELISA plate. The titre of the affinity-purified IgG fraction (1:8,000) was approximately 50% of the serum titre (1:15,000), with the titre of the supernatant only 1:100. The affinity-purified antibodies were approximately one third more dilute compared to the antiserum which together with the loss of lower

Fig. 3.2 SDS/Polyacrylamide gel electrophoresis of affinity-purified anti-GLUT1 antibodies



Samples, containing approximately 5μg protein, of antiserum (1), affinity-purified antibodies (2) or the IgG depleted serum from the affinity purification incubation (3) were prepared as gel samples, boiled for 5min with reductant (DTT) and electrophoresed on a 12% SDS/polyacrylamide gel. The gel was then stained with Coomassie blue, and the proteins visualised. The apparent M_r of the proteins was estimated from the migration of low molecular weight markers shown in lane 4.

Fig. 3.3 Screening ELISA:- GLUT1 anti-C-terminal peptide antibody purification



Antiserum (o), affinity-purified antibodies (•) and the affinity-purification supernatant (▾) were screened for anti-GLUT1 activity by screening ELISA. The ELISA plates were coated with purified GLUT1 protein. The titre of each fraction can be determined from the dilution at half maximal absorbance. The titre of the antiserum was approximately 1:15, 000, of the affinity-purified antibodies 1:8,000 and of the supernatant 1:100.

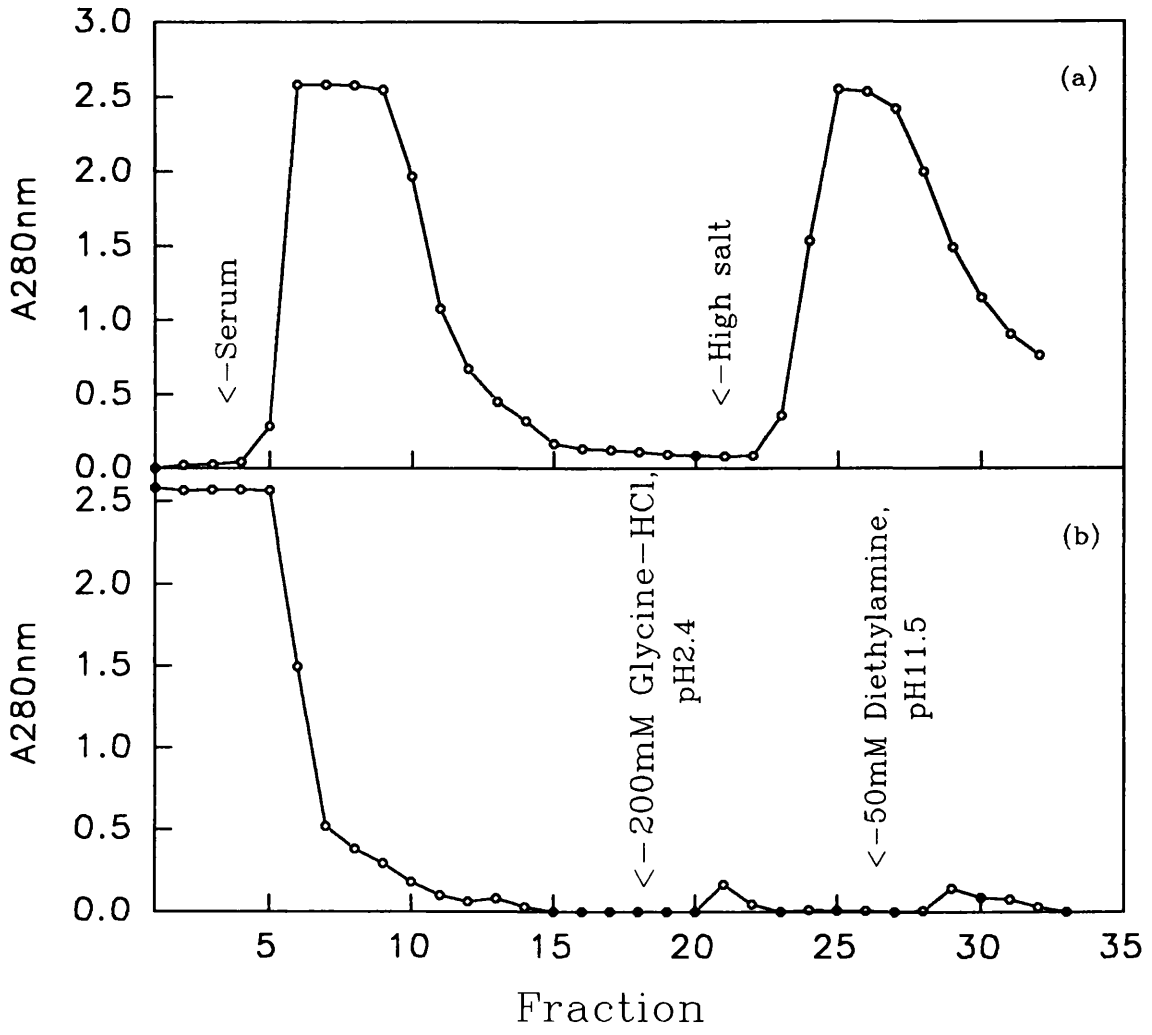
affinity specific IgG during the affinity-purification procedure, probably explains the lower titre. Following affinity-purification of antibodies against regions other than the extreme *C*-terminal, the amount of anti-peptide IgG remaining unbound in the serum following the membrane incubation was often a substantial proportion, suggesting that a smaller fraction of these antibodies bound strongly to the native protein. The fall in absorbance towards the higher concentrations of antibody or antiserum is probably an artefact caused by a strong affinity of the antibodies for the antigen, removing antigen from the plate.

3.4 Preparation of anti-rat liver (GLUT2) glucose transporter antibodies

A peptide corresponding to the *C*-terminal sequence of the rat GLUT2 was provided by Mr. L. Fryer, along with a KHL-GLUT2 peptide conjugate. The conjugate was used to immunise a single New Zealand White rabbit as described in Section 2.1.2, and so to raise an antiserum. The peptide was used to both prepare an affinity column, by conjugation to a Sulpholink matrix, as described in Section 2.1.5.2, and as an antigen for screening ELISA's. The antiserum was found to cross-react with the peptide on ELISA's and to a protein of M_r 66,000 on Western blots of liver membranes (results not shown here, see Section 3.7).

Antibodies were purified from the antiserum using a two step affinity chromatography method, performed as described in Section 2.1.5.2. Serum albumin and proteases were first removed from the antiserum by chromatography on a CM Affi-gel blue column. The IgG eluted first as a broad peak, and the serum albumin and proteases were then eluted using a high salt wash, shown in Fig. 3.4(a). The peak fractions containing IgG were pooled and applied to the affinity column. Following extensive washing to remove non-specifically bound IgG, the specifically

Fig. 3.4 Purification of anti-GLUT2 antibodies



Antiserum raised against the C-terminal peptide of GLUT2 was depleted of serum albumin and serum proteases by chromatography through a CM Affi-gel blue column (a). The IgG-enriched fraction that eluted first was used for the affinity-purification, following which the albumin was eluted from the column using a high salt wash. The specific IgG in the IgG-enriched fraction was affinity-purified on a peptide column of the GLUT2 C-terminal peptide (b), eluting first with 200mM glycine-HCl, pH2.4, then with 50mM diethylamine, pH11.5.

bound, anti-peptide IgG was eluted using first 200mM glycine-HCl, pH2.4, then 50mM diethylamine, pH11.5, as shown in Fig. 3.4(b). Following dialysis the antibodies were collected, the protein concentration determined, and the specificity confirmed as described below.

The titre of the antibodies was determined by screening ELISA's as shown in Fig. 3.3 for GLUT1. The two elution procedures were found to vary in efficiency between different sera, therefore both procedures were always used for the elution. The titre of specific IgG was found to be ≈1:600 compared to 1:4000 for the antiserum and 1:250 for the non-specific IgG. The yield of specific IgG was approximately 130μg/ml serum.

3.5 Antibodies against the adipose/muscle-type (GLUT4), human brain-type (GLUT3), human small intestine-type(GLUT5) and rat liver endoplasmic reticular-type (GLUT7) glucose transporters

Antiserum and affinity-purified antibodies raised against the *C*-terminal peptide of the rat adipocyte/muscle-type glucose transporter (GLUT4), shown in Fig. 3.1, were provided by Mr. L. Fryer. The antibodies had been affinity-purified on peptide columns, and screening ELISA showed that both the antiserum and the purified antibodies had a strong reactivity to the peptide as expected. Antisera raised against the *C*-terminal peptide of human GLUT3 or human GLUT5 were provided by Dr. G. Gould, and antiserum raised against the GLUT7 protein was provided by Dr. A. Burchell.

3.6 Specificity of anti-peptide antisera - ELISA assays

The antisera raised against the GLUT1, GLUT2 and GLUT4 isoforms of the

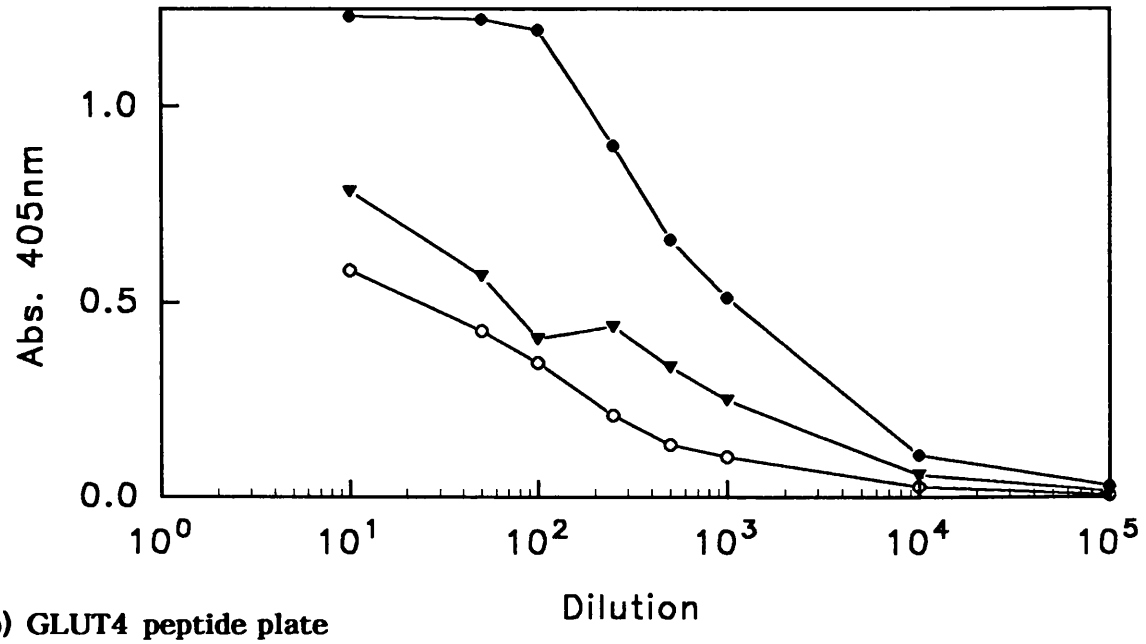
glucose transporter had been shown to react to their respective peptides, indicating that they contained peptide specific antibodies. Furthermore, antibodies raised against GLUT1 and GLUT2 were affinity-purified, and antibodies against GLUT4 were available in an affinity-purified form. However the degree of cross-reactivity between these antisera, raised against specific peptides was not known, therefore this was checked using both screening ELISAs and Western blotting, described in the following section.

To assess the degree of cross-reactivity between the antisera, ELISA plates were coated with the *C*-terminal peptides of either GLUT2 or GLUT4. Each plate was then used to screen the antisera raised against the *C*-terminal peptides of GLUT1, GLUT2 or GLUT4, to determine to what extent the antibodies cross-reacted between the peptides. The screening ELISAs were performed as described in Section 2.1.4.

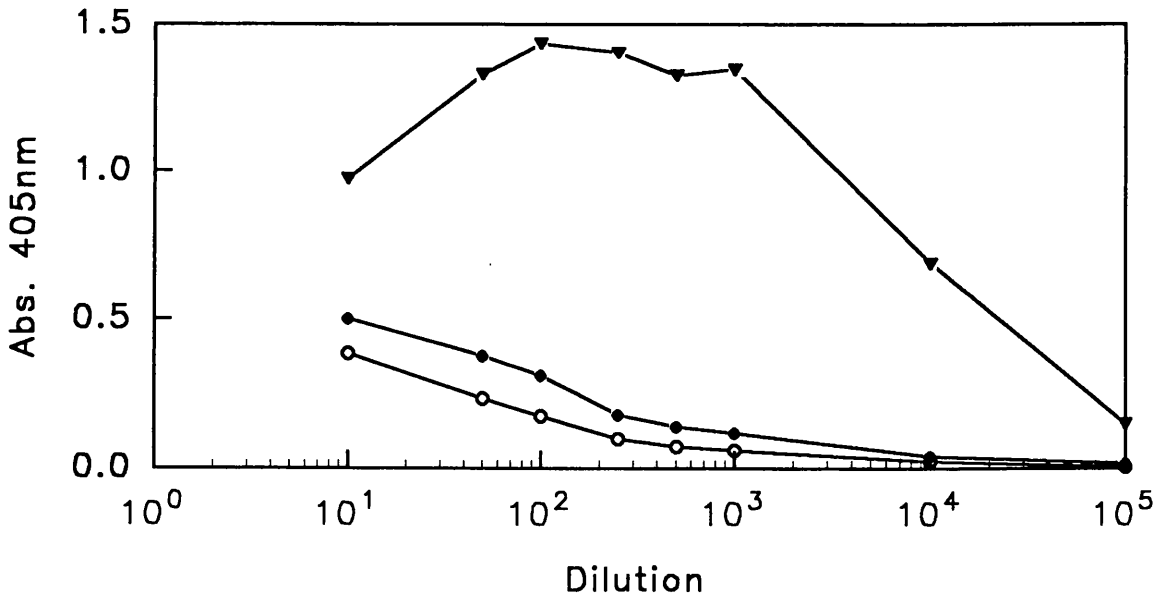
For the GLUT2 peptide coated plate, Fig. 3.5(a), the highest titre of cross-reactive antibodies were seen in the anti-GLUT2 antisera. However, there was also a high cross-reactivity between the antibodies raised against GLUT1 or GLUT4 and the GLUT2 peptide, although the titre of the GLUT2 antiserum was 100-fold higher than that of the GLUT1 or GLUT4 antiserum. On the ELISA plate coated with the GLUT4 peptide, the highest titre of cross-reactive antibodies was seen for the anti-GLUT4 antiserum, Fig. 3.5(b). On this plate only very low levels of GLUT4 peptide cross-reactive antibodies were present in the anti-GLUT1 or anti-GLUT2 antiserum. The titre of the GLUT4 antiserum against the GLUT4 peptide was approximately 1000-fold higher than the titre of the non-specific cross-reactivity of the GLUT1 and GLUT2 antisera. The GLUT1 antiserum showed the lowest degree of cross-reactivity to either the GLUT2 or GLUT4 peptides.

Fig. 3.5 Specificity of antisera :- ELISA

a) GLUT2 peptide plate



b) GLUT4 peptide plate



Antisera raised against the C-terminal peptides of GLUT1 (o), GLUT2 (•) or GLUT4 (▾) were screened by ELISA for cross-reactivity to the peptides of GLUT2 (a) or GLUT4 (b). The standard screening ELISA procedure was used as described in Section 2.1.4.

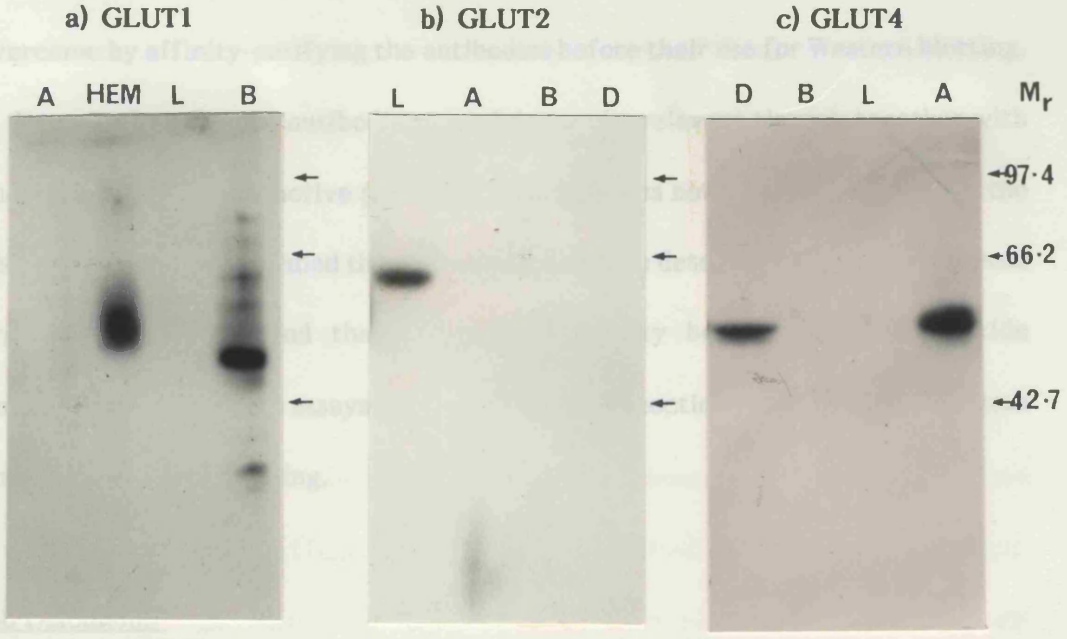
3.7 Specificity of anti-peptide antibodies - Western blotting

The results described in Section 3.6 had shown that while the antisera appeared to have a much greater affinity for the peptide sequence to which they were raised than for other peptides, there was still some degree of cross-reactivity (apparent on ELISAs). As a final check of the antibody specificity, and to ensure that this low degree of cross-reactivity did not affect the detection of the glucose transporter isoforms in mammalian tissues, Western blotting of various tissues and membranes was used.

Samples of various rat and human tissue membranes detailed in the legend to Fig. 3.6 were used to prepare gel samples and electrophoresed on 12% SDS/polyacrylamide gels, as described in Section 2.5. Following transfer to nitrocellulose, the glucose transporters were detected using affinity-purified antibodies raised against the *C*-terminal peptides of GLUT1, GLUT2 or GLUT4, followed by an iodinated second antibody and autoradiography, as described in Section 2.6. Human erythrocyte membranes express only the GLUT1 isoform; rat brain expresses both GLUT1 and GLUT3; rat liver expresses predominantly GLUT2; and rat adipocytes and muscle express predominantly GLUT4 although both these tissues also express lower levels of GLUT1 (review; Gould & Bell, 1990). The rat adipocyte membrane fractions were a gift from Mr. L. Fryer.

The antibodies raised against GLUT1 detected high levels of a protein in human erythrocytes and rat brain, but only low levels in adipose tissue and in liver homogenate (shown in Fig. 3.6(a)), mirroring the reported distribution of the GLUT1 protein (Table 1.1). The anti-GLUT2 antibodies detected glucose transporters at high levels in liver homogenate but no other tissue (Fig. 3.6(b)). The anti-GLUT4 antibodies detected glucose transporters in insulin-sensitive rat adipose tissue and

Fig. 3.6 Specificity of the anti-peptide antibodies:- Western blotting



Various membranes were prepared as gel samples and electrophoresed on 12% SDS/polyacrylamide gels, then the glucose transporters detected by Western blotting as described in Sections 2.5 and 2.6, using affinity-purified antibodies raised against the *C*-terminal peptides of GLUT1 (a), GLUT2 (b) or GLUT4 (c), at 2μg/ml. Samples contained protein-depleted human erythrocyte membranes (HEM: 1μg), rat adipose homogenate (A: (a)25μg, (b)50μg, (c)50μg), rat liver homogenate (L: (a)100μg, (b)50μg, (c)50μg), rat brain homogenate (B: (a)100μg, (b) 50μg, (c)50μg) or rat diaphragm homogenate (D: 50μg).

rat diaphragm but not in human erythrocytes or rat liver (Fig. 3.6(c)). A high level of non-specific background staining was often seen when the antiserum raised against GLUT2 or GLUT4 was used. This contamination could be completely overcome by affinity-purifying the antibodies before their use for Western blotting.

The specificity of the antibodies reactivity to the relevant tissues, together with the absence of cross-reactive proteins in those tissues not predicted to express the relevant proteins, confirmed that the antibodies were detecting the correct glucose transporter isoform, and that the cross-reactivity between the anti-peptide antibodies on the ELISA assays was not affecting detection of glucose transporters following Western blotting.

3.8 Discussion

The antisera raised against the mammalian facilitative D-glucose transport proteins were either prepared in the laboratories of Dr. S.A. Baldwin or kindly donated by other laboratories. Those antisera raised here were more abundant and so characterised in more detail, the specificity of the remaining antisera was assessed by Western blotting alone.

The ELISAs demonstrated that the titre of the antiserum specifically reacting to the peptide against which it was raised was higher than the cross-reactivity to other peptides. It was not clear why the antiserum raised against the GLUT4 (or GLUT1) peptide cross-reacted relatively strongly with the GLUT2 peptide on ELISAs, whereas the antiserum raised against the GLUT2 peptide did not cross-react strongly with the GLUT4 peptide. This could be due to a higher non-specific binding of antibodies within the GLUT4 antiserum, to the GLUT2 peptide or the blocking agent (milk powder). Alternatively, it could reflect a low reactivity of the

GLUT2 antiserum against the GLUT2 peptide, making the level of cross-reactivity of the GLUT1 and GLUT4 antiserum appear high. However, as the cross-reactivity did not affect the specificity of the affinity-purified antibodies on Western blots (Fig. 3.6) the reason for the high cross-reactivity to GLUT2 peptide on ELISAs was not pursued. Antibodies raised against the C-terminal peptides of GLUT1, GLUT2 and GLUT4 were found to detect the protein of the correct apparent M_r in the relevant tissues following Western blotting with no cross-reactivity to other tissues. Further evidence that the protein detected by the anti-GLUT4 antibodies was the insulin-regulatable glucose transporter was gained from the apparent translocation of GLUT4 from the intracellular low density microsomes to the plasma membrane upon insulin-stimulation (James *et al.*, 1989a), (results not shown here, see Fig. 4.2). The results of the Western blotting indicated that although there was cross-reactivity between the antisera towards the peptides on ELISAs, this did not occur to any great extent when Western blotting was used. The antibodies can therefore be used for the detection of specific glucose transporter isoforms in different tissues by Western blotting with confidence.

CHAPTER 4

IDENTIFICATION OF THE GLUCOSE TRANSPORTER(S) IN LACTATING RAT MAMMARY GLAND

4.1 Introduction

In rats the peak of lactational activity, with respect to milk yield, is between days 10-14 post-partum, when the mammary secretory epithelial cell is the predominant cell type in the mammary gland. It was anticipated that glucose transporters involved in the supply of D-glucose to the mammary secretory epithelial cells during lactation, would be most readily detectable at this mid-stage of lactation. Consequently, the following investigations of mammary glucose transporter protein and mRNA expression were made using mammary glands from dams at peak lactation, with a litter size adjusted to 8 pups to minimise any effects of litter size.

Within the mammary secretory epithelial cell there are two membranes across which D-glucose has to move in order to be utilised for lactose synthesis. All substrates for milk production must at some point cross the basal plasma membrane of the epithelial cells, as tight junctions between these cells prevent the passage of substances directly into the milk. In both rat (Threadgold *et al.*, 1982) and mouse (Prosser & Topper, 1986) transport of glucose across the mammary plasma membrane has been shown to occur via a facilitative D-glucose transport system. The mammary plasma membrane system had been identified in preliminary studies of rat as the GLUT1 isoform (Madon *et al.*, 1989). Glucose destined for lactose synthesis must also cross the membranes of the Golgi apparatus into the lumen, as this is the site of lactose synthetase. Madon *et al.* (1990) have also demonstrated

the presence of GLUT1 in a mammary intracellular membrane fraction, enriched in Golgi marker enzymes. This led to the speculation that there was an intracellular glucose transporter facilitating D-glucose entry into the Golgi lumen for use in lactose synthesis. However, results of other workers (White *et al.*, 1981) had indicated that glucose transport into the Golgi lumen occurred through a non-specific proteinaceous pore.

The following experiments were designed to identify and characterise the specific isoform(s) of the glucose transporter present in mammary gland and to investigate their intracellular distribution. Antibodies raised against six of the facilitative D-glucose transporter isoforms (GLUT1-5, review Bell *et al.*, 1990; GLUT7, Waddell *et al.*, 1992) were available, and these antibodies were used to identify the mammary gland glucose transporter(s) by Western blotting. The glucose transporters identified in this fashion were then further characterised by immunoprecipitation of cytochalasin B photolabelled protein, by examining the effect of glycosidase treatment, and by Northern blotting. Finally, the degree of peptide sequence similarity between the mammary glucose transporters and the previously characterised rat GLUT1 glucose transporter isoform was assessed by Western blotting using a panel of antibodies directed against different regions of the GLUT1 sequence.

4.2 Identification of glucose transporter isoform(s) in whole mammary gland homogenate by Western blotting

Six isoforms of the facilitative D-glucose transporters have been identified in various mammalian cells to date (Table 1.1) and one pseudogene. In order to establish which of the facilitative D-glucose transporter isoforms were present in

the mid-lactating rat mammary gland, mammary homogenate was investigated by Western blotting using antibodies raised against the *C*-terminal peptides of five glucose transporter isoforms (GLUT 1-5) and against one purified glucose transporter (GLUT7). The preparation and characterisation of antibodies against GLUT1, GLUT2 and GLUT4 are detailed in Chapter 3. Antisera to three other glucose transporter isoforms, those of the human brain (GLUT3), human small intestine (GLUT5) and rat liver endoplasmic reticulum (GLUT7) were kindly provided by Dr. G. Gould (GLUT3 and GLUT5) and Dr. A. Burchell (GLUT7).

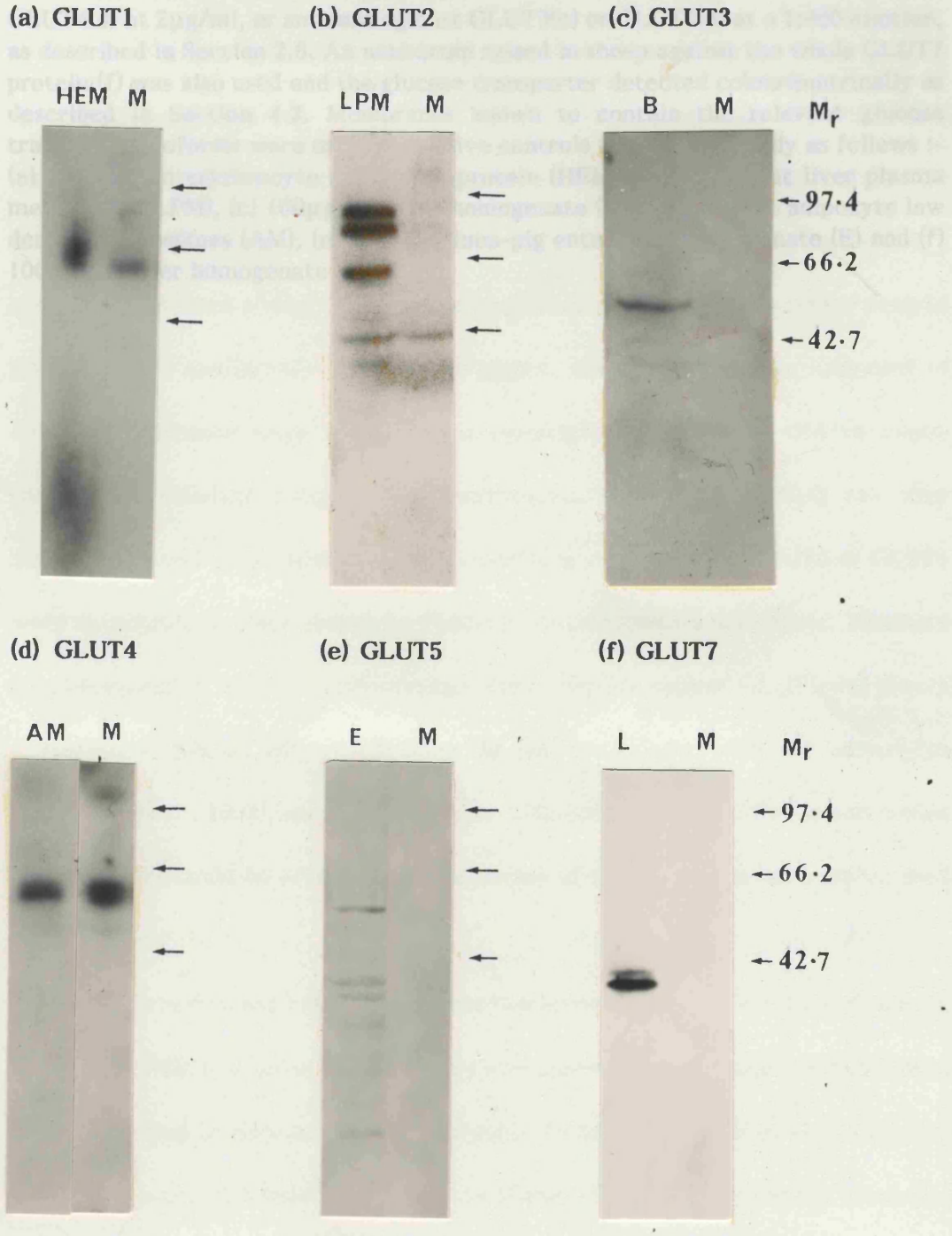
Lactating rat mammary gland was homogenised as described in Section 2.3.1, and the homogenate harvested. Glucose transporters were identified in samples of mammary homogenate following Western blotting as described in Section 2.6, using antibodies raised against their respective *C*-terminal peptides, an iodinated second antibody and autoradiography, with the exception of the antiserum raised in sheep against the purified GLUT7 protein. GLUT7 was detected using a horseradish peroxidase (HRP)-conjugated anti-sheep IgG and the colour development reagent 4-chloronapthol (Sigma colour development reagent) as follows:-

Western blots were incubated in primary antibody (sheep anti-GLUT7 antiserum at a dilution of 1:250 in TTBS/1% low fat milk powder) overnight. Following incubation with the first antibody, blots were washed for 3x 10min with TTBS, then incubated for 2hr with HRP-conjugated goat anti-sheep IgG (Amersham) at a dilution of 1:200 in TTBS/1% low fat milk powder. Following further washing for 3x 10min with TTBS, the blots were incubated with 50ml of the colour development solution (15mg 4-chloronapthol dissolved in 10ml methanol, and added to 40ml TTBS containing 8µl H_2O_2) until a purple colour was detected on the blots, after which the excess reagent was washed off with several changes of distilled H_2O.

Where available, pre-immune serum from the rabbit used to raise the antibodies, or non-specific rabbit IgG, were used as controls for non-specific binding. Tissue homogenate or isolated membranes known to contain the glucose transporter isoform of interest were used on the blots as positive controls for antibody immunoreactivity. Human erythrocyte membranes were used for GLUT1, crude rat liver plasma membranes for GLUT2, rat brain homogenate for GLUT3, rat adipocyte low density microsomes for GLUT4, guinea-pig enterocyte homogenate for GLUT5, and rat liver homogenate for GLUT7. Control membranes were used at between 1-200μg protein, as detailed in the legend to Fig. 4.1, depending upon the intensity of the autoradiographic signal obtained. The adipocyte low density microsomal membranes, prepared according to the method of Cushman & Wardzala, (1980), were a gift from Mr. L. Fryer and the guinea-pig enterocyte homogenate was a gift from Mr. G. Chourimoto. Human erythrocyte membranes and crude liver plasma membranes were prepared as described in Sections 2.3.4 and 2.3.6 respectively, and liver and brain homogenate were prepared from the respective rat tissue by homogenisation in PBS, pH7.4 using a glass Dounce homogeniser.

From Fig. 4.1 it can be seen that the only antibodies to cross-react with the mammary homogenate were either raised against the human erythrocyte glucose transporter, GLUT1, or the insulin-responsive glucose transporter, GLUT4. Both antibodies recognised a single, sharp protein band on mammary homogenate Western blots, with a $M_r$ave 50,000. As only very low levels of GLUT4 were detected in 100μg mammary gland homogenate, a larger protein loading (200μg) was used to produce the autoradiograph of mammary GLUT4 content shown in Fig. 4.1(d). Conversely, less mammary homogenate and a shorter exposure time were required to detect the GLUT1 isoform, 50μg protein being used to produce Fig. 4

Fig. 4.1 Identification of glucose transporter isoform(s) in lactating rat mammary gland homogenate



Samples of mammary homogenate (M: 50μg(a), 100μg(b, c, e, f), 200μg(d)) were electrophoresed on 12% SDS/polyacrylamide gels then transferred to nitrocellulose. The glucose transporter isoforms were identified using affinity-purified antibodies raised against the respective *C*-terminal peptides of GLUT1(a), GLUT2(b) or

Fig. 4.1 Identification of glucose transporter isoform(s) in lactating rat mammary gland homogenate

GLUT4(d) at 2μg/ml, or antisera against GLUT3(c) or GLUT5(e) at a 1:400 dilution, as described in Section 2.6. An antiserum raised in sheep against the whole GLUT7 protein (f) was also used and the glucose transporter detected colourimetrically as described in Section 4.2. Membranes known to contain the relevant glucose transporter isoforms were used as positive controls for each antibody as follows :- (a) 1μg human erythrocyte membrane protein (HEM), (b) 100μg rat liver plasma membranes (LPM), (c) 100μg rat brain homogenate (B), (d) 10μg rat adipocyte low density microsomes (AM), (e) 100μg guinea-pig enterocyte homogenate (E) and (f) 100μg rat liver homogenate (L).

4.1(a). There was no detectable expression of GLUT2, GLUT3, GLUT5 or GLUT7 in the mammary homogenate, although most of the antisera used showed reactivity to the control membranes. For GLUT2 a number of protein bands were often detected around M_r66,000. These bands were not always seen (see Fig. 3.6(b)) and were assumed to derive from partially degraded or aggregated GLUT2. Anti-GLUT3 antibodies detected glucose transporters only in rat brain. The anti-GLUT5 antibodies detected a number of bands in guinea-pig enterocytes, however none of these could be confidently ascribed to a glucose transporter protein. A number of control membranes were also used to investigate GLUT3 and GLUT5 cross-reactivity, including 100μg human erythrocyte membranes, 100μg rat liver membranes and 100μg rat adipose homogenate, however neither GLUT3 or GLUT5 were detectable in these tissues by Western blotting (results not shown). Recently it has been shown that while peptide-specific antibodies against GLUT5 will detect a protein in human adipocytes, they do not cross-react with rat adipocytes (Shepherd *et al.*, 1992), suggesting epitope differences between the rat and human isoforms that could be affecting the detection of GLUT5 in the rat samples used here.

Rat erythrocytes were found to contain low levels of GLUT1 (results not shown). However, contamination of the mammary homogenate with erythrocyte membranes would have had to have been very substantial to account for all of the mammary GLUT1 detected. Extensive washing of the tissue prior to homogenisation precluded such contamination. Furthermore, intact erythrocytes pellet at the low centrifugation speed used to discard the cell clumps following the homogenisation. The pre-immune sera and non-specific IgG failed to detect proteins in any of the membrane and tissue samples.

4.3 Sub-cellular distribution of GLUT1 and GLUT4 in lactating rat mammary gland

Western blotting of glucose transporters in mammary homogenate, shown in Section 4.2, had indicated abundant expression of GLUT1 and low level expression of GLUT4 at mid-lactation. The sub-cellular distribution of these two glucose transporter isoforms in mid-lactating rat mammary gland was next investigated by using differential centrifugation to prepare mammary sub-cellular membrane fractions and Western blotting to detect the glucose transporters, coupled to analysis of membrane purity and cross-contamination of membranes by using marker enzyme activities.

Mammary fractions enriched in plasma membranes, Golgi membranes or milk fat globule membranes were prepared by sub-cellular fractionation of mammary tissue or milk according to the methods in Sections 2.3.1, 2.3.2, and 2.3.3 respectively.

4.3.1 Analysis of mammary sub-cellular membrane fractions using marker enzymes

The enrichments of the plasma membrane and the Golgi membranes in the sub-cellular fractions in comparison to the homogenate were assessed using assays for the marker enzymes 5'-nucleotidase (plasma membrane marker) and galactosyltransferase (Golgi membrane marker). Similarly the degree of contamination of plasma membranes with Golgi membranes, and *vice versa*, could be determined. The milk fat globule membranes were prepared directly from rat milk, and therefore were not contaminated with plasma membranes or Golgi membranes from the epithelial cells.

Plasma membranes prepared as described in Section 2.3.1 were routinely enriched in 5'-nucleotidase activity by approximately 24-fold compared to the activity of the

original homogenate. However, plasma membranes also contained on average 7-fold higher galactosyltransferase activity per mg protein than the homogenate, which indicated there was some contamination of the plasma membrane preparation with the Golgi membrane marker enzyme.

Golgi membranes prepared as described in Section 2.3.2 were routinely found to be enriched 13-fold with galactosyltransferase activity compared to the original homogenate, but also contained an approximately 7-fold enrichment over homogenate with the plasma membrane marker. There was therefore a substantial contamination of the Golgi membranes with plasma membranes. However, the activity of 5'-nucleotidase in the Golgi membrane fraction (≈650 μmol adenosine/min/mg protein) was substantially less than the activity of 5'-nucleotidase in the plasma membrane preparation (≈2500 μmol adenosine/min/mg protein), therefore the Golgi membrane fraction contained less than 30% of the 5'nucleotidase activity of the plasma membrane fraction, a fact which becomes relevant in Section 4.3.2, in relation to the sub-cellular distribution of glucose transporters.

4.3.2 Sub-cellular distribution of GLUT1 and GLUT4 in mammary tissue

The glucose transporter contents of mammary plasma membranes (10μg), Golgi membranes (10μg) and milk fat globule membranes (20μg), were investigated by Western blotting using the methods described in Sections 2.5 and 2.6. Following electrophoresis on 12% SDS/polyacrylamide gels, the proteins were transferred to nitrocellulose and GLUT1 or GLUT4 detected using antibodies raised against their respective *C*-terminal peptides, an iodinated second antibody and autoradiography.

A strong band of labelling was detected on Western blots of mammary plasma

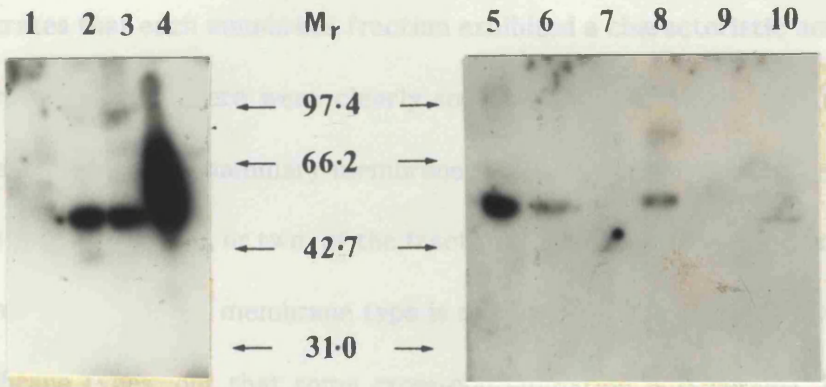
membranes and Golgi membranes using GLUT1, but only a very faint band of labelling in milk fat globule membranes, shown in Fig. 4.2(a). In each membrane the detected protein had an apparent $M_r$ave 50,000. GLUT4 could not be detected in 10μg of mammary plasma membranes or Golgi membranes, shown in Fig. 4.2(b). The GLUT4 content of milk fat globule membranes was not investigated. Both the mammary plasma membranes and Golgi membranes showed substantially increased levels of GLUT1/mg protein compared to the level of GLUT1 in the homogenate detected by Western blotting (shown in Fig. 4.1(a)). This result indicated that both sub-cellular membrane preparation procedures resulted in co-purification from other cellular proteins of both GLUT1 and the relevant marker enzymes.

The intensity of GLUT1 seen in Western blots of Golgi membrane-enriched fractions was very similar to the intensity of GLUT1 seen in the plasma-membrane-enriched fractions. This finding indicated that GLUT1 in the Golgi membrane could not be derived entirely from contamination of the Golgi membranes with the plasma membranes, since, although there is contamination of Golgi membranes with the plasma membrane marker enzyme, the total enzyme activity was less than 30% of the activity of the plasma membrane itself (Section 4.3.1). The absence of GLUT1 in the milk fat globule membranes suggested that either GLUT1 was not present in the apical plasma membrane, or that the milk fat globule membrane proteins were selectively sequestered from the cellular membranes, a possibility discussed in Section 4.9.

4.3.3 SDS/polyacrylamide gel electrophoresis of mammary tissue sub-cellular fractions

To compare the overall protein composition of the mammary sub-cellular

Fig. 4.2 Sub-cellular distribution of GLUT1 and GLUT4 in mammary membranes



Mammary plasma membranes (2,10), Golgi membranes (3,9) (10µg each) or milk fat globule membranes (1) (20µg) were electrophoresed on 12% SDS/polyacrylamide gels and glucose transporters detected by Western blotting as described in Section 2.6, using antibodies raised against the *C*-terminal peptides of GLUT1 (a) or GLUT4 (b). Human erythrocyte membranes (4 : 50µg) and rat adipocyte sub-cellular fractions (10µg each) were used as positive controls for GLUT1 and GLUT4 respectively. Adipocyte membranes were a gift from Mr. L. Fryer and consisted of low density microsomal membranes (5) and plasma membranes (7) from basal adipocytes, and low density microsomal membranes (6) and plasma membranes (8) from insulin-stimulated adipocytes, prepared by the method of Cushman and Wardzala (1980).

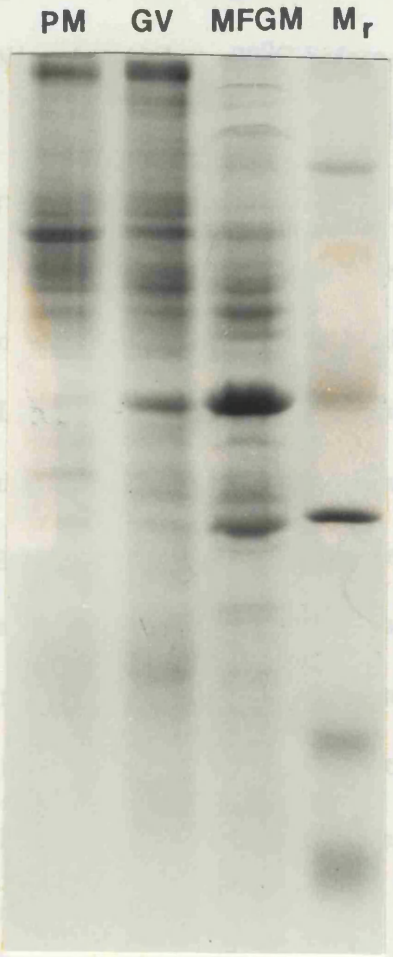
fractions used in the experiments described in Section 4.3.2, mammary plasma membranes (20μg), Golgi membranes (20μg) and milk fat globule membranes (20μg), were made up as gel samples and electrophoresed on 10% SDS/polyacrylamide gels then stained with Coomassie blue as described in Section 2.5.

A typical example of such an SDS/polyacrylamide gel is shown in Fig. 4.3, which illustrates that each membrane fraction exhibited a characteristic and distinct set of protein bands. There were clearly some proteins of an apparently similar M_r present in all three mammary membrane fractions, while other proteins appeared to be confined to one, or two, of the fractions only. This result lends support to the assumption that each membrane type is not severely contaminated with the other membrane types, but that some cross-contamination is occurring. The milk fat globule membranes appeared to contain fewer proteins than the plasma membrane or Golgi membrane fractions, a few proteins being very heavily expressed in the milk fat globule membranes.

4.4 Distribution of GLUT1 in the fractions of a standard mammary plasma membrane or Golgi membrane preparation

One of the major disadvantages of the procedures for preparing mammary plasma membranes and Golgi membranes is that they require different homogenisation conditions, as described in Sections 2.3.1 and 2.3.2. Consequently, it was not possible to prepare both types of membrane from the same starting homogenate and thus investigate the sub-cellular distribution of glucose transporters using a single fractionation procedure. However, in an attempt to throw more light on this distribution, the GLUT1 content and the marker enzyme activities were measured in all the fractions produced during standard plasma membrane and Golgi membrane

Fig. 4.3 SDS/polyacrylamide gel electrophoresis of mammary sub-cellular membrane fractions



Samples of mammary sub-cellular membrane fractions (20μg protein) were electrophoresed on a 10% SDS/polyacrylamide gel, which was stained using Coomassie blue to detect the membrane proteins. The fractions were derived from the mammary plasma membranes (PM), Golgi membranes (GV) and milk fat globule membranes (MFGM) as indicated on the figure. The molecular weight markers are shown in the right hand lane (M_r).

preparations from rat mammary gland.

Samples of all the sub-cellular fractions from a standard mammary plasma membrane (Section 2.3.1) and Golgi membrane (Section 2.3.2) preparation were frozen in liquid N_2 and stored at -70°C before assaying. The nomenclatures used to describe the various fractions are detailed in Fig. 4.4(a) for the plasma membrane preparation and Fig. 4.4(b) for the Golgi membrane preparation. Aliquots (20μg) of each of the cell fractions were made up as gel samples and electrophoresed on 12% SDS/polyacrylamide gels. Following Western blotting onto nitrocellulose, GLUT1 was detected using antibodies raised against the *C*-terminal peptide. The autoradiographs in Fig. 4.5 show the level of GLUT1 in the cell fractions throughout the plasma membrane (a) and Golgi membrane (b) preparations. The increased GLUT1 levels in the final, purified plasma membrane or Golgi membrane fractions over the homogenate were substantial. In order to get a more quantitative estimate of this enrichment, the autoradiographs shown in Fig. 4.5 were scanned by densitometry. The relative intensities of GLUT1 compared to the homogenate for each of the fractions in a plasma membrane preparation are shown in Fig. 4.6(a) and for the fractions in a Golgi membrane preparation, in Fig. 4.6(b) together with the change in activities of the marker enzymes galactosyltransferase and 5'-nucleotidase compared to the homogenate.

The most marked enrichment in plasma membrane markers was seen in the final plasma membrane fraction as expected, where it coincided with the greatest enrichment in GLUT1 density. Similarly the greatest enrichment in the Golgi membrane marker was seen in the final Golgi membrane preparation, and again coincided with the highest density of GLUT1 during this fractionation. These results suggested that for each of the mammary sub-cellular fractionation procedures,

Fig. 4.4 Fractionation stages in standard plasma membrane and Golgi membrane preparations

(a) Plasma membranes

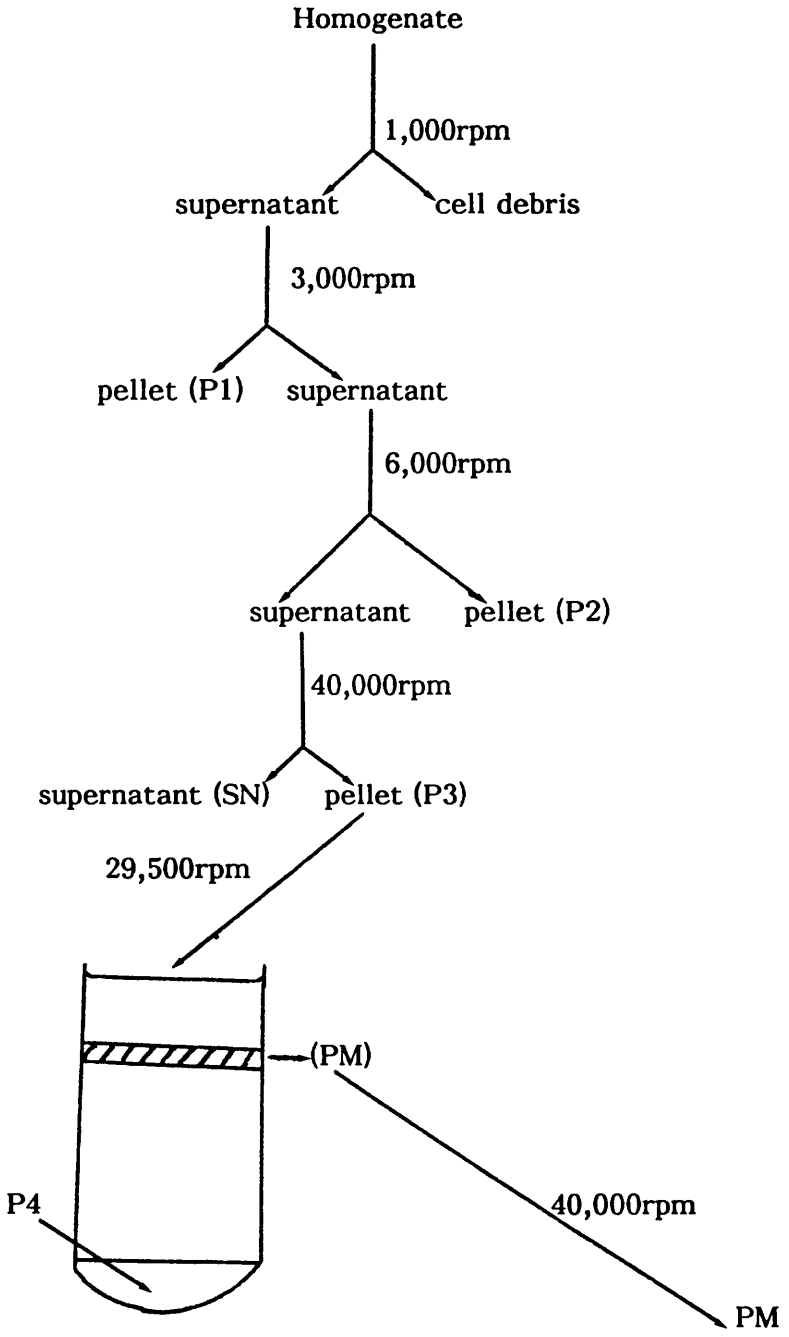


Fig. 4.4(a) shows the fractionation steps in a standard mammary plasma membrane preparation, using an adaptation of the method of Clegg (1981).

Fig. 4.4 Fractionation stages in standard plasma membrane and Golgi membrane preparations

(b) Golgi membranes

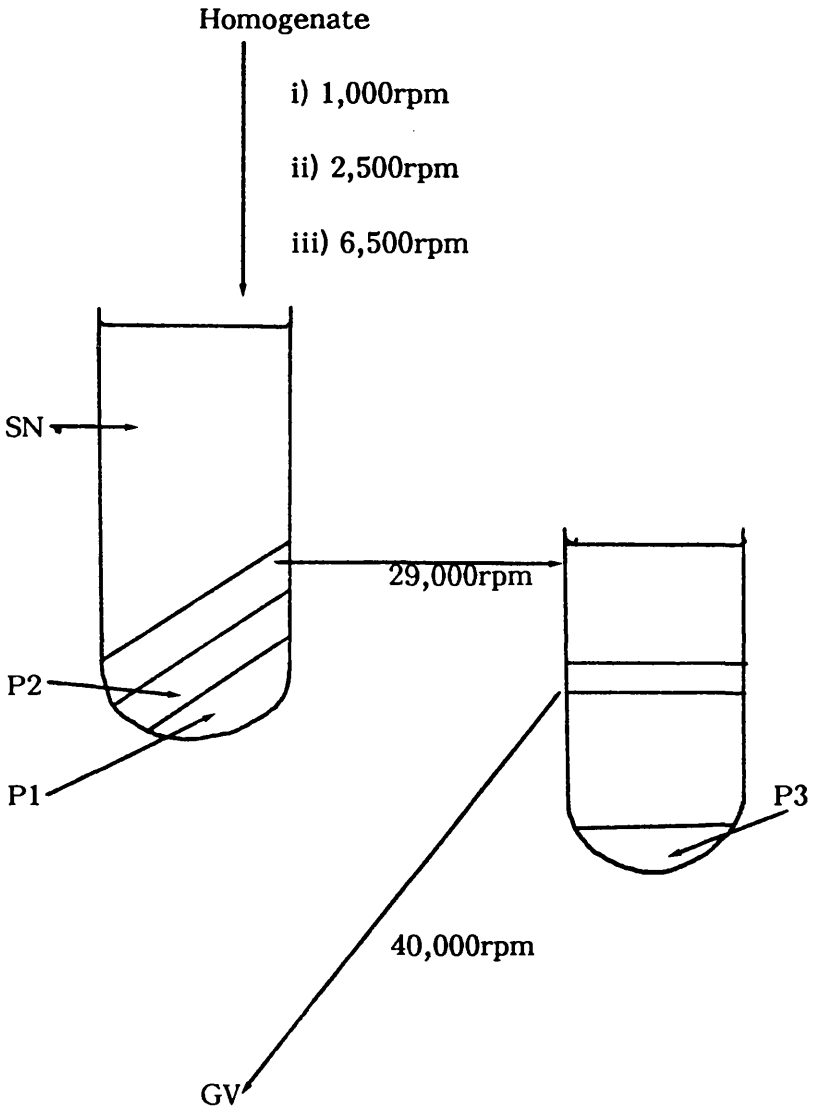


Fig. 4.4(b) shows the fractionation steps in a standard mammary Golgi membrane preparation, using an adaption of the method of West (1981).

Fig. 4.5 Western blots of GLUT1 during mammary plasma membrane and Golgi membrane preparations

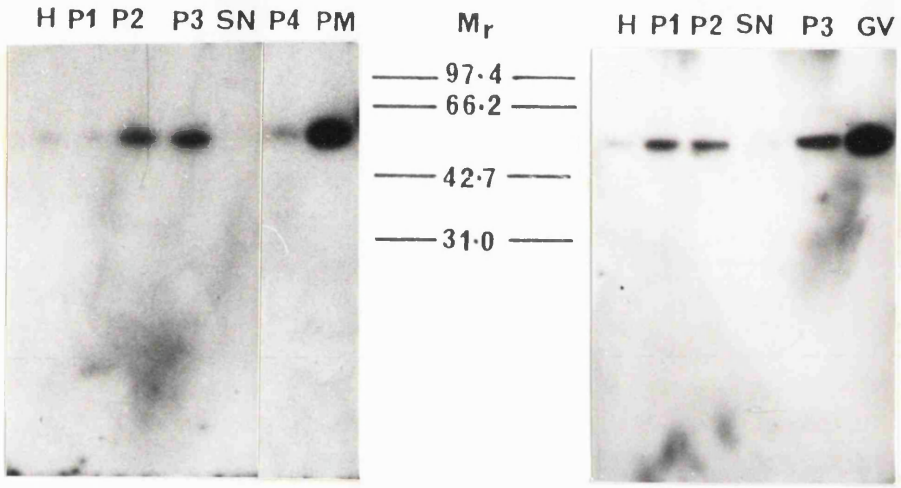
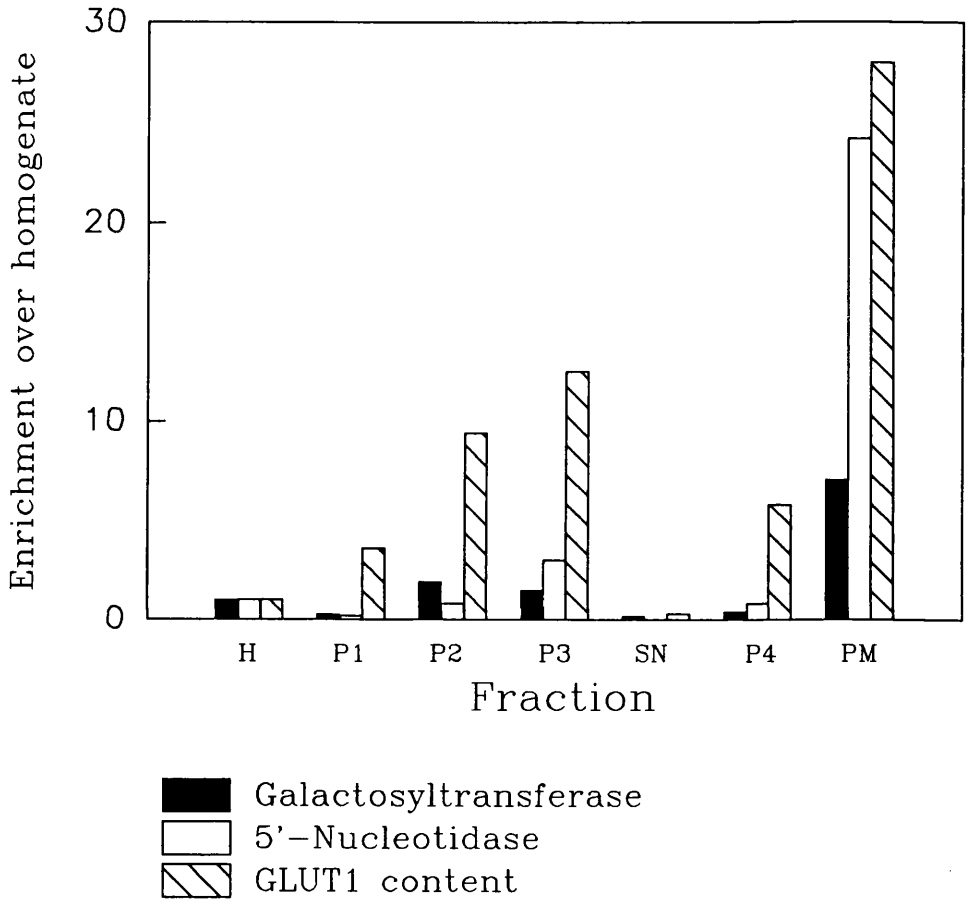


Fig. 4.5 shows autoradiographs demonstrating the level of GLUT1 in the sub-cellular membrane fractions of a standard mammary plasma membrane (a), or a mammary Golgi membrane (b), preparation. Samples (20μg) of each fraction were electrophoresed on 12% SDS/polyacrylamide gels, Western blotted and GLUT1 detected using antibodies raised against the GLUT1 *C*-terminal peptide and an iodinated second antibody. The nomenclature used above for each of the membrane fractions is described in Fig. 4.4.

Fig. 4.6 Characterisation of mammary sub-cellular fractions

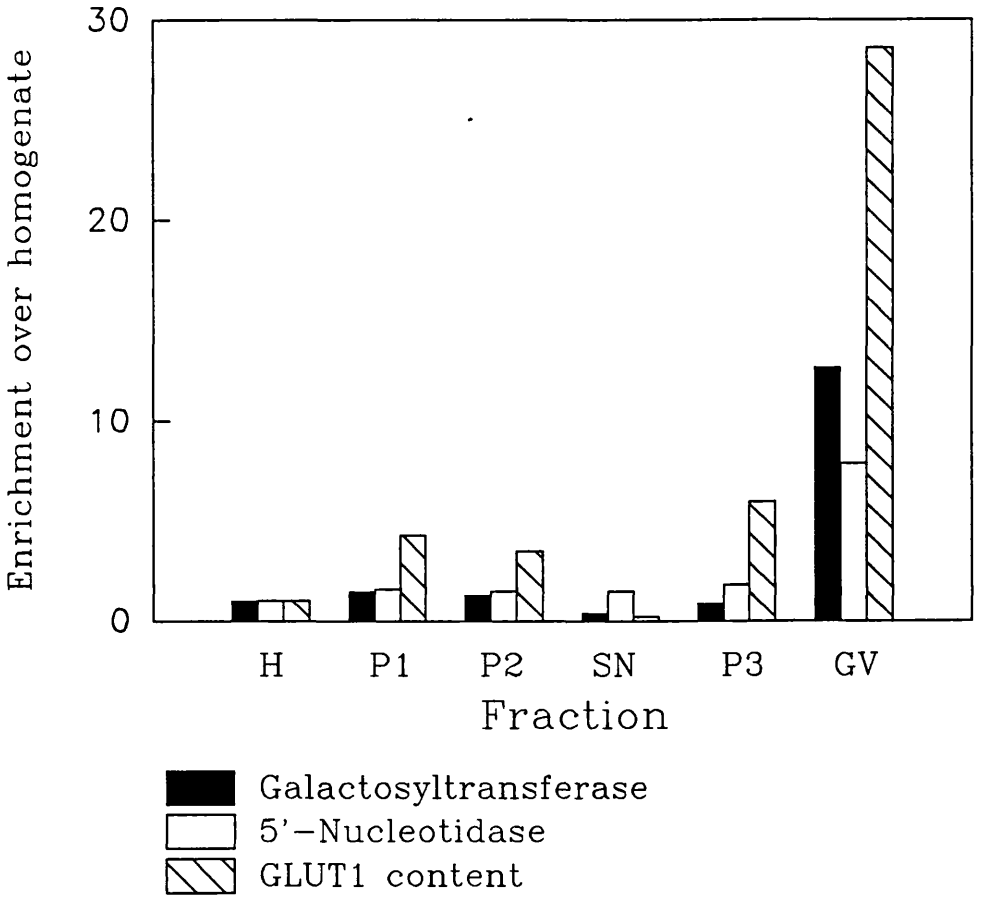
a) Plasma membrane



The enzyme activities of 5'-NT and GTA for each plasma membrane sub-cellular fraction shown in Fig. 4.4(a) were measured as described in Sections 2.4.1 & 2.4.2, and the activity relative to homogenate activity determined. The autoradiograph of sub-cellular GLUT1 distribution, shown in Fig. 4.5(a), was quantified by densitometry and the enrichment in GLUT1 of each fraction relative to the homogenate was also determined.

Fig. 4.6 Characterisation of mammary sub-cellular fractions

b) Golgi membranes



The enzyme activities of 5'-NT and GTA for each Golgi membrane sub-cellular fraction shown in Fig. 4.4(b) were measured as described in Sections 2.4.1 & 2.4.2, and the activity relative to homogenate activity determined. The autoradiograph of sub-cellular GLUT1 distribution, shown in Fig. 4.5(b), was quantified by densitometry and the enrichment in GLUT1 of each fraction relative to the homogenate was also determined.

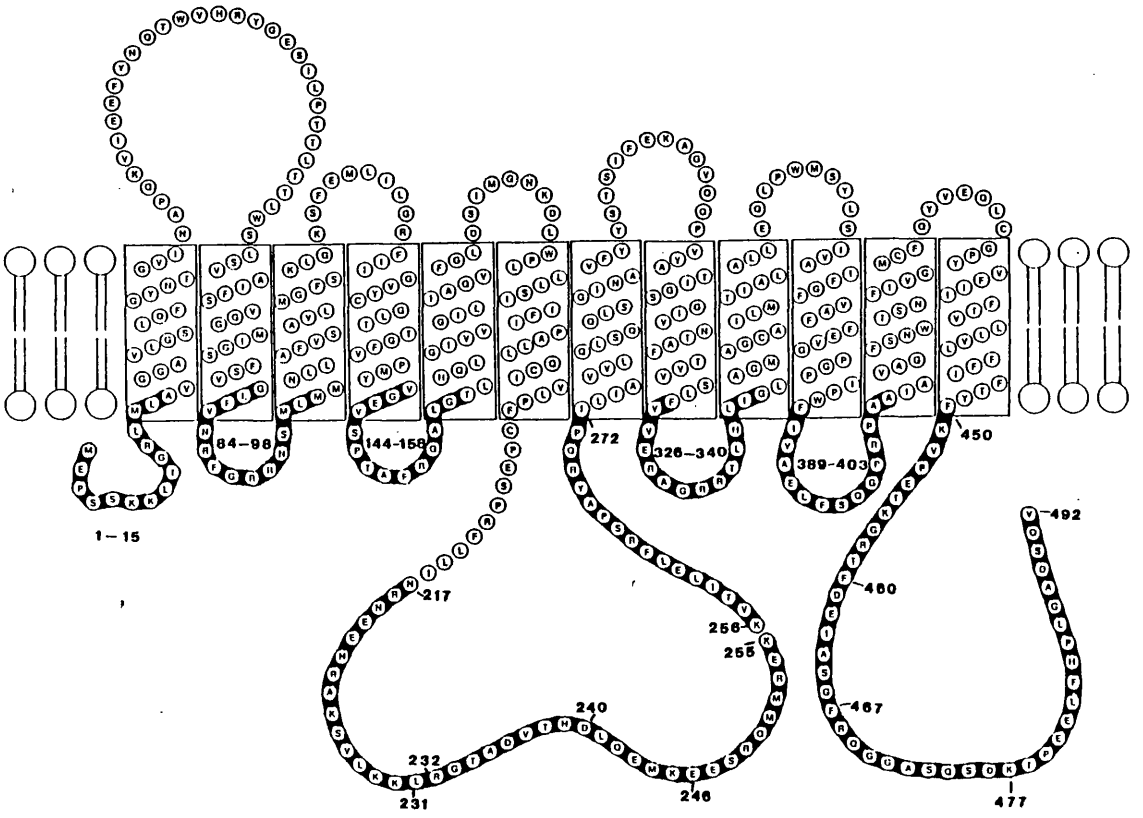
GLUT1 enrichment over homogenate was coincident with the enrichment in either the plasma membranes or Golgi membranes. For the Golgi membranes, these results, when coupled to the actual enzyme activities described in Section 4.3.1 indicate that the "intra-cellular" GLUT1 present in the Golgi fraction is not derived entirely from contamination with the plasma membranes. However, these results do not establish that the intra-cellular GLUT1 is located in those regions of the Golgi apparatus involved in lactose synthesis.

4.5 Cross-reactivity of mammary GLUT1 with a range of anti-GLUT1 peptide antibodies

The glucose transporter identified in mammary sub-cellular fractions (Fig. 4.2) cross-reacted with antibodies raised against the extreme *C*-terminal peptide of GLUT1. To assess the degree of sequence similarity between the mammary glucose transporter and the HepG2/human erythrocyte and rat isoforms of GLUT1, site-specific antibodies raised against a range of GLUT1 peptide sequences were used. These antisera were provided by Dr. A. Davies, with the exception of an antiserum raised against the peptide sequence containing residues 217-232 of human erythrocyte/HepG2 GLUT1, which was provided by Dr. G. Lienhard.

Mammary plasma membranes and Golgi membranes (10μg protein) were electrophoresed on 12% SDS/polyacrylamide gels and Western blotted onto nitrocellulose. GLUT1 was then detected using antibodies raised against various GLUT1 peptide sequences predicted to be cytoplasmic (Mueckler *et al.*, 1985), shown in Fig. 4.7. These sequences included the *N*-terminus, residues 1-15; the small cytoplasmic loops, residues 84-98, 144-158, 326-340 and 389-403; the large cytoplasmic loop, specifically the residues 217-232, 231-246, 240-255, 256-272; and

Fig. 4.7 Peptide sequences of GLUT1 used to raise site-specificantibodies



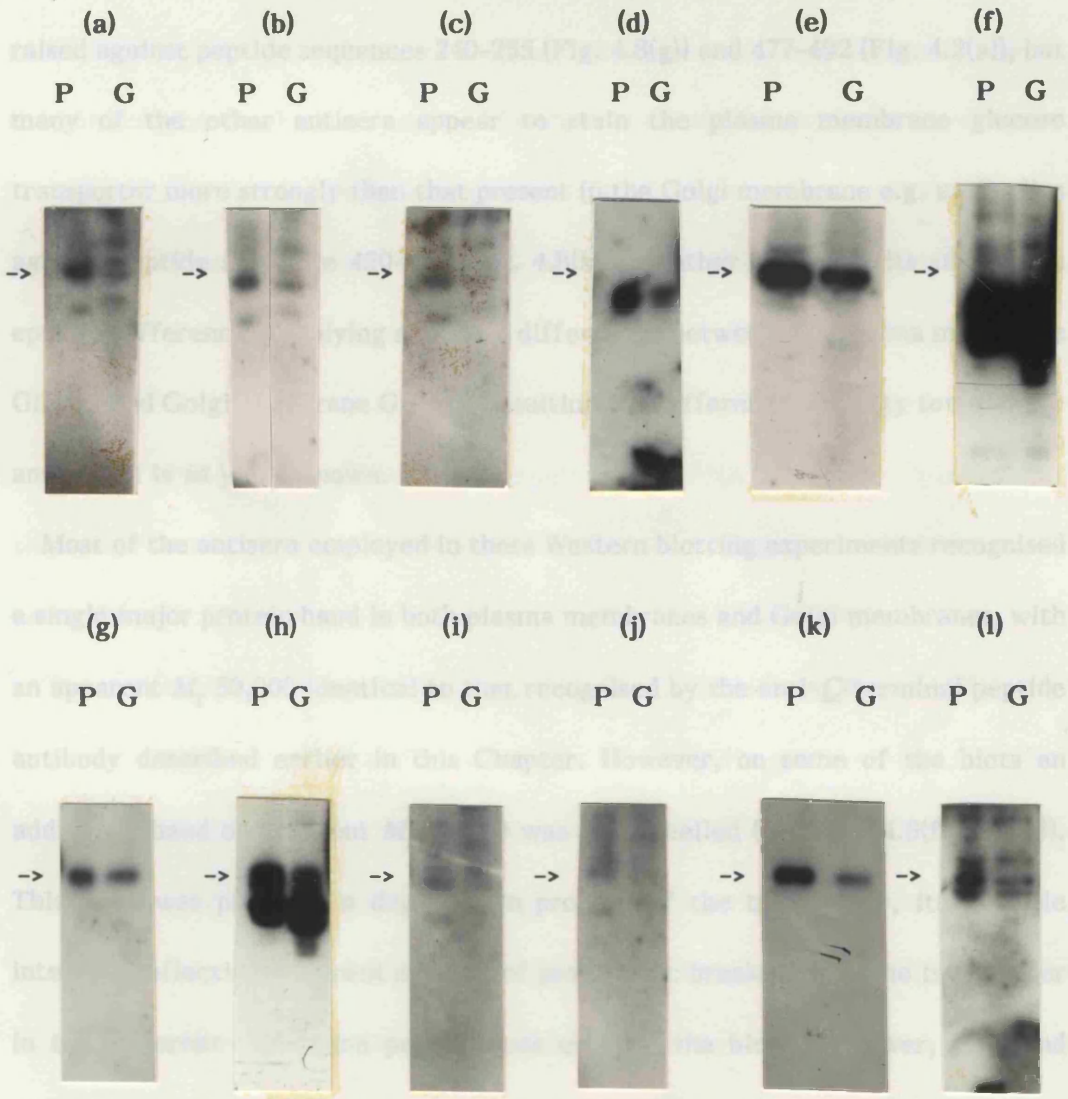
Antibodies had been raised against various peptide sequences of the HepG2/human erythrocyte GLUT1 protein shown above, by Dr. A. Davies. Coloured circles denote an amino-acid difference between the human erythrocyte GLUT1 and rat brain GLUT1. Antisera raised against the rat peptide 217-232, containing a single amino-acid change at 239, was also available.

the entire *C*-terminus, specifically residues 450-467, 460-477 and 477-492, as well as the region 231-246 of the rat brain GLUT1 (identified as rt231-246). The rat sequence contains a single amino-acid change at residue 239 (His(H)→Arg(R)). The antibodies had been characterised by Dr. A. Davies and all had been shown to react to the human erythrocyte GLUT1 to varying degrees (Davies, 1990). Affinity-purified antibodies were available to the sequences 231-246, 240-255, 450-467, 460-477 and 477-492, antisera were available to the remaining regions.

For most antisera, specifically against the *N*-terminal and the small loops, a dilution of 1:100 was necessary to detect GLUT1, due to the low immunoreactivity. As can be seen in Fig. 4.8, this generated high backgrounds. For the remaining antisera, specifically the large cytoplasmic loop sequences 217-232 and 256-272, a dilution of 1:400 was used. The affinity-purified antibodies were used at 2μg/ml. All GLUT1 cross-reactive proteins were detected using iodinated anti-rabbit IgG and autoradiography.

As shown in Fig. 4.8 all of the anti-GLUT1 peptide antibodies or antisera used in Western blotting cross-reacted with the mammary GLUT1 in both plasma membranes and Golgi membranes, and in human erythrocyte membranes (results not shown). This finding implies that the glucose transporters of both Golgi membranes and plasma membranes closely resemble the human/rat GLUT1 proteins in many regions of their sequences. Were the rat mammary and rat brain GLUT1 sequences to be identical, one might expect that anti-human GLUT1 antisera, raised against the various peptides, would vary in the intensity with which they stained blots of mammary membranes. However, the relative intensities of staining exhibited by the plasma membranes and Golgi membranes using the different antisera should remain constant, merely reflecting differences in the transporter content of the two

Fig. 4.8 Cross-reactivity of mammary sub-cellular fractions to site-specific antibodies raised against human erythrocyteGLUT1 peptides



Site-specific antibodies had been raised against peptide sequences shown in Fig. 4.8, within HepG2/human erythrocyte GLUT1 or rat brain GLUT1. Mammary plasma membranes (P) or Golgi membranes (G), containing 10μg protein, were electrophoresed on 12% SDS/polyacrylamide gels, transferred to nitrocellulose and GLUT1 was detected by Western blotting. Antisera raised against the peptide regions 1-15(a), 84-98(b), 143-158(c), 326-340(i), 389-403(j) and rt231-246(f) were used at a dilution of 1:100, antisera against the sequences 217-232(d) and 256-272(h) were used at a dilution of 1:400, and affinity-purified antibodies raised against the sequences 231-246(e), 240-255(g), 450-467(k) and 460-477(l) were used at 2μg/ml. GLUT1 was detected by autoradiography. The GLUT1 is denoted by the position of the arrows.

fractions. This was not always the case; for example, both mammary membrane fractions exhibited roughly the same intensity on blots stained with antibodies raised against peptide sequences 240-255 (Fig. 4.8(g)) and 477-492 (Fig. 4.2(a)), but many of the other antisera appear to stain the plasma membrane glucose transporter more strongly than that present in the Golgi membrane e.g. antibodies against peptide sequence 450-467 (Fig. 4.8(k)). Whether these results stem from epitope differences, implying sequence differences between the plasma membrane GLUT1 and Golgi membrane GLUT1, resulting in a different reactivity towards the antibodies is as yet unknown.

Most of the antisera employed in these Western blotting experiments recognised a single major protein band in both plasma membranes and Golgi membranes, with an apparent M_r 50,000 identical to that recognised by the anti-*C*-terminal peptide antibody described earlier in this Chapter. However, on some of the blots an additional band of apparent M_r 45,000 was also labelled (e.g. Fig. 4.8(f) and (h)). This band was probably a degradation product of the transporter, its variable intensity reflecting different degrees of proteolytic breakdown of the transporter in the different membrane preparations used on the blots. However, a second possibility is that it represents a second glucose transporter only partially similar in sequence to GLUT1 and so recognised to different extents by the antibodies against the different regions of the GLUT1 sequence. Whether or not this is the case, the detection of an M_r 50,000 protein on the blots by all of the anti-GLUT1 antibodies indicates that the sequence similarity of this mammary plasma membrane and Golgi membrane glucose transporter to the human/rat GLUT1 protein extends over most of the cytoplasmic domain of the protein.

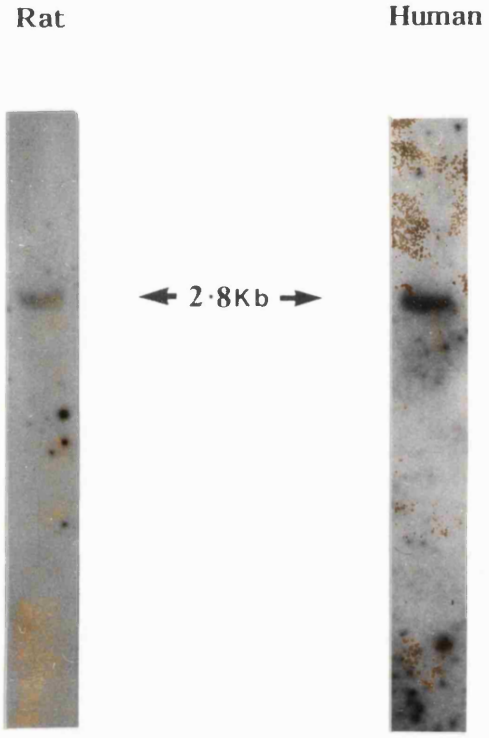
4.6 Northern blotting

The Western blotting results described in Section 4.5 had indicated that a protein similar in sequence to the human/rat GLUT1 was present in both mammary plasma membranes and Golgi membranes. Additional evidence for this similarity was next sought by using Northern blotting to assess the ability of rat mammary mRNA to hybridise with a cDNA probe containing the entire coding region of GLUT1.

Total RNA was prepared from rat mammary gland, and human mammary tumour RNA was provided by Ms. J. Aitken as a control human tissue. Northern blots of rat mammary RNA (35μg) and human mammary tumour RNA (5μg) were hybridised to the entire BamHI fragment of the pSGT GLUT1 insert using the methods described under Section 2.7.

The results of the Northern blotting using GLUT1 cDNA are shown in Fig. 4.9. In both rat mammary RNA and human mammary RNA a single hybridising band of approximately 2.8Kb in size was detected using the GLUT1 probe. This corresponds to the size of the GLUT1 mRNA detected in many rat and human tissues (Mueckler *et al.*, 1985). The exposure time required to detect the putative GLUT1 mRNA in 5μg of human mammary RNA were shorter (between 12-36hr) than the time required to detect GLUT1 mRNA in 35μg rat mammary RNA (up to 10 days). The higher expression of human mammary tumour GLUT1 mRNA than rat mammary mRNA was probably due to the human mammary tumour over-expressing GLUT1, over-expression of glucose transporters being characteristic of mammalian tumour cells (Yamamoto *et al.*, 1990).

Fig. 4.9 Detection of GLUT1 mRNA in lactating rat mammary gland and human mammary tumour



Total RNA from lactating rat mammary gland (35µg RNA) and human mammary tumour (5µg RNA) were electrophoresed on 1.2% agarose/formaldehyde gels, and Northern blotted onto Hybond N. The expression of GLUT1 was determined by hybridisation with a radiolabelled human GLUT1 cDNA probe consisting of the entire BamHI fragment, as described under Section 2.7. Human tumour RNA was a gift from Ms. J. Aitken.

4.7 Cytochalasin B photolabelling and immunoprecipitation of glucose transporters using anti-GLUT1 *C*-terminal peptide antibodies

Results described in Sections 4.2-4.6 had indicated that rat mammary gland expressed a putative homologue of the human/rat GLUT1 glucose transporter. However they had not definitively established that the protein identified with the antibodies was in fact a glucose transporter. The antibodies used to identify the putative transporter on Western blots of mammary tissue, shown in Sections 4.2 and 4.3.2, were raised against the extreme 17 amino-acid *C*-terminal peptide of GLUT1 (residues 477-492). Cross-reactivity between these antibodies and proteins of a similar molecular weight, containing similar peptide sequences and epitopes, but not transporting D-glucose, for example the amino-acid or nucleoside transporters, was therefore possible. In order to definitely establish the identity of the mammary membrane proteins recognised by the antibodies, advantage was taken of the fact that these antibodies are known to recognise human GLUT1 in its native conformation (Davies *et al.*, 1987) and so can be used for immunoprecipitation. Identification of the immunoprecipitated proteins as D-glucose transporters can be made by photolabelling them with tritiated cytochalasin B prior to immunoprecipitation (Kasanicki *et al.*, 1987). Covalent labelling of the transporter by this fungal metabolite, upon irradiation with U.V. light, is specifically inhibited by the substrate, D-glucose, but not by L-glucose.

Photolabelling was performed by the method of Kasanicki *et al.*, (1987). Mammary plasma membranes and Golgi membranes (1.35mg protein) and 250µg human erythrocyte membranes were incubated in glass tubes on ice for 1hr at 1mg/ml in Buffer B (50mM sodium phosphate, 100mM NaCl, 1mM EDTA, pH7.4) containing 0.51µM [^{3}H]cytochalasin B, 10µM cytochalasin E, 1% (v/v) ethanol and

500mM D- or L-glucose. Following incubation, the membranes were transferred to quartz cuvettes in 500µl aliquots, flushed with N_2 and stoppered. Aliquots were irradiated on ice for 10min at 10cm distance using a 100W U.V. lamp. Irradiated membrane samples were transferred to 3.0ml Beckman cellulose propionate ultracentrifuge tubes and the cuvettes rinsed out with a small volume of Buffer B containing 20µM unlabelled cytochalasin B. Samples were centrifuged at 40,000rpm for 30min at 4°C using a Beckman TL-100 benchtop ultracentrifuge and the TLA-100 rotor, and the supernatant discarded. Photolabelled membrane pellets were solubilised at 4°C in 5.4ml Buffer B containing 0.25% (w/v) SDS, 2.5% (v/v) Triton X-100, 1mM PMSF, 0.1mM E-64, 1.5µM Pepstatin A for 1hr. Each solubilised membrane fraction was divided in half, and incubated with either 50µg non-specific rabbit IgG or 50µg affinity-purified anti-GLUT1 *C*-terminal peptide antibodies bound to 25µl Protein A-Sepharose CL-4B, overnight at 4°C. The bound proteins were washed 3x by centrifugation with Buffer B containing 1% (v/v) Triton X-100, 0.1% (w/v) SDS, then once with Buffer B containing 0.1% Triton X-100, 0.01% SDS. Bound proteins were eluted from the Sepharose into 100µl gel sample buffer containing 6M urea. Samples (90µl) were then electrophoresed on 3mm thick, 12% SDS/polyacrylamide gels. Gels were stained with Coomassie blue and the migration of the low molecular weight markers measured. The labelled proteins were detected by slicing the relevant lanes of the gel into 2mm x 1cm slices, drying each slice, then dissolving in 30µl H_2O_2 at 50°C for 8hr, cooling, and adding 5ml Ultima-gold scintillant for scintillation counting.

A narrow band of photolabelled protein was observed, with an $M_r$ave 50,000, in both the mammary plasma membranes and Golgi membranes and a broad band of photolabelled protein, extending between $M_r$ave 45,000-70,000 in the human

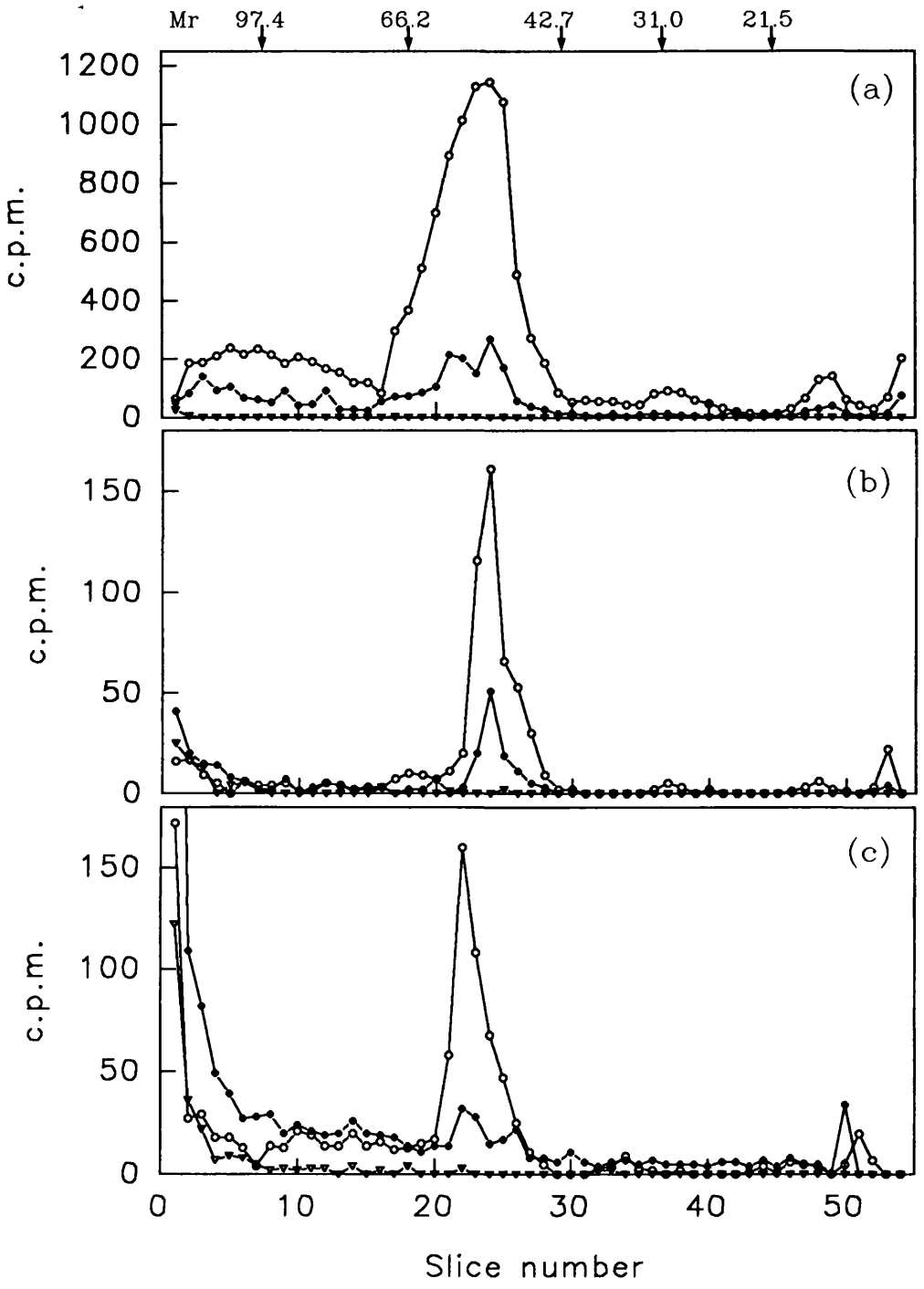
erythrocyte membranes, photolabelled in the presence of 500mM L-glucose, and immunoprecipitated using the antibodies raised against the GLUT1 *C*-terminal peptide (Fig. 4.10). Immunoprecipitation of photolabelled proteins was only achieved when samples were immunoprecipitated using specific anti-GLUT1 *C*-terminal peptide antibodies, and not with the non-specific rabbit IgG. Photolabelling was inhibited approximately 70% by the presence of 500mM D-glucose, compared to the level of photolabelling in the presence of L-glucose, indicating that a D-glucose transport protein was the immunoprecipitated species.

4.8 Endo-ß-galactosidase digestion

The mammary GLUT1 protein identified and characterised in Sections 4.2-4.7 differed from the human erythrocyte GLUT1 in its appearance following denaturing SDS/polyacrylamide gel electrophoresis. Mammary GLUT1 migrated as a sharp band of $M_r$ave 50,000 on Western blots of mammary homogenate or sub-cellular fractions, shown in Fig. 4.1 and Fig. 4.2(a), in contrast to the broad band observed on Western blots of the human erythrocyte GLUT1, which migrated between M_r 40,000 and M_r 70,000, with an apparent $M_r$ave 55,000. In the human erythrocyte the broadness of the band stems from heterogenous *N*-linked glycosylation of the transporter (Gorga *et al.*, 1979). In the following experiment, in order to assess the degree of glycosylation of the mammary GLUT1 proteins, endoglycosidase treatment was used to cleave *N*-linked oligosaccharides from them, and the resultant effects on their apparent M_r investigated by Western blotting.

Duplicate aliquots (100µg each) of mammary plasma membranes, mammary Golgi membranes, and human erythrocyte membranes, prepared as described in Section 2.3.1, 2.3.2 and 2.3.4, were pelleted in an eppendorf microcentrifuge and

Fig. 4.10 Immunoprecipitation of photolabelled GLUT1 from mammary sub-cellular membrane fractions



Human erythrocyte membranes (a), mammary plasma membranes (b) and mammary Golgi membranes (c) were photolabelled with tritiated cytochalasin B by U.V. irradiation in the presence of D-glucose (•) or L-glucose (o), and immunoprecipitated using antibodies raised against the *C*-terminal peptide of

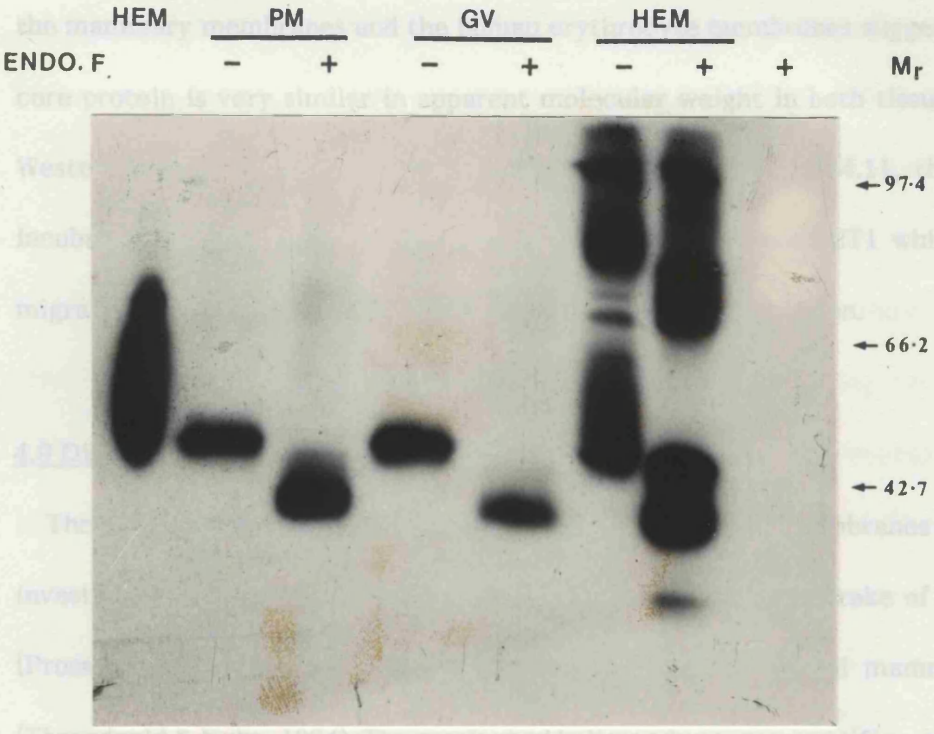
Fig. 4.10 Immunoprecipitation of photolabelled GLUT1 from mammary sub-cellular membrane fractions

GLUT1. Also, membranes labelled in the presence of L-glucose were immunoprecipitated using non-specific rabbit IgG (▾). Immunoprecipitated proteins were electrophoresed on 3mm thick 12% SDS/polyacrylamide gels and the gels stained with Coomassie blue. After the position of the protein markers were noted, the gel lanes were sliced and the slices scintillation counted as described in Section 4.7. The apparent M_r of the photolabelled, immunoprecipitated proteins was determined from the position of the M_r markers.

resuspended in 100mM sodium phosphate, pH6.1 containing 50mM EDTA, 75mM ß-mercaptoethanol, 0.1mM PMSF, 0.05% (w/v) SDS and 1% (v/v) Triton X-100. The membranes were then incubated ± 0.2units of endogalactosidase F (Elder & Alexander, 1982) derived from *Flavobacterium meningosepticum* (Boehringer, Lewes, Sussex, U.K.) for 18hr at 22°C. The reaction was stopped using 20µl of denaturing buffer (85mM Tris-HCl, pH6.8, 1mM EDTA, 7% (w/v) SDS) to give final concentrations of Tris, EDTA and SDS compatible with SDS/polyacrylamide gel electrophoresis. Additional DTT, glycerol and Pyronin Y were then added to obtain normal gel sample concentrations, detailed in Section 2.5. Samples of human erythrocyte membranes (10µg), mammary plasma membranes (30µg) and Golgi membranes (40µg) were then electrophoresed on 12% SDS/polyacrylamide gels and Western blotted onto nitrocellulose. GLUT1 was immunologically detected using antibodies raised against the *C*-terminal peptide of GLUT1, followed by an iodinated second antibody and autoradiography.

Deglycosylation of mammary membrane GLUT1 resulted in a shift in apparent M_r from 50,000 to 38,000, as shown in Fig. 4.11. For the human erythrocyte protein an increase in electrophoretic mobility was also accompanied by a change in appearance; from a broad protein band to a pair of sharper protein bands, of $M_r$ave 45,000 and $M_r$ave 38,000. The $M_r$ave 45,000 band is that usually seen upon endoglycosidase F deglycosylation of the human erythrocyte GLUT1 (Lienhard *et al.*, 1984), however the $M_r$ave 38,000 species has been reported previously (Haspel *et al.*, 1985). It probably represents a conformer of the M_r 45,000 species, and is typically seen if gel samples are heated prior to electrophoresis. The substantial increase in electrophoretic mobility seen following endoglycosidase treatment of GLUT1 in the mammary plasma membranes and Golgi membranes indicates that

Fig. 4.11 Deglycosylation of the GLUT1 from mammary sub-cellular fractions



Mammary plasma membranes (PM), Golgi membranes (GV) and human erythrocyte membranes (HEM) were deglycosylated using endoglycosidase F as described in Section 4.8. Deglycosylated proteins were electrophoresed on 12% SDS/polyacrylamide gels (10μg HEM, 30μg PM and 40μg GV) and Western blotted onto nitrocellulose. GLUT1 was detected using antibodies raised against its *C*-terminal peptide and autoradiography as described in Section 2.6.

these mammary glucose transporters do bear *N*-linked oligosaccharides. However, the sharpness of the bands seen on Western blots even before deglycosylation suggests that these oligosaccharides are less heterogeneous than those attached to the human erythrocyte GLUT1. The deglycosylated $M_r$ave 38,000 of GLUT1 in both the mammary membranes and the human erythrocyte membranes suggest that the core protein is very similar in apparent molecular weight in both tissues. On the Western blot of the human erythrocyte GLUT1 shown in Fig. 4.11, the samples incubated for 18hr at 22°C contained a lot of aggregated GLUT1 which did not migrate through the gel, and can be seen in the higher M_r region.

4.9 Discussion

The transport of D-glucose across mammary plasma membranes had been investigated by various techniques, including the kinetics of uptake of D-glucose (Prosser, 1988) and inhibition of glucose transport in isolated mammary acini (Threadgold & Kuhn, 1984). The results had indicated a stereo-specific, cytochalasin B-inhibitable, temperature-dependent transport mechanism, suggestive of a facilitative D-glucose transport protein, within the mammary epithelial cell plasma membrane. As a family of mammalian facilitative D-glucose transport proteins have been identified and sequenced (Mueckler, 1990) the primary aim of these experiments was to identify the specific, facilitative D-glucose transporter isoform(s) present in lactating rat mammary gland. Preliminary work had demonstrated the presence of GLUT1 in mammary membranes (Madon *et al.*, 1989), later confirmed by results published during the course of this work (Madon *et al.*, 1990; Burnol *et al.*, 1990), as well as the presence of mammary GLUT4 (Burnol *et al.*, 1990). Using antibodies raised against the extreme *C*-terminal peptides of five

of the six GLUT isoforms, and an antibody raised against the purified GLUT7 isoform, the predominant glucose transporter in mammary tissue was found to be GLUT1, with lower levels of GLUT4 also detectable. The K_m for D-glucose of GLUT1 (2-10mM) is within the normal physiological range of plasma glucose, which is between 6-7mM (Page & Kuhn, 1986) during lactation, and as intra-cellular D-glucose is <0.5mM in lactating mammary epithelial cells (Wilde & Kuhn, 1981), the transport of glucose into these cells as catalysed by GLUT1 would be rate-limiting for its utilisation as an energy source, or as a substrate for lactose synthesis. The GLUT4 isoform is expressed exclusively in insulin-responsive tissues, where it translocates between intracellular and plasma membranes in response to insulin stimuli. The mammary gland is not thought to be an insulin-responsive tissue, although it shows insulin sensitivity during pregnancy (Stockdale & Topper. 1966). Assuming low sensitivity of mammary epithelial cells to insulin, particularly during lactation, the GLUT4 identified in mammary gland homogenate was thought more likely to derive from mammary gland adipocytes, than epithelial cells. None of the other glucose transport isoforms were detected in the mammary homogenate by Western blotting. While this alone is not evidence enough to exclude these isoforms from expression in the mammary gland, it does confirm that glucose transport into the mammary epithelial cell occurs primarily through the GLUT1 isoform. Results from Western blotting of rat erythrocyte ghost membranes suggested that it was unlikely that they would contribute much GLUT1 to the results in the mammary gland.

As well as identifying glucose transporters in mammary homogenate, sub-cellular plasma membrane and Golgi membrane fractions of rat mammary gland were found to contain GLUT1, but not GLUT4. Therefore, GLUT4 containing membranes

apparently derived from the adipocytes present within the mammary homogenate did not co-purify with the mammary epithelial cell membranes. The presence of GLUT1 in the mammary plasma membrane confirms the findings of Madon *et al.* (1989), and identifies the putative D-glucose transporter protein proposed to exist by Threadgold *et al.* (1982). The presence of GLUT1 in an intra-cellular membrane fraction enriched with the Golgi membrane marker also confirmed the findings of Madon *et al.* (1989). It is tempting to speculate that these intracellular transporters might be involved in procuring glucose for use in lactose synthesis, but the mere presence of transporters in this sub-cellular fraction cannot be taken as evidence of this involvement. In addition, the conclusion that the transporters are located within the Golgi membranes themselves is obviously highly dependent upon the purity of the Golgi-enriched membrane preparation. Analysis of enzyme activities had indicated that both of the mammary sub-cellular fractions (plasma membrane and Golgi membrane) were contaminated with the other membrane type. Unfortunately it was not possible to isolate plasma membranes and Golgi membranes from the same starting homogenate, in a single procedure. Investigation of the distribution of GLUT1 in relation to the distributions of marker enzyme activities, throughout the entire mammary plasma membrane and a Golgi membrane fractionation procedure yielded the results shown in Section 4.4, and indicated that GLUT1 could be co-purified with both the Golgi membrane marker enzymes and plasma membrane marker enzymes. Although there was contamination of the Golgi membranes with plasma membranes, it was not large enough to account for all GLUT1 identified with the former. The presence of a cell-surface galactosyltransferase has been established in certain epithelial cells (Shaper *et al.*, 1985), where it is postulated to be involved in cell-cell interactions, therefore the

possible existence of plasma membrane galactosyltransferase cannot be ruled out as a source of this activity.

GLUT1 was not detected in the milk fat globule membranes, suggesting a number of possibilities. Either GLUT1 is not present in the apical plasma membrane and/or in the secretory Golgi vesicles, that GLUT1 is present but is rapidly degraded, or recycled, following fusion of the secretory vesicles with the apical plasma membrane, or that the structure of the milk fat globule membrane is highly regulated, containing only the proteins necessary for its function. The presence of a select band of proteins has been shown previously within the bovine milk fat globule membranes (Mather & Keenan, 1975) and the results in Fig. 4.3 suggest that this is also the case for these rat milk fat globule membranes.

The similarity of the mammary glucose transporters to human/rat GLUT1 was investigated both by Western blotting and Northern blotting. Northern blotting (Section 4.6) had shown that GLUT1 mRNA, in total rat mammary RNA, would hybridise to a cDNA probe consisting of the entire BamHI fragment of the HepG2/human erythrocyte GLUT1 sequence, indicating that there was substantial sequence homology. Using antibodies raised against a range of peptide sequences within the cytoplasmic domain of either the HepG2/human erythrocyte GLUT1 or the rat brain GLUT1, and Western blotting, extensive cross-reactivity to the rat mammary GLUT1 was found, suggesting that the peptide sequence similarity extended over most of the cytoplasmic domain of the protein. The signal on Western blots of the mammary plasma membranes and Golgi membranes using the different antibodies was variable, which could indicate epitope differences, however this was not confirmed. Deglycosylation of the mammary GLUT1 with endoglycosidase F, indicated that the protein was heavily glycosylated, with the

core protein electrophoresing with the same mobility as the human erythrocyte GLUT1, suggesting that the core proteins are similar in M_r. The narrow band of labelling normally seen for the mammary GLUT1 protein suggests that it is less heterogeneously glycosylated than the human erythrocyte GLUT1 protein. Following deglycosylation GLUT1 runs as a sharp band of Mr 38,000 or 46,000 for boiled and unboiled SDS/PAGE gel samples respectively. The two protein bands observed for the deglycosylated human erythrocyte GLUT1 were probably the result of different conformers of GLUT1 running within the gel.

Evidence for the D-glucose transport activity of the protein detected by Western blotting in mammary sub-cellular fractions was gained by cytochalasin B photolabelling and immunoprecipitation. GLUT1 in mammary plasma membranes and Golgi membranes could be photolabelled with tritiated cytochalasin B, in a D-glucose-inhibitable manner, and the photolabelled GLUT1 immunoprecipitated using antibodies against the C-terminal peptide of GLUT1, but not non-specific IgG. The presence of D-glucose inhibited the photolabelling by approximately 70% compared to the presence of L-glucose. The $M_r$ave 50,000 of the immunoprecipitated protein corresponded to the $M_r$ave 50,000 protein detected on Western blots of mammary membranes. Thus the antibodies raised against the C-terminal peptide of GLUT1 were proven to cross-react to a mammary D-glucose transport protein.

The overall conclusion from the results shown in this Chapter is that GLUT1 is the predominant mammary glucose transporter isoform, and that the lower expression of GLUT4 probably reflects an adipocyte origin (an assumption validated by work described in Chapter 7). The detection of GLUT4, and to a lesser extent GLUT1, in mammary homogenate was assumed to be affected by the presence of cell types other than the epithelial cells. Among the many cell types present in the

mammary tissue, that are also known to express GLUT1 and/or GLUT4, there are adipocytes which express both GLUT1 and GLUT4, nerve cells (Gerhart *et al.*, 1989) and fibroblasts (Kosaki *et al.*, 1991) both of which express GLUT1, as well as the endothelial cells of the blood capillaries (the expression of GLUT1 in the capillary endothelial cells of brain has been well documented (Kasanicki *et al.*, 1989; Kalaria *et al.*, 1988)). The contribution of cell types other than the mammary secretory epithelium to the detection of glucose transporters in mammary homogenate was investigated more fully in the work described in Chapters 6 and 7.

CHAPTER 5

QUANTITATIVE WESTERN BLOTTING

5.1 Introduction

Prior to this study, R.J. Madon had measured the number of D-glucose-inhibitable cytochalasin B binding sites in rat mammary plasma membranes and Golgi membranes by using Scatchard analysis. This result had indicated the presence of 20 ± 2 pmol binding sites/mg plasma membrane protein and 53 ± 4 pmol binding sites/mg Golgi membrane protein, with K_d values of 259 ± 47 nM and 520 ± 43 nM respectively (Madon *et al.*, 1990a). By comparison, human erythrocyte membranes were found to contain 660 ± 31 pmol binding sites/mg membrane protein, with a K_d of 282 ± 24 nM (Madon *et al.*, 1990a). Assuming one cytochalasin B binding site/glucose transporter these findings suggested a significant difference between the abundance of glucose transporters in the mammary plasma membrane and Golgi membrane fractions, as well as a significant difference in the affinity for cytochalasin B in the Golgi membranes, compared to both plasma membranes and the human erythrocyte membranes. A difference of this magnitude in the abundance of glucose transporters in the two mammary membrane fractions should also be detectable by Western blotting. As shown in Section 4.3.2, only the GLUT1 transporter isoform was immunologically detectable in the mammary plasma membrane and Golgi membrane fractions, where immunoreactivity on Western blots revealed a similar intensity of signal for equal loadings by weight of membrane proteins of each. The fact that the Golgi membrane samples contained a greater quantity of cytochalasin B binding sites, and also, presumably, of transporters, suggested that all of the glucose transporters in the Golgi membranes might not be

immunologically reactive towards the antibodies used in the experiments described in Section 4.2. In order to try and make a more quantitative estimate of the number of immunologically cross-reactive GLUT1 transporters, as opposed to cytochalasin B binding sites, a quantitative Western blotting procedure was developed, using a range of human erythrocyte glucose transporter standards. Using these standards the amounts of immunologically cross-reactive GLUT1 protein in mammary plasma membranes and Golgi membranes were determined. A similar quantitative Western blotting procedure has been reported (Pardridge *et al.*, 1990) to determine the content of GLUT1 in blood-brain barrier membranes. Other factors which might influence the measurement of either the antibody- or the cytochalasin B-binding sites in the mammary membrane fractions were also investigated. For example, it was possible that the Golgi membrane transporter was becoming proteolytically degraded by a process that left the cytochalasin B binding site intact whilst destroying the antibody binding site. It is known that limited tryptic cleavage of the human erythrocyte transporter does not destroy its cytochalasin B binding ability (Cairns *et al.*, 1987). Such degradation could be occurring either within the cells themselves, or during the storage of the membranes following their preparation. To investigate these possibilities, comparative Western blotting was performed on both fresh mammary membranes and membranes that had been stored under various conditions, using antibodies raised against two different regions of the GLUT1 protein, specifically, part of the large, central, cytoplasmic loop and the C-terminal region.

5.2 Scatchard analysis of cytochalasin B binding to protein-depleted human erythrocyte membranes and calculation of GLUT1 content

Human erythrocyte membrane samples were employed as standards of known GLUT1 content in quantitative Western blotting experiments, since these membranes have been well-characterised and are known to contain only the GLUT1 isoform of the glucose transporter. Before use they were depleted of peripheral membrane proteins by the procedure described in Section 2.3.5. Such depletion, in particular of actin, removes all high affinity cytochalasin B binding sites that are unrelated to the glucose transporter and which therefore bind cytochalasin B in a D-glucose-insensitive manner. The concentration of D-glucose-sensitive cytochalasin B binding sites, and thus of transporter, in the membranes was determined by equilibrium dialysis using [4-^{3}H]cytochalasin B, as described by Zoccoli *et al.* (1978).

Equilibrium dialysis was carried out using perspex racks containing 100µl chambers that could be separated into two halves with dialysis membrane. Into one side of the chamber was loaded 40µl of a solution of between 0 and $75x10^{-7}$M unlabelled cytochalasin B containing $5x10^{-8}$M [4-^{3}H]cytochalasin B (13.5Ci/mmol) in 50mM Tris-HCl, pH 6.8. Into the other side was loaded 40µl of a membrane sample (1-2mg protein/ml) also in 50mM Tris-HCl, pH6.8, and ± 600mM D-glucose (final concentration 300mM D-glucose). The racks were sealed and shaken on a horizontal rotary mixer at room temperature overnight. An aliquot (25µl) was removed from each side of the chamber, placed into 4ml Ultima-Gold scintillant, and scintillation counted for 5min.

If the resultant radioactivity measured for the sample from the protein side of the dialysis membrane is 'x' d.p.m., and that for the sample on the buffer side is

'y' d.p.m., then for each given concentration of cytochalasin B [CB], the [bound ligand] can be expressed as:-

Eq. 5.1 $$[\text{bound}] = \{(x-y)/(x+y)\} \times [\text{CB}]$$

In calculating the ratio of bound/free ligand we know that :-

Eq. 5.2 $$x/y = (\text{bound}+\text{free})/\text{free} = (\text{bound}/\text{free}) + 1$$

therefore,

Eq. 5.3 $$\text{bound}/\text{free} = (x/y) - 1$$

A Scatchard plot of bound/free cytochalasin B versus [bound cytochalasin B] was obtained by taking measurements over a range of total cytochalasin B concentrations. From a plot of bound/free versus [bound], the slope of the line ($-1/K_d$) will yield the dissociation constant for binding, and the intercept on the abscissa the total concentration of binding sites (R_T). These conclusions are derived from the following analysis of the binding process :-

At equilibrium the binding of cytochalasin B (ligand, L) to the glucose transporter (receptor, R) can be expressed as:-

Eq. 5.4 $$R + L \rightleftharpoons RL$$

where RL is the bound ligand. Consequently the dissociation constant, K_d, can be

expressed as:-

Eq. 5.5 $K_d = [R][L]/[RL]$

Therefore, in an equilibrium situation, the ratio of bound to free ligand is expressed as:-

Eq. 5.6 $\text{bound/free} = [RL]/[L] = [R]/K_d$

The free glucose transporter concentration [R] is related to the total transporter concentration $[R]_T$ by the equation :-

Eq. 5.7 $[R]_T = [R] + [RL]$

Rearranging, we get the following :-

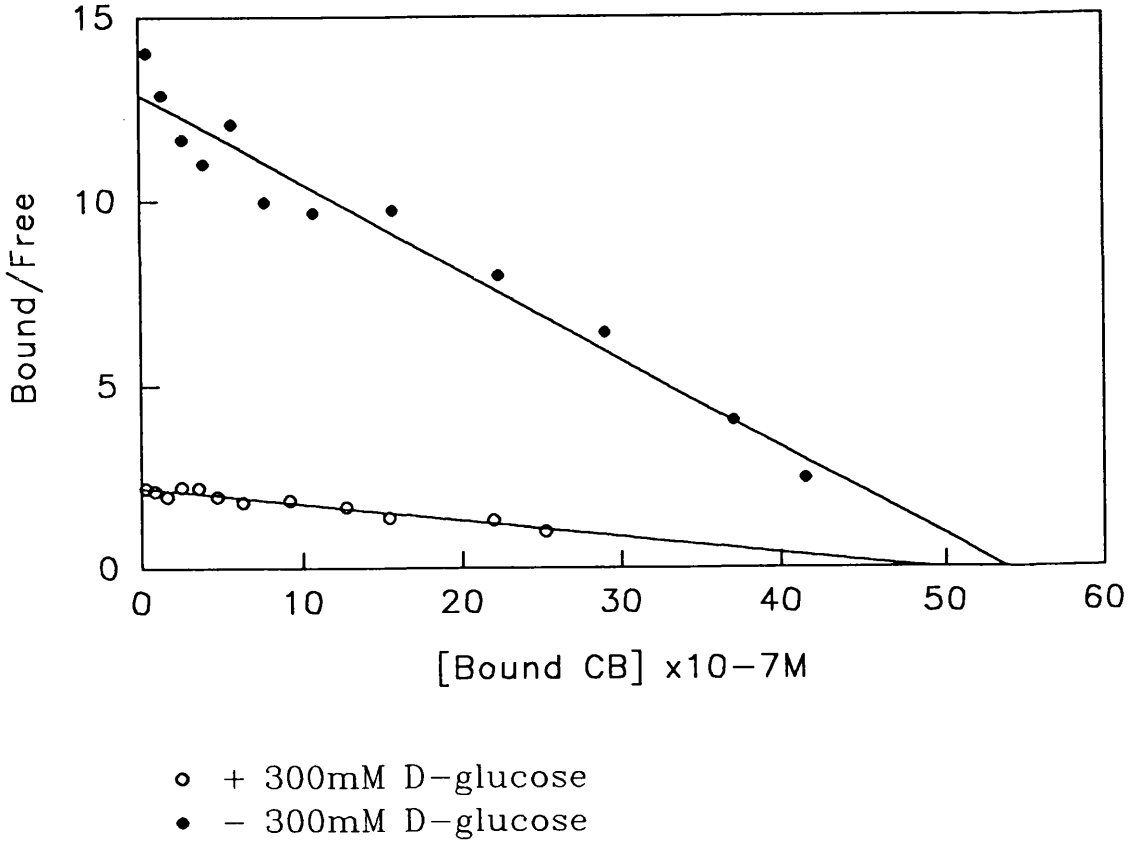
Eq. 5.8 $[R] = [R]_T - [RL]$

Substituting into Eq. 5.6 yields the following :-

Eq. 5.9 $\text{bound/free} = [R]_T/K_d - [RL]/K_d$

As shown in Fig. 5.1 the Scatchard plot of cytochalasin B binding to the human erythrocyte membranes was linear, indicating a single class of binding site. Binding was inhibited by D-glucose as shown in Fig. 5.1. The binding characteristics were

Fig. 5.1 Scatchard analysis of cytochalasin B binding to human erythrocyte membranes



Scatchard analysis of cytochalasin B binding to protein-depleted human erythrocyte membranes, indicating a single class of binding site. Analysis of the binding characteristics determined that the K_d for cytochalasin B binding was 3.67×10^{-7}M, and that GLUT1 constituted 2614pmol/mg membrane protein.

determined by linear regression, and the slope ($-1/K_d$) and the intercept on the x-axis (number of binding sites) calculated. The small amount of non-specific binding was assumed to have little effect on the calculated number of binding sites.

The content of GLUT1 in the protein-depleted human erythrocyte membranes was found to be 2614pmol/mg total membrane protein and the K_d determined as 3.67×10^{-7}M. Using this GLUT1 content, standards containing between 5 and 1,000ng glucose transporter in 10µl of Laemmli gel sample were prepared for SDS/polyacrylamide gel electrophoresis. Gel samples were stored at -20°C for a maximum of 2 weeks. Aliquots of protein-depleted membranes of a known GLUT1 content were stored at -70°C. Neither gel samples or membranes were freeze-thawed more than once before using.

5.3 Quantitative Western blotting

Scatchard analysis of the D-glucose inhibitable cytochalasin B binding to mammary plasma membranes and Golgi membranes had established that the latter contained twice as many cytochalasin B binding sites as there were in the former, whereas Western blots of the two membrane fractions in Section 4.3.2 showed an equivalent degree of immunoreactivity to GLUT1 in both fractions. The following quantitative Western blotting procedure was set up to measure the number of immunologically cross-reactive GLUT1 glucose transporters in both mammary membrane fractions, and so quantify specifically the GLUT1 content. If cytochalasin B were binding to other glucose transporter isoforms, or unrelated proteins, which are not immunologically cross-reactive to GLUT1, quantitative Western blotting should discern between the different cytochalasin B binding sites.

5.3.1 Linearity of the Western blotting signal relative to glucose transporter content

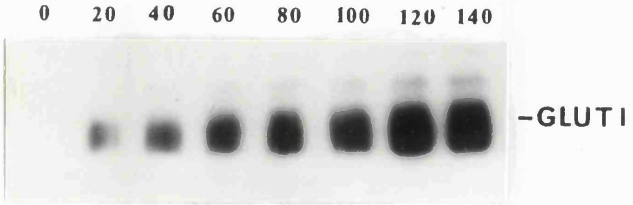
Using the protein-depleted human erythrocyte membrane gel sample standards prepared as described in Section 5.2, a calibration curve of the GLUT1 glucose transporter was set up. By running GLUT1 standards containing between 5-200ng glucose transporter, Western blotting and using a $[^{125}I]F(ab')_2$ anti-rabbit IgG detection system, the relationship between the amount of glucose transporter loaded onto the gel and the resulting signal was examined.

Samples of the GLUT1 standards were loaded onto 12% SDS/polyacrylamide 'mini-gels', and separated by electrophoresis. Following Western blotting onto nitrocellulose and immunodetection of the glucose transporters with an anti-GLUT1 *C*-terminal peptide antibody, followed by an $[^{125}I]F(ab')_2$ anti-rabbit IgG and autoradiography, the glucose transporters in the membranes were quantified in two ways. The density of the bands on the autoradiographs was determined by densitometry using a Chromoscan 3 densitometer at 530nm, and a calibration curve produced using the peak area of each band (in arbitrary units). Also, the radioactivity of each band was determined by aligning the autoradiograph over the radioactive nitrocellulose, and using it as a template to excise the radioactive bands. An equal area of nitrocellulose was cut out for each band, and two bands were taken from a blank part of the blot to serve as background controls. The radioactive pieces of nitrocellulose were placed into glass tubes and γ-counted using an LKB Gamma counter.

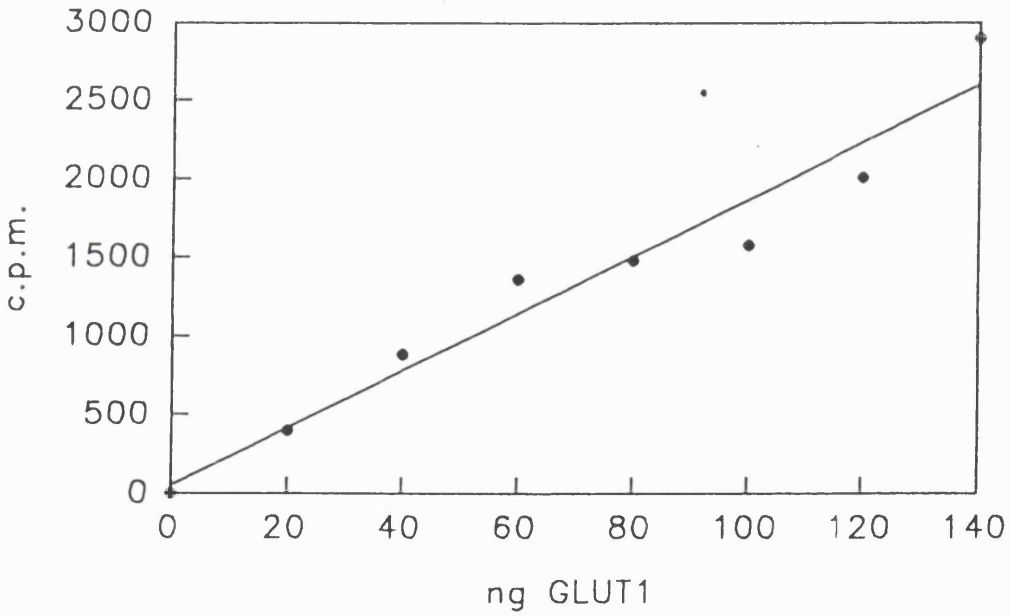
An example of the calibration curve determined using the -counting procedure is shown in Fig. 5.2. As can be seen, the response was linear over the range from 20-140ng of GLUT1. Both the detection methods gave essentially the same result. Although the response curve in Fig. 5.2 only ranges between 20-140ng GLUT1, the

Fig. 5.2 A calibration curve for determination of GLUT1 content in membrane samples by quantitative Western blotting

(a)



(b)



A calibration curve of GLUT1 was constructed using Western blotting, and detected using [^{125}I]F(ab')$_2$ anti-rabbit IgG followed by autoradiography, as described in Section 2.6. The autoradiograph, shown in (a), contains between 0-140ng GLUT1 in protein-depleted human erythrocyte membranes, determined as described in Section 5.2. The calibration curve in (b) was constructed by γ-counting the radioactive bands of (a), and the straight line obtained by linear regression. A similar linear relationship was obtained when the bands were measured densitometrically.

response was found to be linear up to 1μg glucose transporter (results not shown) and, most importantly, over the range of 10-30ng GLUT1, the range most frequently used for the quantitation of the cross-reactive mammary membrane GLUT1 protein.

5.3.2 Quantification of mammary glucose transporters

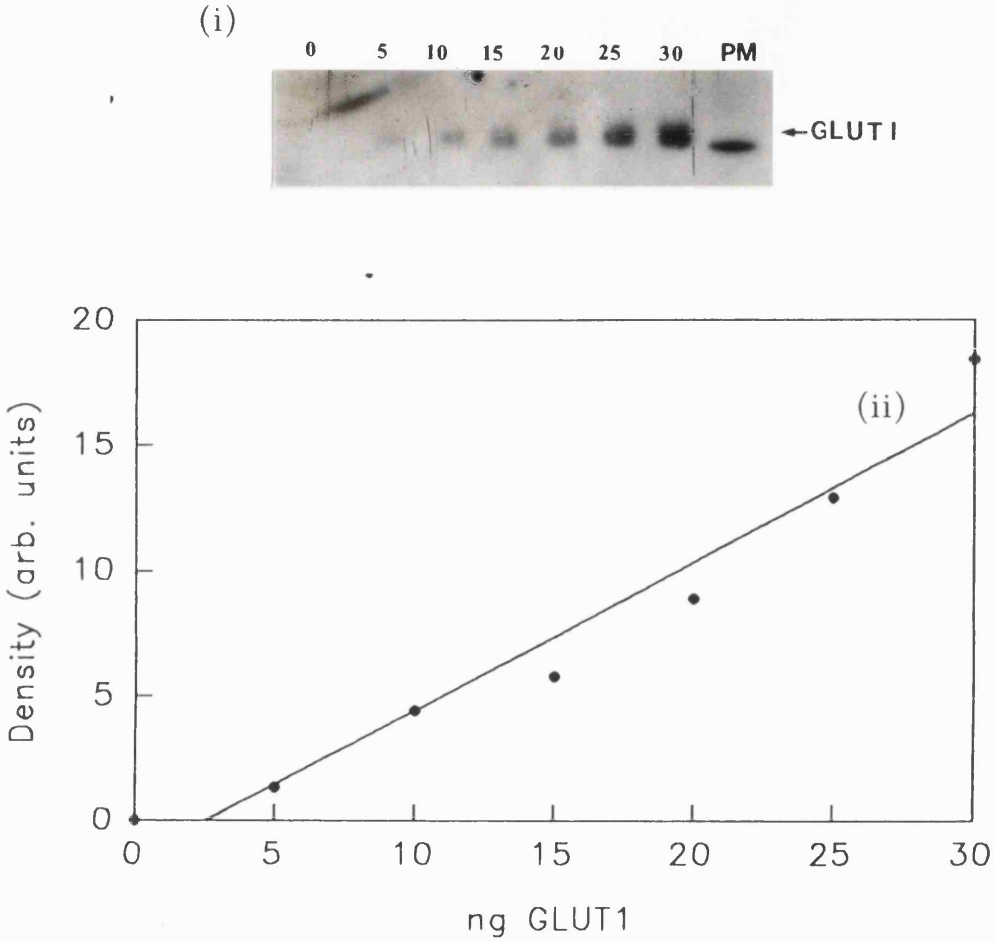
For quantitative Western blotting, 10μg samples of mammary plasma membranes and Golgi membranes were electrophoresed on 12% SDS/polyacrylamide gels alongside erythrocyte membrane standards containing 5-30ng of GLUT1, which were used to construct a calibration curve. Inclusion of standards on the same gel as the mammary membrane samples was found to be necessary because of variations noted in the efficiency of electrophoretic transfer to nitrocellulose, and in the efficiency of immunological staining, between gels run on separate occasions.

The signal obtained from the mammary membranes was evaluated by both densitometry and γ-counting, together with the signal from the standards on the calibration curve. Linear regression was used to obtain the best fit through the points.

Representative quantitative Western blots of both plasma membranes and Golgi membranes are shown in Fig. 5.3 (a) & (b) respectively. Densitometric and γ-counting measurements gave comparable results for both the calibration curves and the pmol GLUT1/mg protein, calculated to be present in the mammary membranes using those calibration curves. A quantitative estimate of the pmol of cross-reactive GLUT1 in both mammary membrane fractions was made for each blot, using the associated calibration curve, assuming an M_r 54,116 for GLUT1 (Mueckler *et al.*, 1985). Table 5.1 shows the mean pmol cross-reactive GLUT1 / mg protein

Fig. 5.3 Representativequantitative Western blots of mammary plasma membranes and Golgi membranes

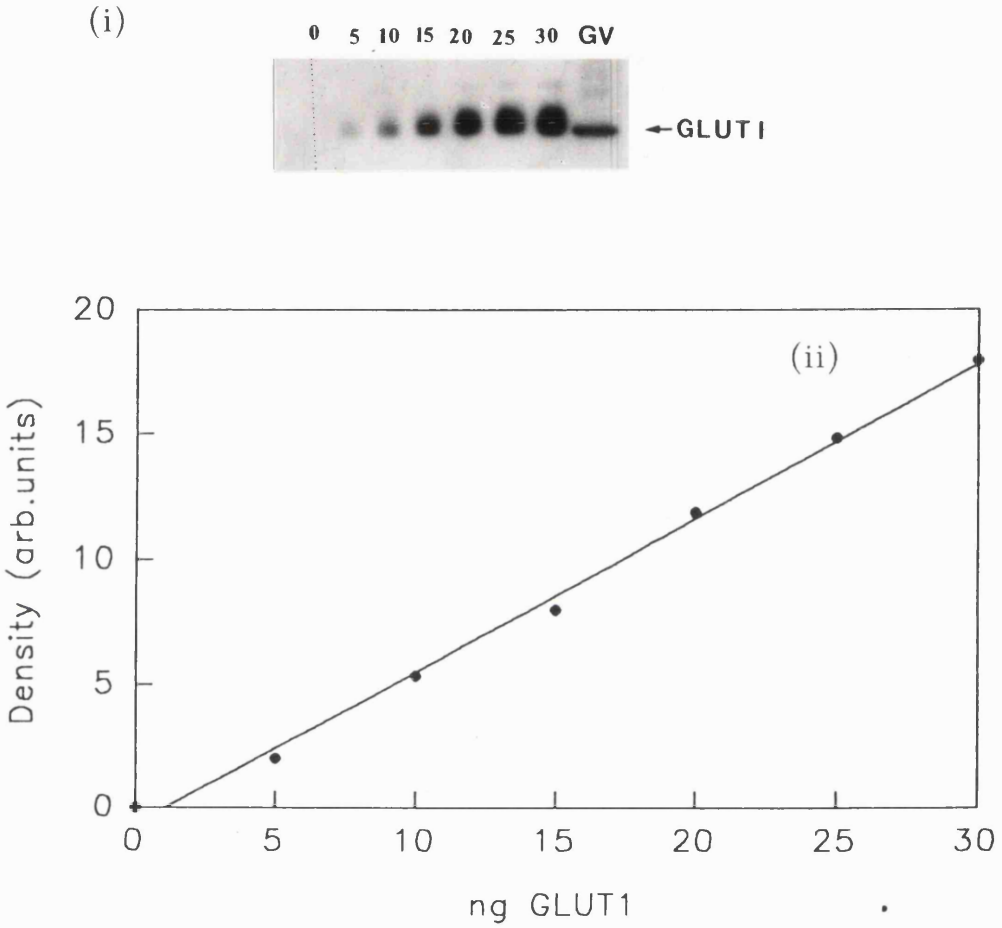
(a) Plasma membrane



This figure shows a quantitative Western blot of mammary plasma membranes. On the autoradiograph (i), lanes 1-7 contain between 0-30ng of GLUT1 in protein-depleted human erythrocyte membranes, as determined in Section 5.2, and lane 8 contains 10μg mammary plasma membranes. Using densitometry a calibration curve of GLUT1 content was constructed (ii), from which the GLUT1 content of the mammary plasma membranes was calculated.

Fig. 5.3 Representative quantitative Western blots of mammary plasma membranes and Golgi membranes

(b) Golgi membranes



This figure shows a quantitative Western blot of mammary Golgi membranes. On the autoradiograph (i), lanes 1-7 contain between 0-30ng of GLUT1 in protein-depleted human erythrocyte membranes, as determined in Section 5.2, and lane 8 contains 10μg mammary Golgi membranes. Using densitometry a calibration curve of GLUT1 content was constructed (ii), from which the GLUT1 content of the mammary Golgi membranes was calculated.

Table 5.1 GLUT1 content of mammary sub-cellular fractions

	#Cytochalasin B			Western Blotting
Membrane	$B_{max.}$ (pmol/mg) (protein)		K_d (nM)	[Transporter] (pmol/mg) (protein)
Human erythrocyte membranes	660 ± 31	(5)	282 ± 24	
Rat mammary plasma membrane	20 ± 2	(5)	259 ± 47	30 ± 10 (3)
Rat mammary Golgi membrane	53 ± 4*	(3)	520 ± 43♦	25 ± 3 (2)

Table 5.1 shows the GLUT1 content of sub-cellular fractions of mammary gland as determined by quantitative Western blotting compared to Scatchard analysis of D-glucose-inhibitable cytochalasin B binding (# binding sites as determined by R. J. Madon). The results presented for the Western blotting were obtained by -counting the radioactive area of the blots, however, values obtained by densitometry of the autoradiographs were not significantly different. Blotting values are means ± S.E.M. for a total of (n) preparations, where each n= an average of >3 blots (total number of blots for plasma membranes = 9, and for Golgi membranes = 7). ♦Significantly different from the K_d values for the human erythrocyte membranes and rat mammary plasma membranes ($p<0.01$), *significantly different from rat mammary plasma membranes ($p<0.001$), Student's unpaired *t*-test.

in the mammary plasma membranes and Golgi membranes determined by the quantitative Western blotting, together with the number of cytochalasin B binding sites measured by R.J. Madon, for mammary membrane fractions prepared under identical conditions. Mammary plasma membranes were found to contain 30 ± 10 pmol GLUT1 / mg protein and mammary Golgi membranes contain 25 ± 3 pmol GLUT1 / mg protein. Three different plasma membrane preparations were quantified, using a total of 9 blots, and two different Golgi membrane preparations using a total of 7 blots.

From the quantitative Western blotting, the mammary membrane fractions appear to have similar numbers of immunologically cross-reactive GLUT1 transporters, whilst the reported number of cytochalasin B binding sites in the Golgi membranes is double that of the plasma membranes (Madon *et al.*, 1990a). The tentative conclusion that can be drawn from these results is that more than one class of cytochalasin B binding site exists in the mammary Golgi membranes and this possibility will be discussed in Section 5.6.

5.4 Western blotting using antibodies against a different region of the GLUT1 protein

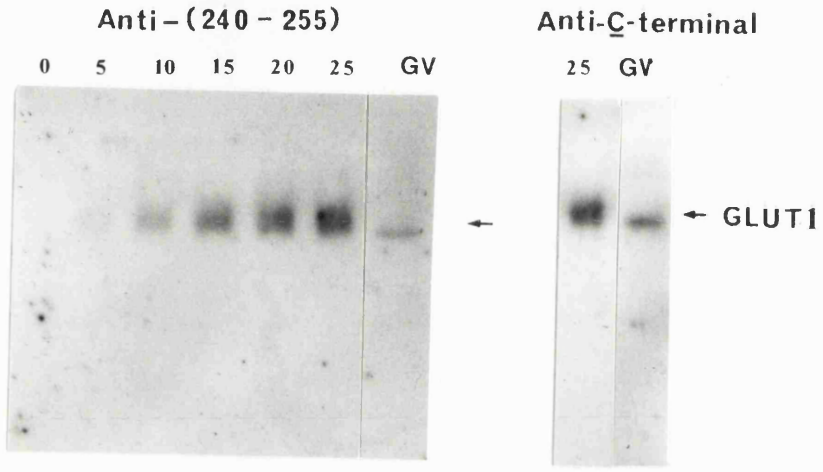
The antibodies used for the quantification of Western blots in Section 5.3.2 were raised against the *C*-terminal peptide sequence (residues 477-492) of the GLUT1 glucose transporter. A possible explanation for the apparent difference between the number of cytochalasin B binding sites and the immunologically cross-reactive GLUT1 detected in the mammary Golgi membranes could be that the *C*-terminal peptide epitopes used to raise the antibodies were being degraded in the Golgi membrane transporters. Additional quantitative Western blotting experiments were

therefore performed using a different antibody, raised against residues 240-255 in the large, central, cytoplasmic loop of GLUT1. The use of this antibody to detect mammary glucose transporters was described in Section 4.5.

Western blotting was performed as described in Section 5.3.2 except that the primary antibodies were raised against either GLUT1 residues 240-255 or the *C*-terminal peptide. The resulting autoradiographs were again quantified using densitometry and -counting. Fig. 5.4 shows Western blots of mammary Golgi membranes, together with 25ng GLUT1 in human erythrocyte membrane standards, visualized using anti-(240-255) peptide antibodies or the anti-*C*-terminal peptide antibodies. The intensity of staining seen on blots of both the human erythrocyte membrane samples and the mammary membrane samples was approximately the same using either antibody to detect GLUT1. Quantification of the mammary Golgi membrane GLUT1 using the *C*-terminal peptide antibodies gave 16.5pmol GLUT1/mg protein, while using the anti-(240-255) antibodies 18.9pmol GLUT1/mg protein was found (blots not shown). This suggested that there was no significant degradation of the GLUT1 *C*-terminal compared to the central cytoplasmic loop of GLUT1 in the mammary Golgi membranes. Therefore the loss of the *C*-terminus could not account for the lower level of immunologically detectable GLUT1 compared to cytochalasin B binding sites in the Golgi membranes.

Also shown in Fig. 5.4 are the relative intensities of GLUT1 in mammary plasma membrane samples (10μg) or human erythrocyte membranes containing 25ng GLUT1 also detected using both the anti-(240-255) peptide antibodies or the anti-*C*-terminal peptide antibodies. Quantitative Western blotting of mammary plasma membrane GLUT1 using antibodies against the *C*-terminal peptide gave 35.6pmol GLUT1/mg protein compared to 43.5pmol GLUT1/mg protein detected using anti-

Fig. 5.4 Western blotting using antibodies against a different region of GLUT1



Human erythrocyte membrane standards containing between 0-25ng GLUT1 and 10μg of mammary Golgi membranes (GV) were Western blotted and GLUT1 labelled using antibodies against the residues 240-255. GLUT1 was also labelled in human erythrocyte membranes containing 25ng GLUT1 and 10μg of mammary Golgi membranes using anti-*C*-terminal peptide antibodies. In both cases GLUT1 was detected using an iodinated second antibody and autoradiography.

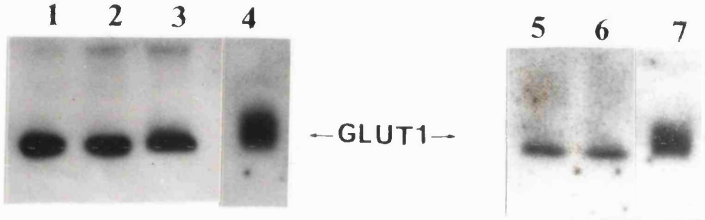
(240-255) antibodies. This result showed no significant difference in the concentration of GLUT1 in the mammary plasma membranes detected using antibodies raised against the central cytoplasmic loop of GLUT1, compared to the *C*-terminus.

5.5 Investigation of the effect of storage conditions on the detectable GLUT1 content of mammary membranes

To look at the effect of membrane storage on the stability of GLUT1, equal amounts (10μg protein) of mammary plasma membranes and Golgi membranes were Western blotted either immediately following preparation or after storage at -70°C. Mammary plasma membranes and Golgi membranes were electrophoresed on 12% SDS/polyacrylamide gels, Western blotted and the glucose transporter detected using anti-GLUT1 *C*-terminal peptide antibodies followed by an iodinated anti-rabbit IgG. The autoradiographs in Fig. 5.5 show mammary plasma membranes either freshly prepared, stored at -70°C for 48hr, or stored at -70°C for 6 months, electrophoresed at the same time as a single sample containing 15ng GLUT1. Also shown in Fig. 5.5 are mammary Golgi membranes either freshly prepared, or stored at -70°C for 6 months, again electrophoresed at the same time as a single sample containing 15ng GLUT1.

No significant decrease in the GLUT1 content of either the mammary plasma membranes or Golgi membranes was found following storage at -70°C. This observation strongly suggested that there was no significant proteolytic degradation of the glucose transporter in either of the mammary membrane fractions during storage.

Fig. 5.5 Western blots illustrating the effectof storage on the immunoreactivity of the mammary membrane GLUT1



The GLUT1 contents of samples (10μg) of mammary plasma membranes, either freshly prepared (1), or stored at -70°C for 48hr (2) or 6 months (3), and samples (10μg) of mammary Golgi membranes, either freshly prepared (5) or stored at -70°C for 7months (6) were determined by Western blotting using antibodies raised against the GLUT1 *C*-terminal peptide. Also shown are 15ng of GLUT1 (4,7) in protein-depleted human erythrocyte membranes. All bound antibodies were detected using an iodinated second antibody and autoradiography.

5.6 Conclusion

The quantitative Western blotting procedure developed for the GLUT1 glucose transporters was a reproducible and simple way to compare the levels of the GLUT1 protein in different membrane preparations. Following Western blotting, both the density of the band obtained on the autoradiograph, and its radioactivity, were linear in relation to the amount of human erythrocyte membrane standards loaded onto the gel.

For the mammary plasma membrane and Golgi membrane the GLUT1 content measured by the quantitative Western blotting procedure was found to be 30 ± 10 pmol/mg protein and 25 ± 3 pmol/mg protein (Table 5.1) respectively. For the mammary plasma membranes this value was very similar to the concentration of 20 ± 2 pmol D-glucose-inhibitable cytochalasin B binding sites / mg protein found by R.J. Madon. However, for the Golgi membranes the concentration of immunologically-detectable GLUT1 is approximately half that of the concentration of D-glucose-inhibitable cytochalasin B binding sites, which has been reported to be 53 ± 4 pmol / mg protein (Madon *et al.*, 1990a). This disparity might arise merely from the fact that different mammary membrane preparations were used for the cytochalasin B binding measurements and for the Western blots. Unfortunately the very large amount of material required for the cytochalasin B binding assays precluded repetition of both types of measurements on the same preparations. However, the membranes used for the Western blotting were prepared in an identical fashion to, and in the same laboratory as, those that had been used by R.J. Madon, and there was little variation between the preparations as judged from analysis of membrane marker enzymes.

A second explanation for the detection of less than expected numbers of glucose

transporters in Western blots of Golgi membranes might have been selective proteolytic degradation of the transporters in this fraction. However, this is unlikely because Western blotting against Golgi membranes stored under the conditions used to store those membranes used by Madon *et al.* (1990a)for the cytochalasin B binding measurements, as well as blotting using an antibody to a central cytoplasmic loop of GLUT1 showed no evidence to suggest preferential GLUT1 degradation in the Golgi membranes. Unfortunately quantitative Western blotting using antibodies raised against regions of the GLUT1 protein not susceptible to proteolytic cleavage, such as the small cytoplasmic loops, could not be performed because these antibodies had been shown in Section 4.5 to stain Western blots of mammary membrane fractions only weakly.

It is known that cleavage of the *C*-terminal domain and the large, central, cytoplasmic loop of the GLUT1 protein reduces the affinity for cytochalasin B without affecting the binding capacity (Cairns *et al.*, 1987). Substantial cleavage of either the *C*-terminus or the central cytoplasmic loop would show up as a change of M_r on the Western blots, and cleavage of both regions would result in a reduced immunoreactivity of the mammary membranes. At no time were bands of a lower M_r than that of the mammary GLUT1 ($M_r$ave 50,000) seen using antibodies against either the *C*-terminal or the 240-255 peptides, although occasionally higher M_r bands were seen indicating that there were aggregates of the glucose transporter present. Similarly, storage of the mammary membranes caused no substantial decrease in the amount of antigenic GLUT1 glucose transporter compared to fresh membranes. The membranes used for determination of the immunoreactive GLUT1 glucose transporter were, therefore, assumed to be equivalent to those used in the determination of the cytochalasin B binding sites by Madon *et al.* (1990a).

The conclusion tentatively drawn from the quantitative Western blotting was that there are one, or more, types of cytochalasin B binding site present in the mammary Golgi membrane-enriched fraction, other than the GLUT1 glucose transporter. The cytochalasin B binding measurements made by R.J. Madon on the Golgi membranes were performed in the presence of cytochalasin E, which is a compound related to cytochalasin B, and which is an inhibitor of cytochalasin B binding to peripheral membrane proteins such as the polymerising ends of actin filaments, but not to GLUT1. The presence of cytochalasin E during the cytochalasin B binding measurements, together with the D-glucose-sensitivity of the binding indicated that it is reasonable to assume that the extra binding is to a different isoform of the glucose transporter family. The results of Western blotting lactating mammary gland for the five other characterised facilitative D-glucose transporters, shown in Fig. 4.1, had indicated that only GLUT4 was also present in crude homogenate. However GLUT4 is not present in Golgi membrane fractions (Section 4.3.2) or in isolated epithelial cells (see Section 7.5). Therefore, the cytochalasin B binding sites probably do not correspond to one of the known isoforms.

There was also a significant difference in the K_d of cytochalasin B binding to the mammary Golgi membranes, K_d 520 ± 43 nM, compared to the mammary plasma membrane and the human erythrocyte membranes, K_d 259 ± 47 nM and K_d 282 ± 24 nM respectively (Madon *et al.*, 1990a). Again this indicates that there could be a second type of cytochalasin B binding site present in the Golgi membranes. However, the presence of a substantial quantity of GLUT1 in the Golgi membranes, as revealed by quantitative Western blotting, should result in the detection of a component of approximate K_d 280nM (Golgi membrane GLUT1) in the cytochalasin

B binding experiments on Golgi membranes which, in conjunction with a second transporter species of a higher K_d, should give rise to biphasic Scatchard plots. Such behaviour was not observed by Madon *et al.* (1990a). Given the evidence that the intrinsic activity of the glucose transporters can be altered (Section 1.9.3) it is also possible that the Golgi GLUT1 protein is modified so that its affinity for cytochalasin B is halved, and its affinity for the antibodies is also reduced. In this case the 'apparent' reduction in the number of antibody binding sites could reflect the decrease in the affinity of the antibodies for the Golgi GLUT1 protein.

CHAPTER 6

IMMUNOCYTOCHEMICAL LOCALISATION OF THE MAMMARY GLUCOSE TRANSPORTER

6.1 Introduction

The mammary gland is a highly developed and complex tissue containing a number of different cell types. Therefore, although the glucose transporter content of whole mammary gland homogenate and sub-cellular fractions is likely to reflect that of the most abundant cell type, the secretory epithelial cell, a significant contribution from other cell types to the results discussed in previous chapters cannot be ruled out. As well as the secretory epithelial cells and myoepithelial cells, less frequent cell types include the capillary endothelial cells, fibroblasts, adipocytes and nerve cells. The effect of adipocytes on the detected levels of the mammary glucose transporters was of most interest since adipocytes are known to express both the GLUT1 and GLUT4 isoforms of the glucose transporter. The expression of both these isoforms by mammary tissue has been described by Burnol *et al.* (1990), although the cell types involved were not specifically identified. It was suggested that the GLUT4 and some of the GLUT1 isoform identified using Western blotting of mammary homogenate originated from contamination of the epithelial cell population with mammary adipocytes. Capillary endothelial cells express high levels of GLUT1 in some tissues where glucose is vital, for example mammalian brain (Kasanicki *et al.*, 1989; Kalaria *et al.*, 1988), and similar findings have been reported for the perineurial sheath of peripheral nerves (Froehner *et al.*, 1988). Therefore, the possible contribution of other cell types to the total glucose transporter pool in mammary gland was investigated.

Immunocytochemistry was used to detect glucose transporters within the lactating mammary gland, at the level of the light microscope. It was hoped to distinguish between different cell types, and identify those which express the highest levels of the GLUT1 and GLUT4 isoforms of the glucose transporter. It was also hoped that immunocytochemistry would be able to immunolocalise the glucose transporter within the mammary epithelial cells to distinguish whether the apical or baso-lateral plasma membrane face of the secretory epithelial cells contained the protein, and whether there were any transporters in an intracellular location. The lack of GLUT1 in the milk fat globule membrane determined by Western blotting, shown in Section 4.3.2, had indirectly suggested that GLUT1 was not present in the apical plasma membrane.

The detection method used for immunocytochemistry was a silver-enhanced immunogold technique, where a gold-conjugated anti-rabbit IgG was used to detect anti-glucose transporter *C*-terminal peptide antibodies bound to the glucose transporters in tissue sections. The gold signal is intensified using silver enhancement and detected by light microscopy. Various tissues were used, including mammary gland, brain and liver, all of which were prepared by perfusion fixation of the whole rat.

6.2 Perfusion fixation

The perfusion fixation technique required two people, one holding an appropriate animal licence, and an assistant, and was performed by Dr. P. Bagley and Mr. C. Nolan at the MRC toxicology laboratories, Carshalton, London, during which I assisted.

Tissues were prepared by *in vivo* perfusion fixation of Porton-derived Wistar rats

(250-300g) between days 4-7 of lactation. Female rats were ether-anaesthetized and the heart freed from surrounding tissue, whilst the vascular connections were left intact. Heparin (500i.u.) was injected into the vascular system, through the left ventricle, to inhibit blood clotting. The aorta was cannulated through the left ventricle and the blood washed out through a small slit in the right ventricle by perfusion using 0.9% saline at a pressure of 120mmHg. *In vivo* fixation, with either neutrally buffered 4% formaldehyde or formal-acetic (4% formaldehyde, 2% acetic acid), was maintained at a pressure of 120-140mmHg for 12min, in a fume hood. The efficiency of perfusion and fixation was estimated by the changing colour of the liver, an even blanching indicating a successful perfusion. The whole body was left in fixative overnight at 4°C, and the following day the tissues of interest were dissected free, cut into small pieces and embedded in paraffin wax. The brains were sliced coronally before embedding.

Embedding in paraffin wax was performed by processing tissue blocks in 80% methanol for 1hr, 90% methanol for 1hr, five changes of absolute methanol for 1hr each, chloroform for 1hr and finally chloroform overnight. The sections were then embedded into pure PARAPLAST paraffin wax at 56°C, using two 1hr incubations without vacuum and two incubations for 1hr under vacuum. The blocks were then cast and cooled rapidly. Tissues embedded in paraffin wax in this way were stable indefinitely and stored at room temperature.

Sections of mammary tissue (6μm) were cut using a microtome, warmed on a 45°C water bath and picked up on slides coated with the adhesive gelatin. Some sections were stained with haematoxylin and eosin (H&E) to determine maintenance of tissue morphology. The mammary tissue, as judged by a veterinary report on the H&E stained sections, was found to be well maintained in most of the samples

prepared. The tissues with a poor morphology following fixation were not used for later immunodetection of the glucose transporters.

6.3 Localisation of glucose transporters by immunogold silver staining

The major motive for the immunocytochemistry was to identify the cell types expressing the various isoforms of the glucose transporters within the lactating rat mammary gland. The two isoforms of most interest were GLUT1 and GLUT4, as both were expressed in mammary tissue as judged by Western blotting, Section 4.2. As a control for the GLUT1 antibodies rat brain sections were used, as brain is enriched in the GLUT1 isoform of the glucose transporter (Bagley *et al.*, 1989) in particular the capillaries at the blood-brain barrier (Kasanicki *et al.*, 1989; Kalaria *et al.*, 1988). The following results are divided into sections dealing with the expression of various isoforms of glucose transporters in different tissues from mid-lactating rats.

Paraffin-embedded sections mounted on gelatin-coated slides were de-paraffinised with 2 changes of xylene, for 90sec each, and rehydrated through a series of 2x 100%, 90%, 80% and 70% ethanol incubatios, then rinsed with PBS (Sörensen's phosphate buffer: 0.1M sodium phosphate, pH7.4, containing 0.225% cold-water fish skin gelatin, 0.8% sodium chloride and 0.1% sodium azide). Normal goat serum was applied at a 1:5 dilution in PBS for 20min to block non-specific binding, the excess tipped off, and primary antibody applied at a 1:200 dilution for antiserum and at 50μg/ml for purified antibodies, for 1hr. Sections were washed extensively in PBS, and the second antibody, a gold conjugated anti-rabbit IgG (Auroprobe LM GAR G5; Janssen Life Science Products (U.K.)) applied at a dilution of 1:50 in PBS for 40min. Sections were again washed extensively with PBS, rinsed

with distilled water, then silver-enhanced according to the protocol supplied with the Intense M silver enhancing kit (Janssen Life Science Products), for 20min.

6.3.1 Brain from lactating rat

The presence of glucose transporters within the brain of lactating rats was investigated using affinity-purified antibodies against the *C*-terminal peptides of the GLUT1 and GLUT4 isoforms, and also antisera raised against either the GLUT1 *C*-terminal peptide or purified GLUT1 protein. As controls, pre-immune serum and normal rabbit IgG (Sigma) were used.

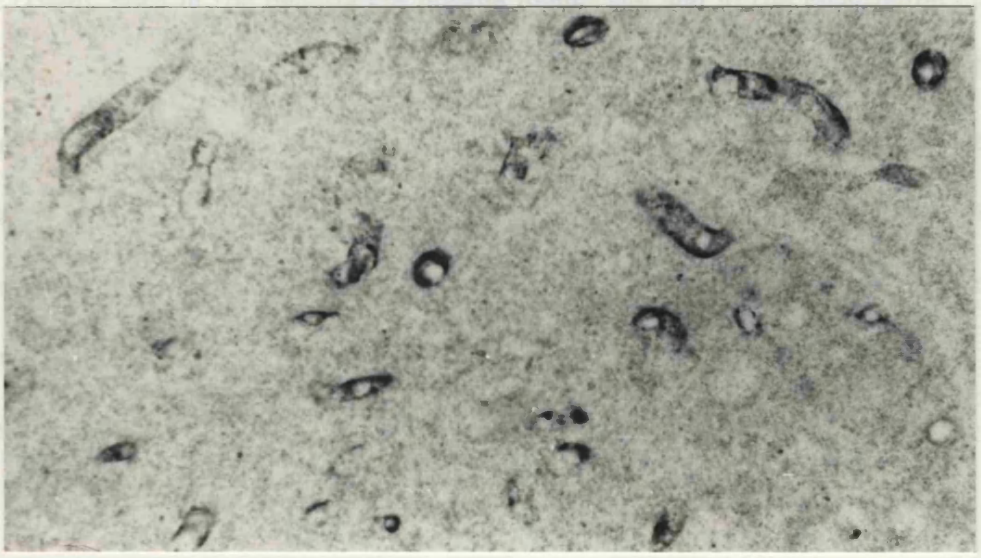
The distribution of GLUT1 in rat brain neocortex detected using the anti-GLUT1 *C*-terminal peptide antibodies, shown in Fig. 6.1(a), was found to be restricted to the microvessels forming part of the blood-brain barrier, visualised at a 40x magnification. The GLUT1 distribution in coronal sections of brain, visualised at a lower magnification (10X), had an intensity and pattern of staining corresponding to that seen by Bagley *et al.* (1989) (results not shown). The antiserum raised against the purified GLUT1 protein gave the same level of staining as the anti-GLUT1 *C*-terminal peptide antibodies. Pre-immune serum, antibodies raised against the GLUT4 *C*-terminal peptide (results not shown) and control rabbit IgG, shown in Fig. 6.1(b), gave no staining in rat brain.

6.3.3 Liver from lactating rat

The distribution of GLUT2 was investigated in liver sections, taken at the same time as the mammary tissue, to ensure that the anti-GLUT2 *C*-terminal peptide antibodies would react against the intact GLUT2 protein. Non-specific IgG was used as a control antibody. The method of staining has been described in Section 6.3. The

Fig. 6.1 Localisation of GLUT1 in rat neocortex

(a) GLUT1



(b) Rabbit IgG

The GLUT1 glucose transporter was localised in the rat brain neocortex by immunocytochemistry using the silver-enhanced immunogold technique, detailed in Section 6.3. GLUT1 detected using antibodies raised to the GLUT1 *C*-terminal peptide (a) was restricted to microvessels, while non-specific rabbit IgG (b) gave no detectable labelling. (40x mag.)

results in Fig. 6.2(a) show that the GLUT2 staining was restricted to the sinusoidal surfaces of the liver cells adjacent to the blood supply, as reported by Thorens *et al.* (1990), both in the hepatocyte plasma membrane and in the endothelial cells. The non-specific IgG (Fig. 6.2(b)) did not stain the tissue. These results confirm that the anti-GLUT2 antibodies react with the liver isoform of the glucose transporter, and that the antibodies raised against the GLUT2 *C*-terminal peptide recognise the intact transporter protein in its native state. These antibodies could therefore be used to look for GLUT2 in the mammary gland by immunocytochemistry.

6.3.3 3T3-L1 adipocytes

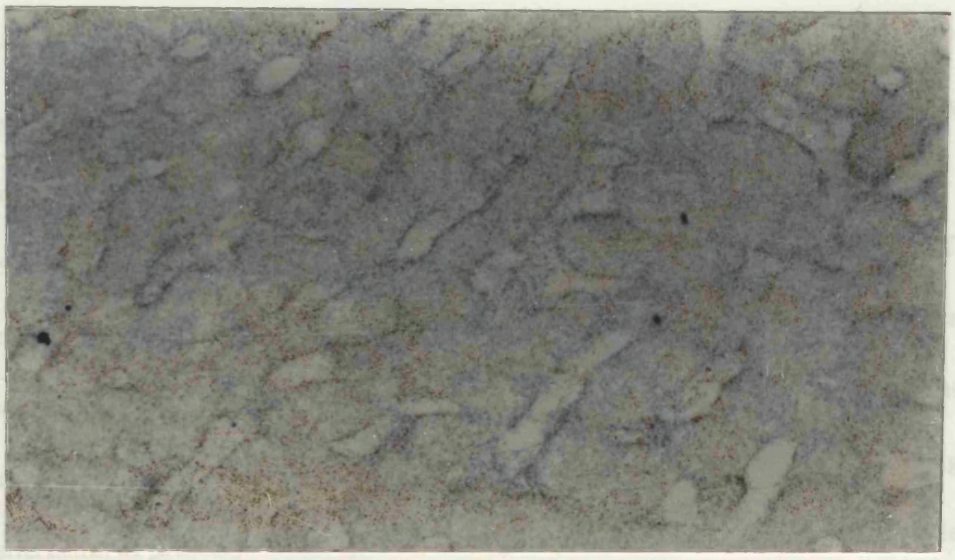
The distribution of GLUT4 in 3T3-L1 adipocytes, in both the basal and insulin-stimulated state, was investigated by Dr. Kan, to ensure that the antibodies raised against the *C*-terminal peptide of GLUT4 would recognise the intact protein. Antibodies at a dilution of 10μg/ml were found to stain a translocatable GLUT4 protein in paraformaldehyde-fixed, Triton X-100 permeabilised 3T3-L1 adipocytes, which had been grown on coverslips. GLUT4 was detected using an fluoroisothiocyanate-conjugated anti-rabbit IgG second antibody, diluted 1:50, and immunofluorescence, visualised using a Bio-Rad confocal microscope. These results confirmed that the anti-GLUT4 *C*-terminal peptide antibodies recognise and bind to the intact GLUT4 protein (results not shown).

6.3.4 Lactating rat mammary gland

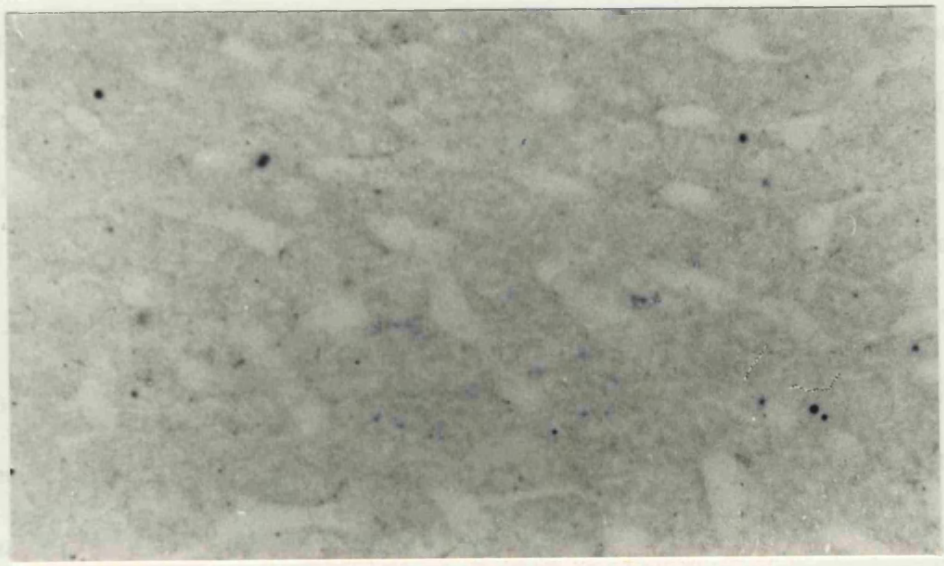
The distribution of GLUT1, GLUT2 and GLUT4 in the lactating rat mammary gland was investigated using antibodies to the *C*-terminal peptides of these

Fig. 6.2 Localisation of GLUT2 in rat liver

(a) GLUT2



(b) Non-specific IgG



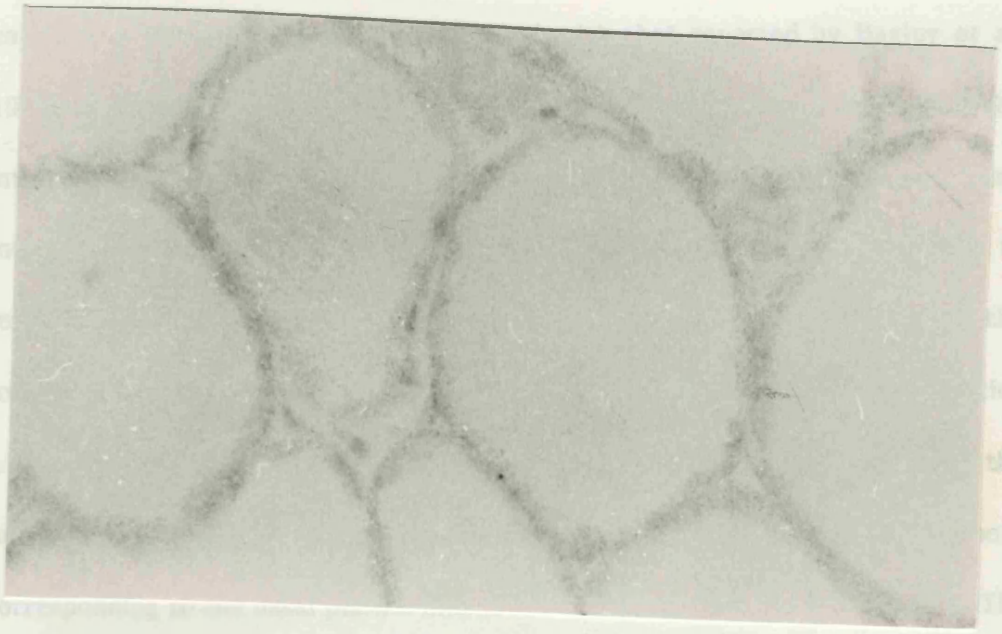
The GLUT2 glucose transporter was localised in the rat liver using the method described in Section 6.3. GLUT2 was detected using antibodies raised against the GLUT2 *C*-terminal peptide (a). Non-specific rabbit IgG (b) was used as a control antibody. GLUT2 was localised in the membranes adjacent to the sinusoidal space. (40x mag.)

transporter isoforms. From the results described in Sections 6.3.1-6.3.3, all of these antibodies had been shown to react to the native form of the glucose transporters, by immunocytochemistry of tissues known to express the specific isoforms. Both GLUT1 and GLUT4 had been shown to be present in mammary gland using Western blotting in Section 4.2. The method used for the mammary gland immunocytochemistry is described in Section 6.3.

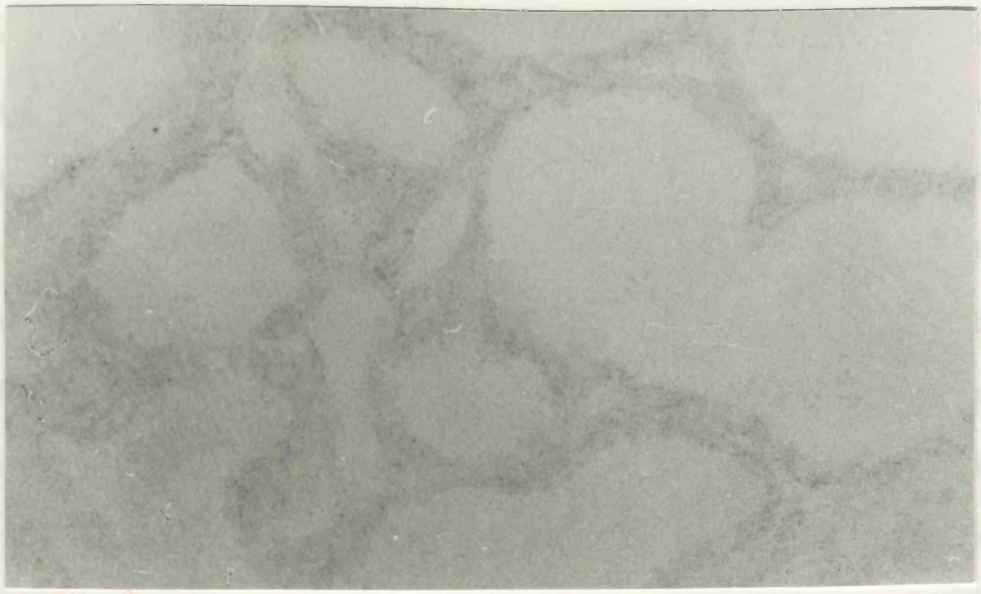
The photographs in Fig. 6.3(a) show that there is detectable expression of GLUT1 in the lactating mammary gland. The extent of labelling shown in Fig. 6.3(a) was characteristic of that obtained using the affinity-purified anti-GLUT1 *C*-terminal peptide antibodies. The intensity of staining was never very high despite the high concentration of primary antibody used. Using antiserum raised against the purified GLUT1 protein the intensity of staining was no higher than when using the antibodies against the *C*-terminal peptide (results not shown). The most intense labelling appeared around the periphery of the alveoli, presumed to be the basal epithelial cell plasma membrane, schematically illustrated in Fig. 1.2. The cytoplasm also appeared more densely stained using the specific antibodies, than when the non-specific IgG was used, although it was not possible to distinguish any intra-cellular structures. No intense staining was seen in the space between the alveoli. The pre-immune serum, control rabbit IgG (Fig. 6.3(b)), anti-GLUT4 antibodies or anti-GLUT2 antibodies gave no detectable labelling. Unfortunately, it was not possible to distinguish adipocytes in the mammary tissue. However, from the sections it can be seen that the majority of cells in the tissue are secretory epithelial cells, which implies that contamination with other cell types is minimal.

Fig. 6.3 Localisation of GLUT1 in lactating rat mammary gland

(a) GLUT1



(b) Non-specific IgG



The GLUT1 glucose transporter was localised in mammary gland using the method detailed in Section 6.3. The distribution of GLUT1 was detected using antibodies raised against the GLUT1 *C*-terminal peptide (a). The most intense labelling is restricted to the interstitial surface of the secretory alveoli, probably corresponding to the basal plasma membrane of the epithelial cells. There was also a higher staining in general over the surface of the cytoplasm, compared to the non-specific rabbit IgG (b). (40x mag.)

6.4 Conclusion

The distribution of GLUT1 observed in the rat brain using the immunogold silver-staining method, shown in Fig. 6.1, agreed with that reported by Bagley *et al.* (1989) suggesting that the methodology was working in these fixed rat tissues. The anti-GLUT1 antibodies were used at a concentration of 50μg protein/ml and antisera at a dilution of 1:200, which is higher than the concentration required to detect GLUT1 in the brain, as reported by Bagley *et al.* (1989), but was the concentration required to detect GLUT1 in mammary gland, as described in Section 6.3.4. Using immunogold silver-staining, low levels of GLUT1 were detected in the rat mammary gland, apparently concentrated around the outside of the alveoli, corresponding to the basal plasma membrane of the secretory epithelial cells. The level of staining was far lower than in the rat brain microvessels. The concentration of GLUT1 in bovine brain microvessels is approximately 12pmol/mg protein, using Scatchard analysis of cytochalasin B binding, or 11pmol GLUT1/mg protein using quantitative Western blotting (Pardridge *et al.*, 1990), and in the mammary plasma membranes approximately 20pmol/mg protein (Table 5.1). Therefore the level of staining in the mammary gland would be expected to be higher, however, this is not the case. It is possible that the bovine membranes were less highly purified than the rat plasma membranes, which could in part account for the difference in intensity upon immunocytochemistry. However it was also found, during work described in Section 7.5, that separation of the litter from the lactating female down-regulates the mammary GLUT1 protein within 24hr. The rats used in the immunocytochemistry experiments had been separated from their litters for up to 6hr, therefore there could be some down-regulation, decreasing the level of GLUT1 detected in the mammary cells. The distribution of GLUT1 in the

mammary gland served to exclude the possibility that a significant proportion of the GLUT1 isoform within the mammary homogenate identified by Western blotting originated from a source other than the epithelial cell. As well as the apparent localisation of GLUT1 to the basal plasma membrane of the epithelial cells, there was a generally higher level of staining over the cytoplasm observed using antibodies against GLUT1 compared to non-specific IgG. It was not feasible to precisely locate the transporter within the cells, although the higher cytoplasmic staining could infer the presence of an intracellular GLUT1. The immunogold silver-staining method has also been used by Erdmann & Binas (1991) to localise a growth inhibitor in bovine mammary tissue, indicating that the method itself works well in mammary tissue. Further investigation of GLUT1 distribution in mammary epithelial cells at both the light microscope and transmission electron microscopic level is needed to precisely localise the GLUT1.

GLUT4 was not detected in either adipose tissue within the mammary gland or in the mammary epithelial cells. Unfortunately, a control tissue for the GLUT4 isoform taken from the same rats as the brain, liver and mammary gland was not available at the time of this study, therefore the immunoreactivity of the antibodies to the native GLUT4 protein under these fixation conditions could not be checked. However, the results described in Section 6.3.3 indicate that the anti-GLUT4 antibodies detect GLUT4 in fixed immortal cell lines. Also preliminary work with the anti-GLUT4 antibodies had shown that they are selectively removed by incubation with an adipocyte membrane fraction in a competitive ELISA assay (personal communication, Mr. L. Fryer), indicating that the antibodies are capable of binding to the native GLUT4 transporter. Therefore, the lack of GLUT4 detection in the mammary gland supports the suggestion that this isoform is not

expressed in the mammary epithelial cells.

GLUT2 was also not detected in lactating rat mammary gland, confirming the results of Western blotting in Section 4.2. Two glucose transporter isoforms, GLUT2 (predominant) and GLUT1 (increased levels after fasting), are expressed in hepatocytes, however only GLUT2 is readily detected in liver (Hacker *et al.*, 1991). The presence of GLUT2 on the sinusoidal membrane of hepatocytes demonstrated in Section 6.3.2 is consistent with the results obtained by Thorens *et al.* (1990), further confirming the specificity of the anti-GLUT2 antibodies.

CHAPTER 7

EXPRESSION OF GLUCOSE TRANSPORTERS DURING PREGNANCY, LACTATION AND LITTER REMOVAL

7.1 Introduction

Regulation of the expression of glucose transporters in mammary epithelial cells is of particular interest as these cells are uniquely responsible for the synthesis and secretion of milk constituents. In the work described in this Chapter it was decided to investigate GLUT1 and GLUT4 expression in the epithelial cells during development and differentiation of the tissue from a virgin animal, through pregnancy and lactation until involution. Therefore these experiments were designed to detect glucose transporters in relation to the lactogenic activity of the mammary gland, particularly through the differentiation of the mammary epithelial cells.

The results obtained from Western blotting experiments, performed on crude homogenate of whole lactating rat mammary gland (Section 4.2), had indicated that GLUT1 and GLUT4 are both present. The increased abundance of GLUT1 per mg protein, and scarcity of GLUT4, on Western blots using equivalent loadings of mammary sub-cellular fractions, shown in Section 4.3, and immunocytochemistry of lactating rat mammary gland shown, in Section 6.3.4, which had found only GLUT1 in mammary epithelial cells, indicate that GLUT1 is the predominant glucose transporter in mammary epithelial cells and that the GLUT4 detected in mammary gland homogenate by Western blotting is probably derived from mammary adipocytes.

The expression of GLUT1 and GLUT4 was investigated both in whole mammary

gland homogenate and in isolated mammary epithelial cells. It was considered necessary to purify the epithelial cells from the mammary tissue to minimise the effect of the changing predominance of cell types within the mammary tissue on the detection of glucose transporters. In particular, it was necessary to minimise contamination of mammary epithelial cell glucose transporters with those present in the mammary adipocytes.

The development of the mammary gland from a virgin state to peak lactation involves a high degree of differentiation, from a largely quiescent tissue composed almost entirely of adipocytes, to a highly active tissue composed largely of milk-synthesizing, secretory epithelial cells. The glucose transporter content of homogenate of virgin rat mammary gland should, therefore, be strongly influenced by the predominance of adipocytes. Adipocytes isolated from other fatty tissues in the rat are known to express high levels of GLUT4 relative to GLUT1, a typical ratio of the two isoforms being 9:1 (Zorzano *et al.*, 1989). Homogenate of mid-lactating rat mammary gland should, in contrast, reflect the glucose transporters present in the secretory epithelial cells which constitute >70% (Kraehenbuhl, 1977) of the mammary cells at this stage, and therefore, those glucose transporters specifically involved with lactation. Investigations by Burnol *et al.* (1990) of rat mammary gland glucose transporters had demonstrated a high expression of GLUT4 in the mammary gland of virgin rats compared to a low expression of GLUT1. While it was not confirmed, it was assumed that this result reflected the higher adipocyte content of mammary tissue from virgin rats. Conversely, following the mammary development, in late pregnancy and peak lactation, Burnol *et al.* (1990) had shown high expression of mammary GLUT1 and a low expression of GLUT4. My own results, shown in Fig. 4.2, indicated that GLUT1 was selectively expressed in rat

mammary homogenate compared to GLUT4 at peak lactation. In this chapter the results of experiments investigating the expression of GLUT1 and GLUT4 throughout pregnancy and lactation, both in mammary homogenate and in epithelial cells, are described. In addition, the expression of GLUT1 was investigated in mammary epithelial cells from rats at peak lactation following a 24hr litter-removal. This treatment causes increased milk retention in the mammary gland and inhibits further lactation, mimicking, to some extent, the changes in mammary function at involution.

7.2 Mammary epithelial cell and homogenate preparation

Burnol *et al.* (1990) had demonstrated that expression of GLUT1 and GLUT4 in the mammary gland was dependent upon the development and differentiation of the mammary tissue. A more detailed study of the changes in expression of mammary GLUT1 and GLUT4 during mammary gland development was performed by myself, using mammary homogenate and epithelial cells, described in the following experiments.

Female rats at various stages of pregnancy and lactation were killed by cervical dislocation and the mammary tissue removed. Specifically, mammary tissue was removed from rats at day 12 (P12) and day 20 (P20) of pregnancy, day 2 (L2) and day 10 (L10) of lactation, day 10 of lactation following a 24hr litter removal (LR) and from virgin rats which had failed to mate (FTM). The parametrial adipose tissue was also removed from the rats, frozen immediately in liquid N_2 and stored at -70^{o}C for the experiments described in Section 7.7. During the dissection of the pregnant rats, foetuses were checked for size and development to confirm the stage of pregnancy. Mammary epithelial cells were then isolated from the mammary

tissue and used to investigate GLUT1 and GLUT4 expression using Western blotting.

Mammary tissue removed from P20, L2, L10 and LR rats weighed between 6.5g and 14.0g. Most of this tissue (6.0g) was used to isolate epithelial cells by the collagenase digestion procedure described in Section 2.2. The remaining tissue was stored at -70^{o}C, prior to homogenisation. Mammary tissue from the P12 rats weighed less than 6.0g, therefore, all of the tissue was required for isolation of epithelial cells. Progress of the collagenase digestion was followed by observing drops of the digest under a low power light microscope, and the digestion was stopped when clumps of 10-15 cells were seen. Following the epithelial cell preparation, cells were inspected under a low power light microscope to determine adipocyte content. Epithelial cells were seen as small clumps of secretory or pre-secretory epithelium. No adipocytes were seen in epithelial cell preparations from any developmental stage. Whether this was due to the adipocytes perishing during the rigorous shearing required to break down the connective tissue of the mammary gland, or floating in the supernatant during the washing steps was not determined. Epithelial cell clumps were assumed to retain the myoepithelial cells and to resemble the acini prepared by Grigor *et al.* (1988). Epithelial cells were pelleted for 30sec at 14,000g and frozen in liquid N_2.

Mammary tissue from FTM rats was composed almost entirely of adipose tissue. Therefore no attempt was made to isolate the epithelial cells, and the entire glands were used to prepare homogenate. Homogenate of mammary gland was prepared by thawing mammary tissue from FTM, P20, L2, L10 and LR rats into 25mM HEPES, pH7.4 containing protease inhibitors (0.2mM PMSF, 4mM EDTA, 1μM Leupeptin, 1μM Pepstatin A, 1μM Aprotinin and 1μM E-64) and chopping finely. Chopped tissue (500mg) was diluted into 2ml 25mM HEPES, pH7.4 containing

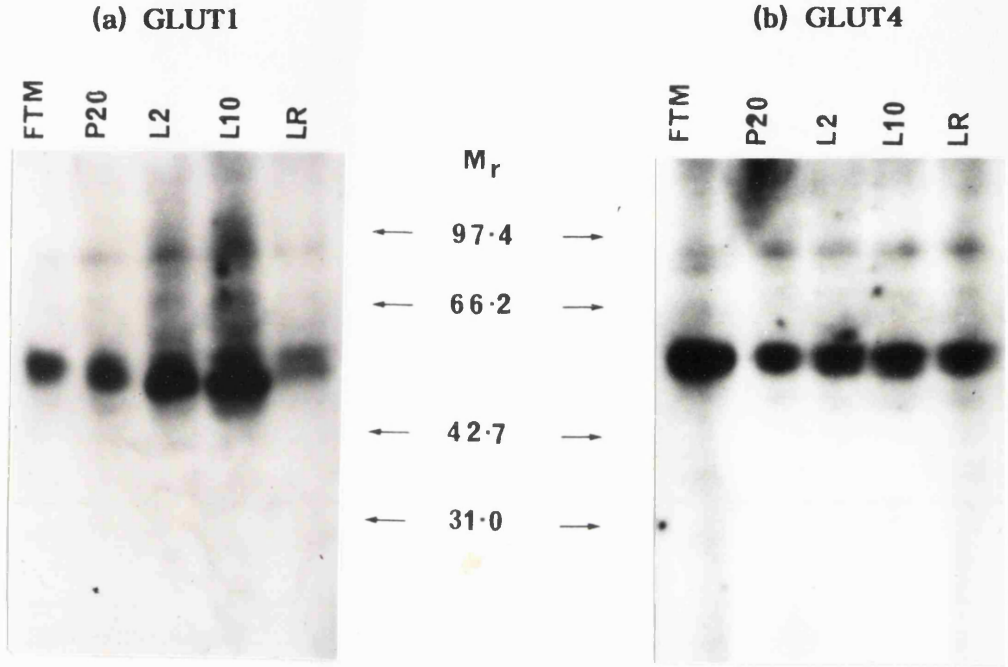
protease inhibitors and homogenised using a 1cm diameter Polytron homogeniser at 9000rpm for 3x 20sec, on ice. The homogenate was centrifuged in a Sorvall bench-top centrifuge at 1,000rpm for 2min at 4°C to pellet any large lumps of tissue debris. The supernatant, designated homogenate, was carefully removed and stored frozen at -70°C.

7.3 Levels of GLUT1 and GLUT4 in mammary homogenate

Results shown in Section 4.2 indicated that GLUT1 and GLUT4 were present in the lactating rat mammary gland, with GLUT1 the predominant isoform. Developmental changes in the expression of both GLUT1 and GLUT4 were therefore investigated in mammary gland homogenate using Western blotting.

Mammary homogenates, prepared as described in Section 7.2, were made up as gel samples and 200µg electrophoresed on 10% SDS/polyacrylamide gels. Following Western blotting onto nitrocellulose, the blots were probed with antibodies raised against the *C*-terminal peptides of either GLUT1 or GLUT4, followed by an iodinated second antibody. The resulting autoradiographs, shown in Fig. 7.1, revealed changes in the expression of GLUT1 and GLUT4 in mammary gland homogenate. The expression of GLUT4 during development of the mammary gland, shown in Fig. 7.1(b), decreased substantially from the high level in the virgin mammary gland, to a lower level in late pregnant mammary gland. The expression of GLUT4 then remained constant at the resulting lower level for the duration of lactation and following a 24hr litter-removal. In contrast, the expression of GLUT1 in the mammary gland, shown in Fig. 7.1(a), increased steadily from a basal level in the virgin rat mammary gland, to a maximum level at day 10 of lactation. Following litter-removal for 24hr at peak lactation, the expression of GLUT1 in the

Fig. 7.1 GLUT1 and GLUT4 levels during mammary gland development



Mammary homogenate (200μg) was electrophoresed on 10% SDS/polyacrylamide gels, Western blotted onto nitrocellulose and glucose transporters detected using antibodies raised against the *C*-terminal peptides of GLUT1 (a) or GLUT4 (b), followed by an iodinated second antibody and autoradiography. Mammary homogenate was prepared from virgin (FTM), late-pregnant (P20), early- (L2) and peak-lactating (L10) rats, and peak-lactating rats following a 24hr litter-removal (LR).

mammary gland decreased to a level equivalent to that seen in the virgin animal.

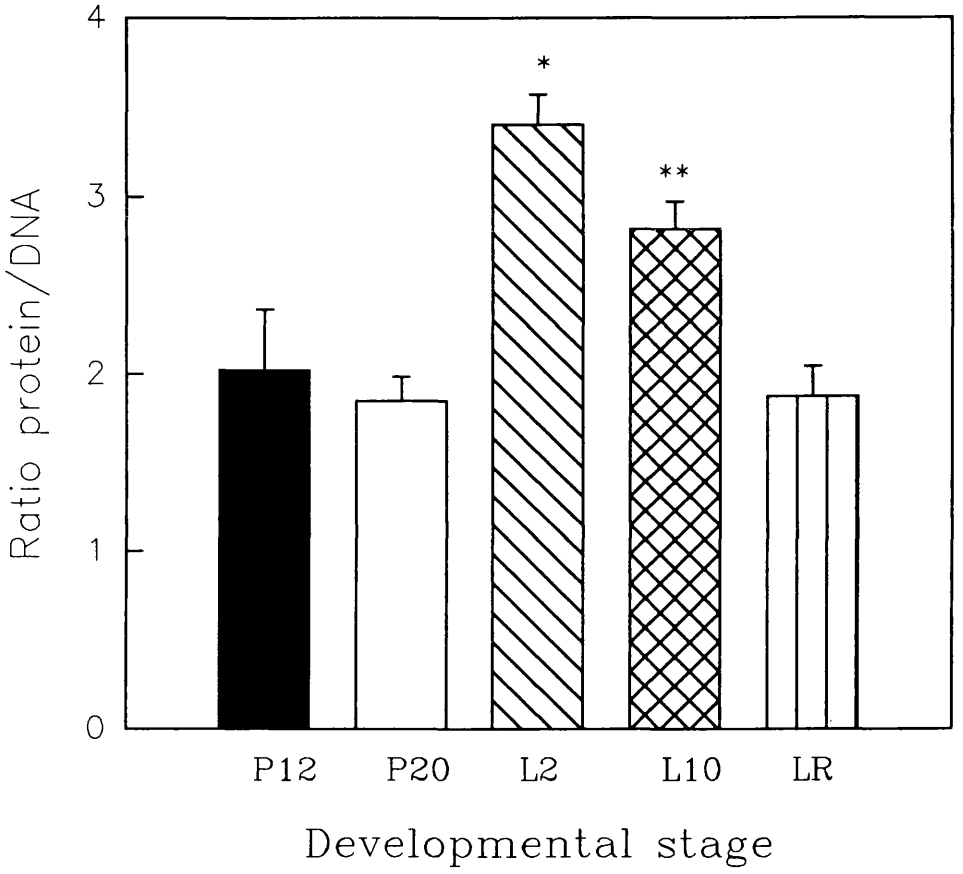
7.4 Ratio of protein / DNA in mammary epithelial cells during mammary development

In order to investigate changes in the expression of GLUT1 and GLUT4 in the epithelial cells during development and differentiation of the mammary gland, it was desirable to express the results on a per cell basis. Direct counting of epithelial cells in the preparations to be used for Western blotting was not possible because they were present as cell clumps. One readily estimated measure of cell number is the total cellular protein. However, lactogenesis is associated with the rapid initiation of milk-specific protein synthesis and so the protein content of the mammary cells might change during differentiation. Therefore, to assess whether the total protein content of mammary epithelial cells does alter during mammary gland development the ratios of protein / DNA in the cell preparations described in Section 7.2 were determined.

Frozen epithelial cell pellets were resuspended and thawed in 200µl PBS, pH7.4 containing protease inhibitors, and homogenised thoroughly in a 1ml glass Dounce homogeniser on ice. Samples of cell homogenate were then assayed for protein and DNA concentration as described in Sections 2.9 and 2.8 respectively.

The ratios of protein / DNA in the epithelial cells was determined and are shown in Fig. 7.2. The figures in Fig. 7.2 are the mean ± S.E.M. of (n) epithelial cell preparations, as detailed in the figure. The highest ratios of protein / DNA were seen during lactation, significantly higher than at early pregnancy (L2, $p<0.01$; L10, $p<0.02$). There was no significant difference between the ratio of protein / DNA in pregnancy and following a 24hr litter-removal.

Fig. 7.2 Ratio of protein / DNA in mammary epithelial cells



The ratio of protein concentration / DNA concentration in mammary epithelial cells isolated from mammary glands of early- (P12) and late-pregnant (P20), early- (L2) and peak-lactating (L10) rats and peak-lactating rats following a 24hr litter removal (LR) were determined. The ratios are expressed as means ± S.E.M. for n epithelial cell preparations where n = 5 for P12 and n = 4 for the remaining developmental stages. The protein/DNA ratios of the lactating rats were significantly higher than those of the pregnant rats (* $p<0.01$; ** $p<0.02$; Student's unpaired *t*-test). However, there was no significant difference between the ratio of protein/DNA of the pregnant and litter-removed rats.

The increase in the protein / DNA ratio immediately following parturition (day 2 of lactation) coincided with the onset of lactation and probably reflected synthesis of both the milk proteins, and the proteins involved in synthesis of other milk-specific compounds, for example the enzymes involved in triglyceride and lactose synthesis. The protein / DNA ratio had fallen slightly by day 10 of lactation, but still remained at a higher level than during pregnancy.

Following a 24hr litter-removal at peak-lactation the ratio of protein / DNA in the epithelial cells fell to a level significantly lower than that seen in peak-lactating rats kept with their litters. Despite the large amount of milk present in the mammary gland, the protein content of the epithelial cells had clearly fallen.

7.5 Developmental changes in mammary epithelial cell glucose transporter levels relative to cell number

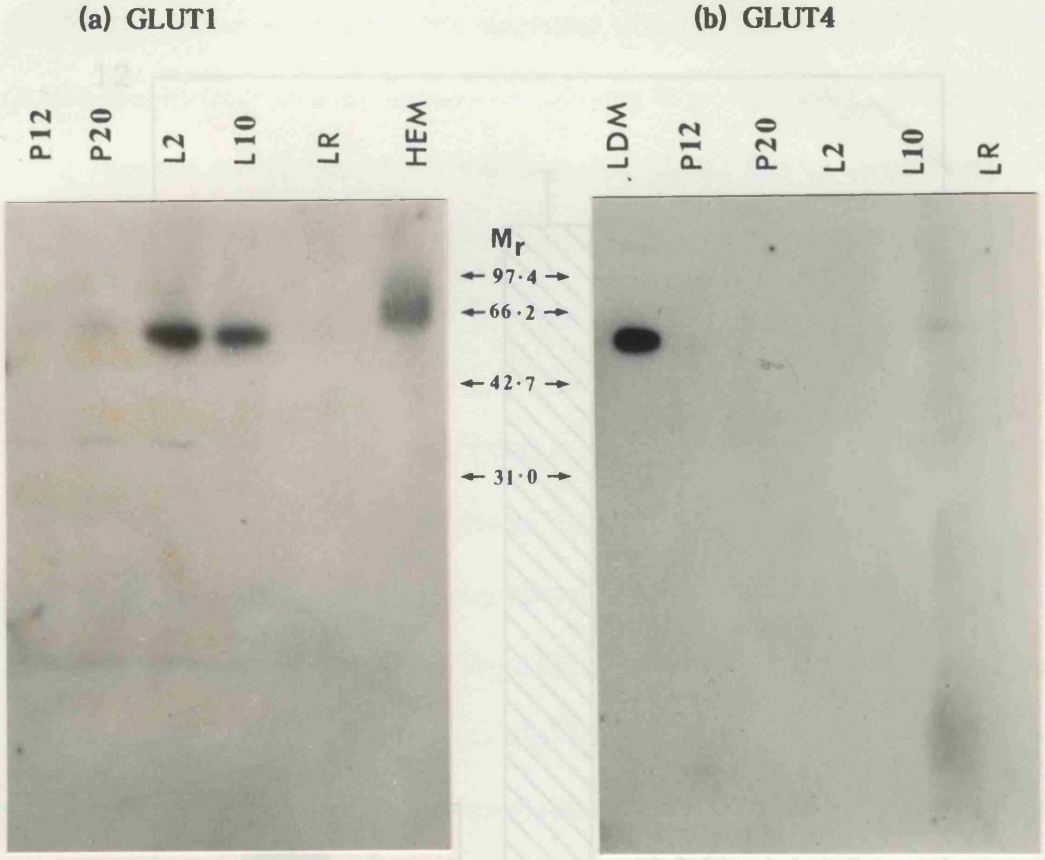
Mammary epithelial cell homogenates prepared as described in Section 7.4, and containing equal amounts of DNA, were used to prepare SDS/polyacrylamide gel samples. Following vigorous vortexing, the cellular DNA was sheared by passing the samples repeatedly through 21g, then 23g, needles. Samples containing 40μg DNA (between 100-200μg protein) were electrophoresed on 10% SDS/polyacrylamide gels and blotted onto nitrocellulose. Samples of protein-depleted human erythrocyte membranes (prepared as GLUT1 standards as described in Section 5.3, containing 100ng GLUT1) and rat adipocyte low-density microsomal membranes (20μg protein, a gift from Mr. L. Fryer) were also electrophoresed as positive controls for GLUT1 and GLUT4 respectively. Equivalent blots were probed with antibodies raised against the *C*-terminal peptides of GLUT1 or GLUT4.

GLUT4 was not detected in any of the mammary membrane epithelial cell

samples (Fig. 7.3(b)), although the anti-GLUT4 antibodies detected GLUT4 in the adipocyte membranes. The amount of epithelial cell protein loaded onto the blots was between 100-200μg. In contrast to the absence of detectable GLUT4 in these epithelial cell samples, GLUT4 was readily detectable in samples of a comparable size (200μg) from total mammary gland homogenate, as illustrated in Fig. 7.1(b). These findings indicate that GLUT4 is not expressed at significant levels in the epithelial cells at any stage of mammary development. It follows that the GLUT4 detected by ourselves and by Burnol *et al.* (1990) in total mammary gland homogenate (Fig. 7.1(b)) is probably derived from mammary gland adipocytes. In contrast to GLUT4, GLUT1 was found to be expressed in mammary epithelial cells, and the autoradiograph shown in Fig. 7.3(a) demonstrates that this expression is developmentally regulated. GLUT1 was undetectable during early pregnancy, and only visible at very low levels by late pregnancy. However, by the second day of lactation there was a substantial increase in the level of the GLUT1 protein. The highest levels were seen during early lactation, and levels were approximately 50% of the maximum at day 10 of lactation. Removal of the litter for 24hr at peak lactation resulted in an almost total loss of GLUT1 from the epithelial cells. A more quantitative estimate of the level of GLUT1 expression was determined by densitometry, the results of which are shown in Fig. 7.4.

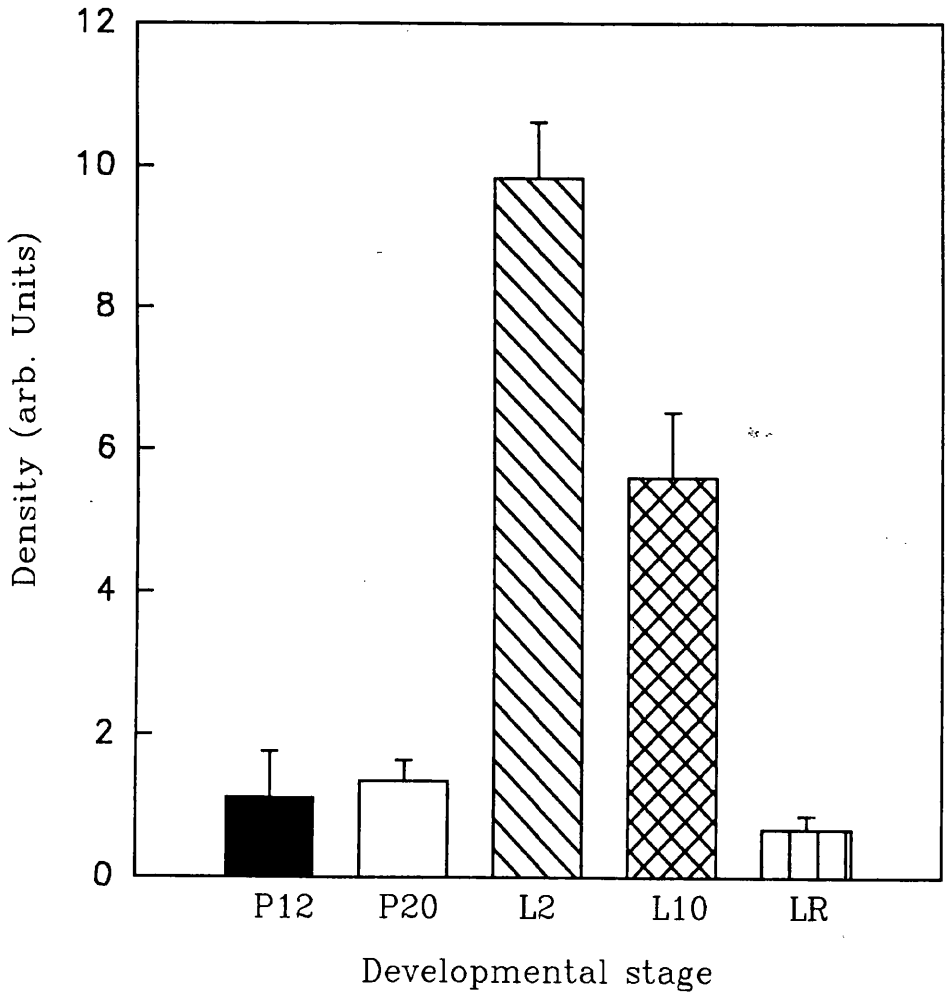
To ensure that the changes in GLUT1 expression seen in mammary epithelial cells in Fig. 7.3(a) were not an artefact of GLUT1 *C*-terminal peptide degradation, equivalent Western blots were probed with an antiserum raised against the entire purified human erythrocyte glucose transporter, used at a dilution of 1:500. These antibodies are known to cross-react with other regions of GLUT1 protein as well as with the extreme *C*-terminus (Davies, 1990). Fig. 7.5(a) shows that anti-GLUT1

Fig. 7.3 GLUT1 and GLUT4 levels in mammary epithelial cells during mammary development - expression relative to cell number



Mammary epithelial cell homogenates containing 40μg DNA were electrophoresed on 10% SDS/polyacrylamide gels and Western blotted onto nitrocellulose. Glucose transporters were detected using antibodies raised against the *C*-terminal peptides of GLUT1 (a) or GLUT4 (b), followed by an iodinated second antibody and autoradiography. Epithelial cells were isolated from P12, P20, L2, L10 and LR rats as detailed in the legend to Fig. 7.2. Also shown in (a) are human erythrocyte membranes (HEM) containing 100ng GLUT1 determined as shown in Section 5.3, and in (b) 20μg rat adipocyte low density microsomal membranes (LDM, gift of Mr. L. Fryer).

Fig. 7.4 Quantification of the changes in mammary epithelial cell GLUT1 during mammary gland development



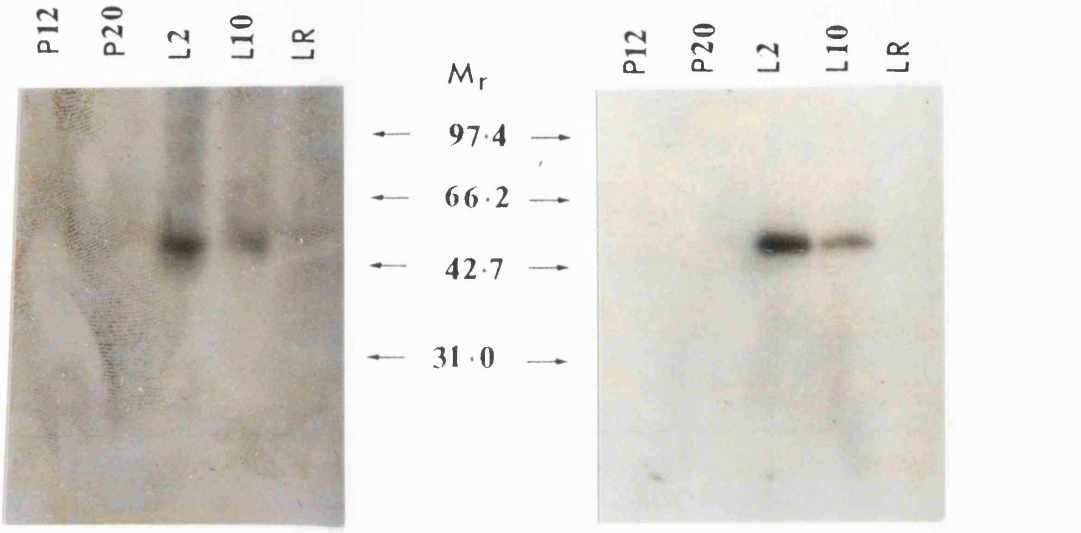
The density of the GLUT1 bands detected by autoradiography of Western blotted mammary epithelial cells, isolated from P12, P20, L2, L10 and LR rats as detailed in the legend to Fig. 7.2, were determined. The results shown are the mean ± S.E.M. for n = 4 animals at each stage. Using Student's unpaired *t*-test, the level of GLUT1 in the lactating rat mammary epithelial cells was significantly higher (L2, $p<0.001$; L10, $p<0.0025$) than the P12 and P20 levels. There was no significant difference between GLUT1 in the epithelial cells of pregnant and litter-removed rats.

antibodies only detected GLUT1 in the lactating (L2 and L10) rat mammary epithelial cells, showing the same pattern of expression as detected with the anti-GLUT1 *C*-terminal antibodies. This confirmed that the low levels of detectable GLUT1 in cells from pregnant animals or following litter-removal, as shown in Fig. 7.3(a) were not due to the presence of degraded GLUT1 lacking an antigenic *C*-terminal.

The results described in Section 7.4 showed that the ratio of protein / DNA varied in the epithelial cells during development, hence the reason for using samples containing fixed amounts of DNA. For the sake of completeness, the level of GLUT1 relative to total cellular protein was also examined. Mammary epithelial cell homogenate gel samples containing 150μg protein were electrophoresed on 10% SDS/polyacrylamide gels, Western blotted and probed with antibodies raised against the *C*-terminal peptide of GLUT1. As Fig. 7.5(b) shows, expression of GLUT1 in mammary epithelial cells, when expressed relative to a constant amount of cellular protein, followed the same overall pattern as GLUT1 expression when the results were expressed relative to cell number.

The results shown in this section indicate that expression of the mammary GLUT1 protein is regulated during mammary development. There is known to be a developmental control on the uptake of D-glucose into the mammary tissue, with a 13-fold increase between mid-pregnancy and mid-lactation (Prosser & Topper, 1986), which would correlate to the increase seen in GLUT1 expression. It appears that under conditions where high glucose uptake into the mammary gland is not required, i.e. when the rat is not lactating, either during pregnancy or due to litter-removal, the expression of the GLUT1 protein in mammary epithelial cells is switched off, and any pre-existing GLUT1 rapidly degraded. When glucose is

Fig. 7.5 Levels of GLUT1 in mammary epithelial cells during mammary development



GLUT1 was detected in mammary epithelial cells (P12, P20, L2, L10 and LR rats as detailed in the legend to Fig. 7.2) by various means. Samples of epithelial cell homogenate equivalent to 40µg DNA (a), or 150µg protein (b) were Western blotted and GLUT1 detected using antibodies raised against the purified human erythrocyte GLUT1 protein (a), or against the GLUT1 C-terminal peptide (b), an iodinated second antibody and autoradiography.

required for lactation, GLUT1 protein expression is switched on.

7.6 Expression of mammary GLUT1 mRNA relative to the expression of mammary actin mRNA

The syntheses of a number of mRNAs are initiated in mammary gland during late gestation and early lactation, especially of milk-specific proteins (Rosen *et al.*, 1975). Using a total RNA fraction prepared from pellets of mammary epithelial cells, isolated as described in Section 7.2, the expression of GLUT1 mRNA was investigated relative to the level of mRNA for a housekeeping gene, mammary actin mRNA. The expression of rat ß-actin mRNA was kindly measured by Dr. C. Bingle, using a rat actin riboprobe.

Epithelial cell pellets, frozen as described in Section 7.2, were thawed directly from liquid N_2 into 1ml of guanidine isothiocyanate and used to prepare total RNA according to the method described under Section 2.7. Total RNA from rat forebrain was also prepared and used as a positive control for the GLUT1 cDNA probe. The concentration of RNA was estimated from the absorbance at 260nm of 1µl diluted into 1ml deionised water and the degree of contamination with protein estimated from the ratio of A_{260}/A_{280}. Samples of mammary RNA (20µg) were electrophoresed on formaldehyde/agarose gels and Northern blotted onto Hybond N nylon membranes. Further samples (5µg) of mammary RNA and brain RNA were slot blotted directly onto Hybond N.

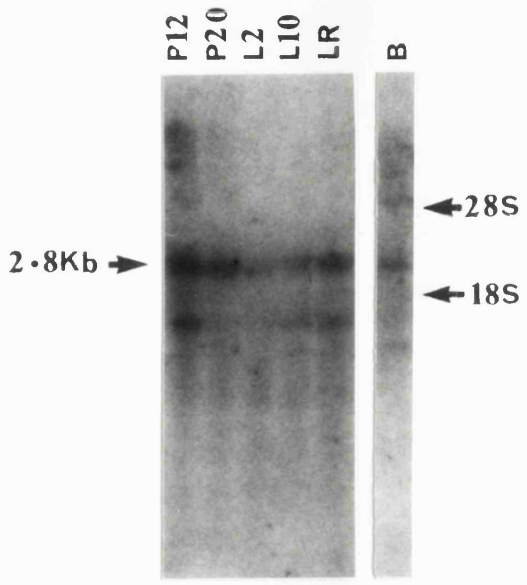
Hybridisation was performed as described in Section 2.7.7. Both the Northern blots and slot blots were first hybridised with a cDNA probe for GLUT1, at a concentration of 10^5-10^6cpm/ml at 42°C overnight. Following autoradiography the blots were stripped of hybridised cDNA. Stripped blots were autoradiographed to

check that the radioactive probe had been successfully removed from the blot, then re-probed, by Dr. C. Bingle, using a riboprobe to rat ß-actin mRNA, and again developed by autoradiography.

The intensity of the signal obtained using the GLUT1 cDNA probe, for both the rat brain RNA and rat mammary RNA, was found to be extremely low compared to the same blots probed with the actin riboprobe. Approximately 11% of the rat brain mRNA bases are mismatched compared to the human HepG2 GLUT1 mRNA, as determined from the published DNA sequences (Birnbaum *et al.*, 1986 and Mueckler *et al.*, 1985 respectively), which could lower the degree of hybridisation.

An example of the Northern blotting obtained using the GLUT1 cDNA probe is shown in Fig. 7.6. Two bands of hybridisation were seen, one running at approximately 2.8kB, assumed to be the intact GLUT1 mRNA (Birnbaum *et al.*, 1986), and a second, smaller mRNA species running below the 18S rRNA. Other RNA transcripts have been shown to hybridise to the GLUT1 cDNA (Charron *et al.*, 1989) however, as the stringency of hybridisation was relatively high, it was assumed that the smaller mRNA bands were related to the GLUT1 mRNA transcripts, possibly being partial transcripts. The varying intensity of the bands seen in Fig. 7.6 probably reflected an uneven loading of RNA onto the gel, as judged from the intensity of the 23S and 18S rRNA species when stained with EtBr and viewed under U.V. light, the positions of which are noted on the right hand side of the figure, and from the intensity of the actin RNA bands following labelling with the actin riboprobe. All estimations of GLUT1 mRNA expression during mammary development were compared to the expression of actin mRNA. A comparison of actin signal obtained following hybridisation and the intensity of the rRNA following EtBr staining indicated that actin was an appropriate house-keeping

Fig. 7.6 Northern blotting of mammary epithelial cell GLUT1 mRNA during mammary development



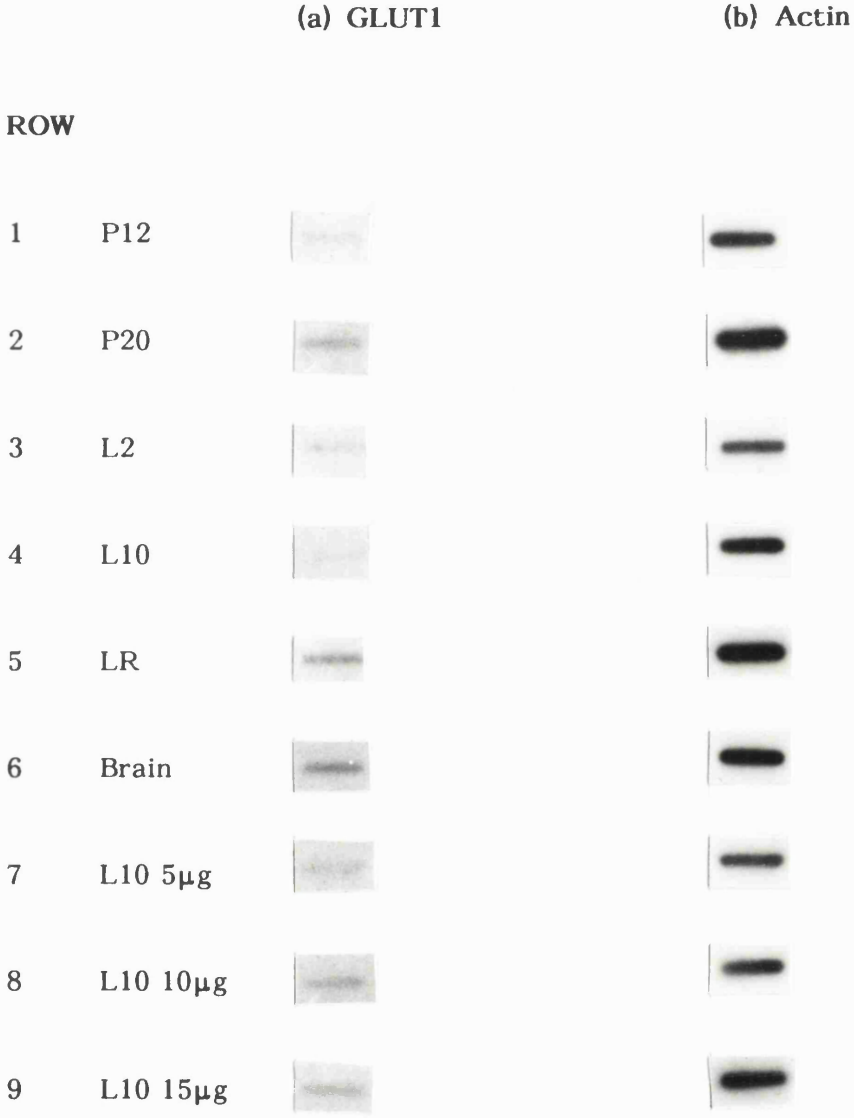
The expression of GLUT1 mRNA in mammary epithelial cells was investigated by Northern blotting. Samples of mammary total RNA, estimated as 20μg, were Northern blotted onto Hybond N. The Northern blots were hybridised to a radiolabelled HepG2 GLUT1 cDNA probe and detected by autoradiography. Total RNA was prepared from rat brain, $\lambda^{(B)}$ and mammary epithelial cells of P12, P20, L2, L10 and LR rats, detailed in the legend to Fig. 7.2. The position of the 28S and 18S ribosomal RNAs were determined by staining the blots with EtBr.

gene to use for the mammary gland.

Developmental changes in the expression of GLUT1 mRNA were more easily investigated using the slot blots, where the signal obtained from the hybridisation was contained within a well-defined area. The relative intensities of GLUT1 mRNA hybridisation signal at the different stages of mammary development were estimated relative to the actin RNA. The slot blot shown in Fig. 7.7(a) demonstrated that GLUT1 mRNA was present in mammary epithelial cells throughout pregnancy and lactation, and following a 24hr litter-removal. The expression of actin mRNA, also shown in Fig. 7.7(b), was used to normalise the expression of GLUT1 mRNA. GLUT1 mRNA expression during mammary gland development followed the same pattern as actin mRNA, suggesting that the different labelling intensities seen for GLUT1 in Fig. 7.7(a) resulted from different RNA loadings rather than specific changes in the expression of GLUT1 mRNA. Unfortunately the GLUT1 slot blots and Northern blots were either too faint, or the background was too high, to enable them to be scanned by densitometry to confirm this. GLUT1 mRNA expression was only investigated in two epithelial cell preparations from each developmental stage (a second similar set of results are not shown). In none of these epithelial cell preparations were the developmental changes in GLUT1 protein expression, shown in Section 7.5, reflected in the expression of GLUT1 mRNA, all mammary RNA containing a detectable level of GLUT1 mRNA.

To ensure that the signal from hybridisation, detected by slot blotting mammary gland RNA, using probes for both actin mRNA and GLUT1 mRNA, was linear with respect to the amount of RNA (by weight) loaded onto the Hybond N, samples of 5, 10 and 15μg total RNA from day 10 lactating mammary gland were also loaded

Fig. 7.7 Slot blotting mammary epithelial cell RNA



The expression of GLUT1 mRNA in mammary epithelial cells during development of the mammary gland was compared to the expression of ß-actin mRNA. Rat brain, and mammary RNA samples (≈5µg RNA) from the developmental stages detailed in the legend to Fig. 7.2, were slot blotted directly onto Hybond N. The expression of GLUT1 was determined by hybridisation to a radiolabelled HepG2 GLUT1 cDNA probe and autoradiography (a). The slot blots were then stripped, and reprobed using a riboprobe to rat ß-actin by Dr. C. Bingle (b). Rows 7-9 contain 5µg, 10µg and 15µg rat mammary epithelial cell RNA from peak-lactating rats, respectively.

onto the slot blots and probed. As shown in Fig. 7.7(a) and (b), rows 7-9, the intensity of the signal obtained in these slots appeared to be linearly related to the amount of RNA loaded, confirmed for the ß-actin probe by densitometric scanning of the bands.

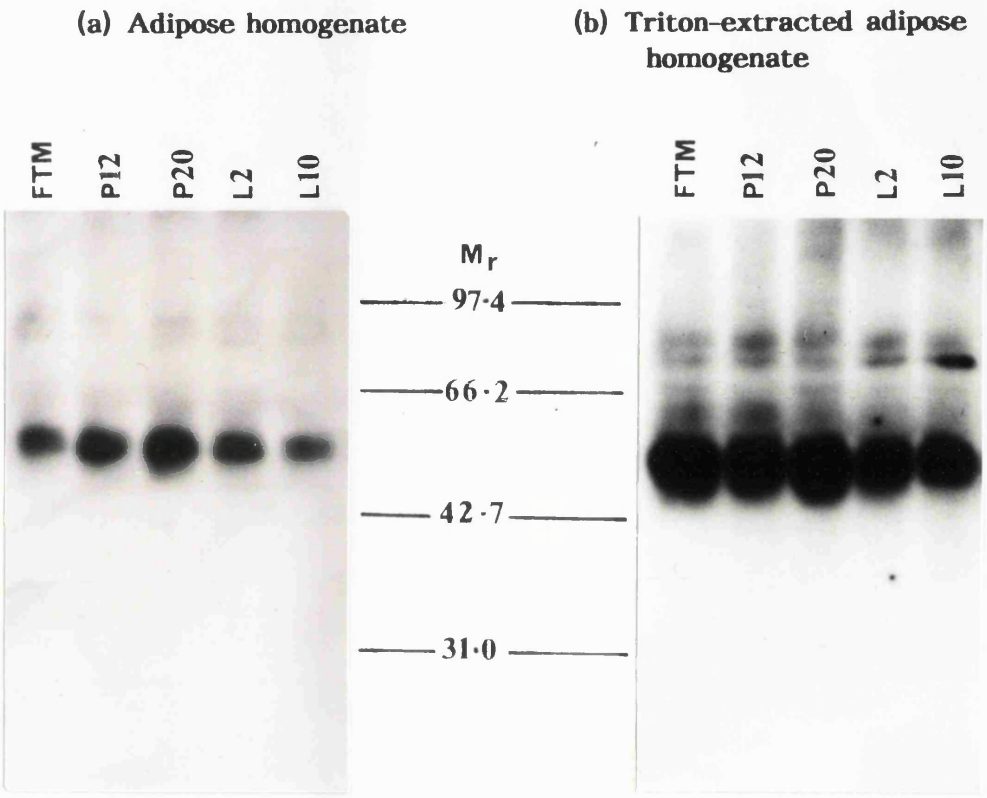
7.7 Levels of GLUT1 and GLUT4 in rat adipose tissue

Parametrial adipose tissue from the pregnant and lactating rats, killed as described in Section 7.2, had been frozen in liquid N_2 and stored at -70°C until required. Samples of adipose tissue, between 1.0-1.5g, were used to prepare adipose tissue homogenate and Triton X-100 extracts as described in Section 2.3.7.

Aliquots of both the adipose homogenate and the Triton-extract were assayed for protein by the method described in Section 2.9, and gel samples containing 200µg protein were electrophoresed on 10% SDS/polyacrylamide gels. Following Western blotting, GLUT4 was detected using antibodies raised against the *C*-terminal peptide, an iodinated second antibody and autoradiography.

The expression of GLUT4 in the adipose tissue homogenates and Triton-extracts are shown in Fig. 7.8; a similar pattern of GLUT4 was observed in both. In the adipose tissue preparations there was a decrease in the GLUT4 level during lactation, compared to the level during pregnancy. However, as only two animals had been used for the investigation, it was not possible to make a statistical analysis of GLUT4. The higher GLUT4 level seen during pregnancy compared to FTM rats (Fig. 7.8(a)) suggested that the adipose GLUT4 levels are regulated during pregnancy and lactation. However, as the same results were not seen in the Triton-extracts (Fig. 7.8(b)), this subject requires a more detailed investigation. The advantage of using the Triton-extracted adipose tissue was the relative ease of

Fig. 7.8 Levels of GLUT4 in rat adipose tissue during pregnancy and lactation



Adipose tissue was isolated from FTM, P12, P20, L2 and L10 rats, described in the legend to Fig. 7.2. Samples of parametrial adipose tissue homogenates containing 200μg protein (a) and of adipose homogenates extracted with 1% Triton X-100, also containing 200μg protein (b), were electrophoresed on 10% SDS/polyacrylamide gels and Western blotted onto nitrocellulose. GLUT4 was detected using antibodies raised against the *C*-terminal peptide of GLUT4, followed by an iodinated second antibody and autoradiography.

measuring volumes compared to the adipose homogenate, in which the interference of large amounts of fat was often a problem. However, it is possible that the Triton-extraction selectively removed sub-sets of adipose membranes, therefore the results obtained with the Triton-extracts are open to question.

7.9 Discussion

It is possible to isolate epithelial cells from the mammary adipocytes by collagenase digestion in a suitable medium (Topper *et al.*, 1975). In the experiments detailed in this chapter, isolating epithelial cells from pregnant and lactating rat mammary gland was found to remove most, if not all, of the adipocytes so reducing the effect of the adipocyte cell population on the measurement of GLUT1 and GLUT4 in the milk-producing epithelial cells. Changes in expression of GLUT1 and GLUT4 in epithelial cells during the development of the mammary gland would, therefore, reflect more faithfully the changes associated directly with lactogenesis and lactation. Comparing GLUT1 and GLUT4 levels present in the epithelial cells to that in the mammary homogenate, allowed measurement of the relative levels of GLUT1 and GLUT4 expression in the epithelial cells and other cell types, particularly adipocytes, removed from the tissue during the epithelial cell purification.

The expression of GLUT1 and GLUT4 was first investigated in whole mammary gland homogenate, using mammary glands from rats at various stages of pregnancy and lactation compared to virgin animals. During the course of my study, the results of similar investigations were published by Burnol *et al.* (1990) who demonstrated a developmental change in the levels of GLUT1 and GLUT4 in the rat mammary gland. These authors reported the finding of little or no GLUT1 in

mammary homogenate of virgin rats, but of high levels of GLUT1 in late pregnancy and at peak lactation. Conversely they reported high levels of GLUT4 in virgin rat mammary gland, which fell at late pregnancy and remained low at peak lactation. The results of my own work partly confirmed the findings of Burnol *et al.* (1990), but also showed some differences. As shown in Fig. 7.1(b), the level of GLUT4 in the mammary homogenate was highest in glands from virgin rats but fell to a low level during pregnancy, where it remained during lactation. This result was consistent with the changing predominance of cell types in the mammary gland from adipocytes in the virgin gland to epithelial cells at late pregnancy and during lactation, assuming that little or no GLUT4 is expressed by the mammary epithelial cell (an assumption validated by results shown in Section 7.5). The expression of GLUT1 in mammary homogenate, shown in Fig. 7.1(a) demonstrated that there was very little increase in GLUT1 expression at late pregnancy compared to the expression in virgin rat mammary gland. However, between late pregnancy and early lactation there was a substantial increase in mammary GLUT1, the expression of which continued rising, reaching its highest observed level at mid-lactation. My results suggested an induction of GLUT1 expression around the time of parturition and lactogenesis. The main difference between my results and those of Burnol *et al.* (1990) was in the relative expression of GLUT1 in mammary gland at late pregnancy. Burnol *et al.* (1990) reported much higher levels of GLUT1 for pregnant than for virgin rat mammary gland, whereas I had found roughly equal levels of expression in both virgin and late pregnant animals. However, the fact that Burnol *et al.* (1990) took rats at day 21 of pregnancy whereas my rats were taken at day 20 of pregnancy might explain this difference, given the proximity of parturition and lactogenesis in 21-day pregnant rats. Lactogenesis is initiated during the final

day of pregnancy, in particular lactose synthesis initiates 12-24hr prior to parturition (Threadgold & Kuhn, 1984). If the induction of high levels of mammary GLUT1 expression coincides with the onset of galactopoiesis in mammary tissue, mammary epithelial cells from pregnant animals in which lactogenesis had not yet initiated might only express low levels of GLUT1, equivalent to virgin animals, whereas epithelial cells from rats closer to parturition, in which lactogenesis had been initiated, would have begun expressing higher levels of GLUT1. If this is the case, then the difference between my own results and those obtained by Burnol *et al.* (1990) could have been caused by their rats being closer to parturition than my own.

In order to investigate the expression of glucose transporters specifically involved in lactation, epithelial cells were isolated from mammary glands and used for Western blotting experiments to quantify GLUT1 and GLUT4. Under low power light microscopy, epithelial cells isolated from animals at different stages of pregnancy and lactation had characteristic differences. The medium from cells of early and late pregnancy were found to contain few lipid droplets, whereas when lactating mammary tissue was used, the medium and the cells were full of lipids.

GLUT4 was not detectable in mammary epithelial cells from any stage of pregnancy or lactation. Epithelial cells from early and late pregnant rats were also found to express little or no GLUT1. There was a substantial induction of the GLUT1 expression seen at day 2 of lactation, which was assumed to coincide with lactogenesis, and which fell to approximately 50% of the day 2 level by day 10 of lactation. The decrease in GLUT1 seen between day 2 and day 10 of lactation was unexpected, as previous results using the mammary homogenate had shown that GLUT1 expression continued rising between day 2 and day 10 of lactation. The

number of epithelial cells in the mammary gland is known to increase until day 5 of lactation (Shipman *et al.*, 1987), therefore there are fewer epithelial cells in the mammary gland at early lactation compared to peak lactation. This difference in the ratio of epithelial tissue to stromal tissue might be affecting the detection of GLUT1 in the mammary homogenate. The difference could also reflect a variability in the protein content of the mammary tissue and the mammary epithelial cells during lactation. For example, a higher level of protein in the mammary tissue at early lactation could produce a decrease in the relative level of GLUT1 compared to the expression of homogenate protein, while the expression of GLUT1 in the epithelial cell is high. Conversely at peak lactation the epithelial cell expresses less GLUT1 but a lower content of tissue protein could result in a higher apparent expression of GLUT1 when measured relative to homogenate protein concentration. The level of particulate proteins, especially casein, in the mammary homogenate will significantly effect the protein content.

Isolation of mammary epithelial cells by collagenase and hyaluronidase digestion has been shown to remove some cell-surface glycoproteins (Ceriani *et al.*, 1978). This occurs primarily through proteolytic cleavage, catalysed by protease contamination of the hyaluronidase, as the collagenase is otherwise protease-free. While it was possible that isolation of the epithelial cells was resulting in the partial cleavage of cell surface GLUT1 or GLUT4, this was considered unlikely for a number of reasons. It is unlikely that proteolytic cleavage of epithelial cell GLUT1 would only occur in cells taken from specific stages of development. Furthermore, in the erythrocyte, GLUT1 is known to be very resistant to proteolytic cleavage on its extra-cellular domain (Cairns *et al.*, 1987). The expression of GLUT4 was markedly reduced in mammary homogenate from late pregnant and lactating rats,

implying its expression is confined to the adipocytes.

Burnol *et al.* (1990) had shown little or no GLUT1 mRNA in the mammary gland of virgin and mid-pregnant rats (day 16), however, there was a high expression of GLUT1 mRNA in 21 day pregnant rat mammary gland which fell by day 3 of lactation and was undetectable at peak lactation. In my own work, GLUT1 mRNA was detected in equivalent mammary epithelial cell preparations as those used for the protein investigations, using a cDNA probe to the human HepG2 GLUT1. This probe showed poor hybridisation efficiency to the rat mRNA, even when the stringency conditions were lowered (result not shown). However, by comparing the expression of mammary GLUT1 mRNA to mammary ß-actin mRNA, it was possible to make some observations on mammary GLUT1 mRNA expression during mammary development. In the experiments shown in Section 7.6, GLUT1 mRNA was found to be present in mammary epithelial cells at all stages of pregnancy, lactation and following a 24hr litter-removal and compared to the expression of ß-actin mRNA, appeared not to change.

Removal of the litter for 24hr at peak lactation was found to completely remove GLUT1 protein from the epithelial cells. Similarly the level of GLUT1 protein in the mammary homogenate fell to a level lower than that seen in virgin rats. This suggested that the GLUT1 protein had been completely removed within 24hr after the litter had been removed. This probably reflects the fall in glucose uptake rates by isolated mammary acini of mid-lactating rats prepared following a 48hr litter-removal shown by Prosser & Topper (1986). Litter removal could precipitate hormonal changes, whereby removal of the suckling stimulus leads to a decrease in the secretion of lactogenic hormones, or by the retention in the mammary alveoli of a milk-specific inhibitory factor causing feed-back inhibition of GLUT1 protein

synthesis. In either case, the results suggest that continued expression of GLUT1 is dependent upon the continuance of active lactation and that a mechanism exists in the mammary epithelial cells for the rapid removal of GLUT1 under conditions where high levels of glucose transport are not required. Litter-removal is associated with milk retention in the mammary tissue and lowered plasma prolactin concentrations (Agius *et al.*, 1979, Burnol *et al.*, 1983) which affect a number of galactopoietic functions, including casein synthesis. Much of the work relating to litter-removal has involved the related subject of milking frequency. It is known that lower milking frequency reduces milk yield in goats by inhibiting galactopoiesis (Knight & Wilde, 1987).

During lactation the physiology of the rat is uniquely altered, with the bulk of the plasma nutrients, in particular glucose, directed towards the mammary gland and away from those tissues normally associated with glucose storage, such as adipose tissue. Evidence exists that plasma insulin levels are lower during lactation (Agius *et al.*, 1979), probably due to slight hypoglycaemia, as insulin secretory activity of the pancreatic ß-cells in response to glucose is unimpaired (Madon *et al.*, 1990b). Western blots of parametrial adipose tissue were used to see what effect lactation had upon the expression of the insulin-responsive D-glucose transporter, GLUT4. When adipose homogenate was investigated, higher levels of GLUT4 were found in pregnant rats than were seen in the FTM or lactating rats (Fig. 7.8(a)), however this result was difficult to confirm using the Triton-extracted adipose homogenate (Fig. 7.8(b)). Therefore, the validity of the observed changes in the adipocyte GLUT4 levels during pregnancy and lactation requires more detailed investigation, as does the expression of adipocyte GLUT1. There is some evidence for reciprocal changes in the activity of adipocytes and mammary

epithelial cells during lactation, for example in the ability to synthesize triglycerides (Ros *et al.*, 1990). It was noted that receptors for the main lactogenic hormone, prolactin, have not been detected in adipocytes, however this does not preclude indirect prolactin effects, through other hormones or other receptors.

A number of conclusions were drawn from this study on the expression of GLUT1 and GLUT4 during rat mammary gland development and differentiation under normal physiological conditions. It seems certain that the mammary epithelial cells expressed only the GLUT1 isoform of the glucose transporter and then only when actively synthesizing and secreting milk. The GLUT4 isoform was presumed to be expressed in the mammary gland adipocyte population. One or more regulatory systems were acting to control the expression of GLUT1, switching it on at lactogenesis, and switching it off again when lactation became inhibited. The most likely mechanism for the regulation of GLUT1 in the mammary gland is via the actions of the lactogenic hormones. The influence of hormones on GLUT1 expression in the rat mammary gland was therefore investigated in more detail and the results of those experiments are shown in Chapter 8.

CHAPTER 8

HORMONAL CONTROL OF MAMMARY GLUCOSE TRANSPORTER EXPRESSION *IN VITRO* AND *IN VIVO*

8.1 Introduction

The changing levels of GLUT1 detected in mammary epithelial cells during the *in vivo* development and differentiation of the mammary gland (described in Chapter 7) suggests that the synthesis and/or degradation of the transporter is developmentally regulated in this tissue. GLUT1 protein levels during pregnancy and following a 24hr litter-removal were depressed relative to the GLUT1 levels found during lactation (Section 7.5), although GLUT1 mRNA was still present. The largest increase in the level of GLUT1 was observed at lactogenesis, occurring between day 20 of pregnancy and day 2 of lactation, presumably either immediately preceding, or following, parturition. The apparent constancy of GLUT1 mRNA levels indicated that regulation was probably post-transcriptional, either at the level of translation or transporter degradation. The loss of GLUT1 protein, but not mRNA, from epithelial cells within 24hr of litter-removal indicated that a means for the rapid degradation of GLUT1 exists in mammary cells, and it is possible that the protein undergoes a rapid turnover throughout the period of lactation.

The mechanism(s) controlling GLUT1 synthesis and degradation could be endocrine, autocrine and/or paracrine. The probable hormonal candidates for controlling the expression of GLUT1 in mammary gland during lactation are prolactin and growth hormone. Prolactin is the major lactogenic and galactopoietic hormone in rats, however the galactopoietic role of growth hormone increases as lactation continues. The amount of prolactin binding to isolated mammary

epithelial cells mirrors the pattern of GLUT1 expression shown in Section 7.5, increasing from a basal level during pregnancy to a high level at lactogenesis, and slowly declining as lactation progresses (Hayden *et al.*, 1979). This similarity between prolactin binding and GLUT1 expression suggested a possible relationship between the two. The effects of prolactin on milk protein synthesis (Emerman *et al.*, 1977; Razooki-Hasan *et al.*, 1982), lactose synthesis (Oppat & Rillema, 1988), phospholipid synthesis (Rillema *et al.*, 1990) and upon the ultrastructural differentiation (Mills & Topper, 1970) of the mammary gland at lactogenesis have been well documented *in vitro* using explant cultures. Both primary epithelial cell cultures and mammary explants are widely used to look at hormone effects on mammary differentiation. Mammary explant cultures were used in the following experiments to study glucose transporter expression during hormonally induced differentiation of mid-pregnant mammary explants at lactogenesis, under controlled conditions.

In peak-lactating animals, lowering the plasma levels of galactopoietic hormones has been shown to inhibit lactation. Simultaneous treatment of rats with an inhibitor of prolactin secretion, bromocryptine (CB 154 or 2-bromo-α-ergocryptine) (Knight *et al.*, 1986) and an antiserum raised against rat growth hormone (anti-rtGH) will inhibit milk production, hence mimicking involution of the mammary gland (Madon *et al.*, 1986). Bromocryptine suppresses the secretion of prolactin from the pituitary gland and in conjunction with the anti-rtGH antiserum has been shown to cause a 90% reduction in the amount of GLUT1 detectable in mammary plasma membranes (Fawcett *et al.*, 1991). Whether the decrease in plasma membrane GLUT1 is related to a redistribution of this protein to an intracellular membrane site, similar to the translocation events controlling cell-surface levels

of the insulin-regulatable GLUT4 transporter isoform in adipocytes, or to complete degradation of cellular GLUT1 is as yet unknown. The experiments described in this chapter were therefore performed to examine the effects of lactogenic hormones on mammary epithelial cell GLUT1 expression.

8.2 Levels of GLUT1 and GLUT4 in explants of mid-pregnant mammary gland

To investigate levels of mammary gland GLUT1 and GLUT4 during terminal differentiation of mammary epithelial cells at lactogenesis, explants of mid-pregnant rat mammary gland were used. Both GLUT1 and GLUT4 were studied to compare the effects of various combinations of insulin, cortisol and prolactin on the expression of these proteins over the culture period. Previous *in vitro* work had shown that while insulin and cortisol were required for the maintenance of mid-pregnant mouse mammary explants *in vitro*, prolactin was necessary to induce terminal differentiation, suggesting that prolactin was the minimum requirement for induction of lactation. Each experiment was performed on explants of mammary tissue from a single mid-pregnant rat. The mammary explanting technique is reviewed by Dils & Forsyth (1981). I am indebted to Dr. C. J. Wilde for assistance in cutting the explant tissue and advice on explant techniques.

8.2.1 Mid-pregnant rat mammary explant culture

Culture media were obtained from Gibco or Northumbria Biologicals Limited unless otherwise stated. All procedures were carried out under sterile conditions.

Mid-pregnant rats were killed by cervical dislocation and the abdominal and sub-inguinal mammary tissue removed into culture medium on ice for the duration of the cutting procedure. Culture medium was composed of Medium 199 (with Earles

salts, without glutamine or $NaHCO_3$) buffered with 13.5mM HEPES, pH 7.4 and containing 2μg/ml fungizone (amphotericin B), 50U/ml Penicillin G, 50μg/ml streptomycin, 2mM glutamine, 0.225% $NaHCO_3$. The mammary tissue was pinned onto cork mats and mammary explants (approximately 1mm cubed, visualised using a dissecting microscope) dissected from the tissue. Explants were plated out onto stainless steel grids at 30 explants/well in 6-well culture plates and maintained in medium until the cutting was finished, the medium meniscus just covering the top of the explant tissue. When the cutting was complete and the explants plated out, the medium was replaced with fresh medium, containing either no hormones (NH), 5μg/ml insulin and 100ng/ml cortisol (IC) or 5μg/ml insulin, 100ng/ml cortisol and 1μg/ml prolactin (ICP). Insulin (bovine pancreas) was prepared as a concentrated stock (100μg/ml) in 0.1M NaOH, cortisol (hydrocortisone-21-acetate) was prepared as a concentrated stock (2mg/ml) in ethanol and prolactin (sheep pituitary luteotropic hormone) was dissolved as a concentrated stock (100μg/ml) in H_2O. Insulin and cortisol stocks were filter sterilised, whereas the prolactin stock was prepared under sterile conditions but <u>not</u> filter sterilised. Explants were cultured in the appropriate media for a maximum of 72hr, replacing the media every 24hr. At 24hr intervals two wells of explants were harvested. Time 0 explants were taken at the time of explant cutting and plating out. Harvested explants were blotted dry, placed into tared cryotubes and weighed, before being frozen in liquid N_2 and stored at -70°C.

8.2.2 Levels of GLUT1 and GLUT4 in explants of mid-pregnant rat mammary gland

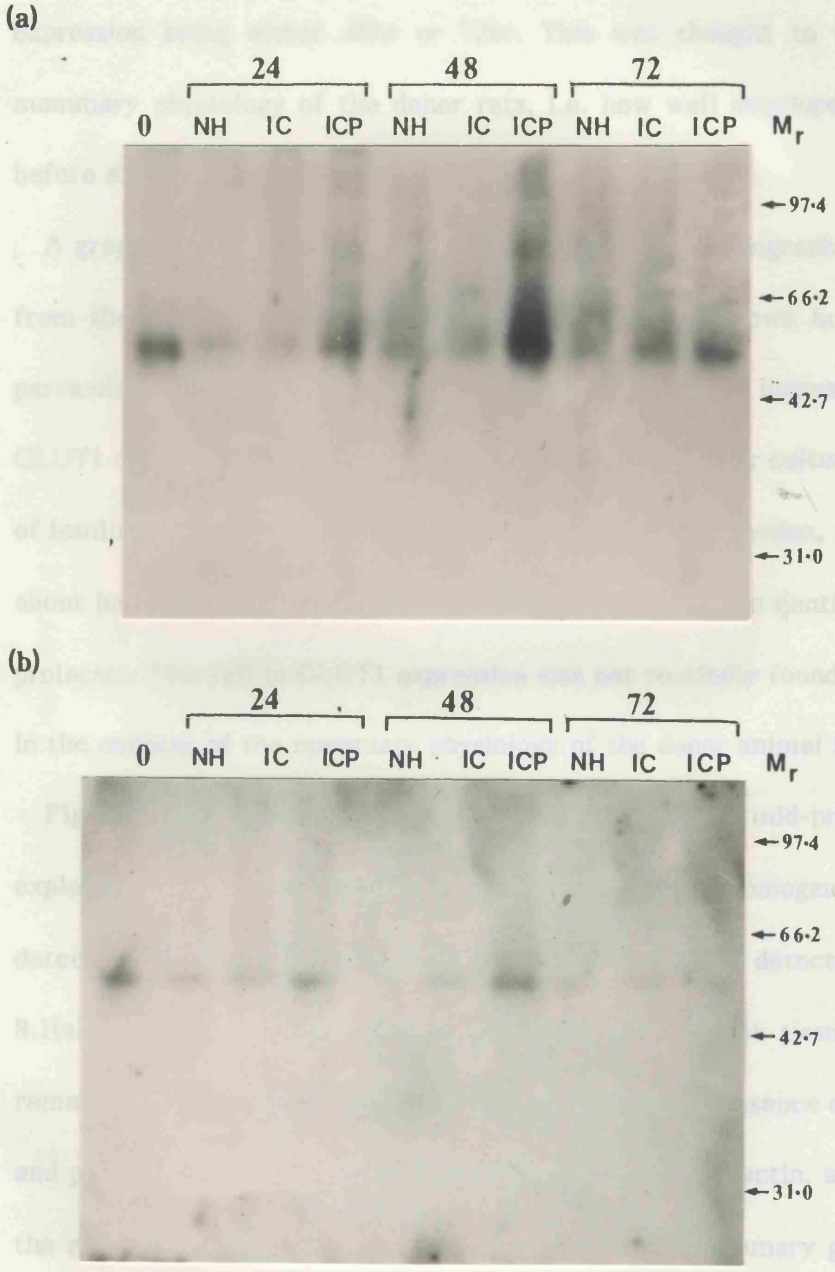
Cryotubes equivalent to a single well of 30 explants were thawed into 1.0ml PBS, pH7.4 containing 0.2mM PMSF on ice. Each pellet was homogenised by 10-15

strokes of a glass Dounce homogeniser. Aliquots of the explant homogenate were used for DNA and protein assays as described in Sections 2.8 and 2.9 respectively.

Aliquots of explant homogenate equivalent to 20μg DNA were placed into 1.5ml Beckman eppendorf tubes, and centrifuged at $100,000g_{av}$ using a Beckman TL-100 benchtop ultracentrifuge for 30min at $4^{o}C$. The supernatants were discarded and the membrane pellets resuspended in gel sample buffer containing 6M urea. Gel samples equivalent to 5μg DNA (between 83-140μg protein) were electrophoresed on 10% SDS/polyacrylamide gels and Western blotted onto nitrocellulose. Glucose transporters were detected using affinity-purified antibodies raised against the C-terminal peptides of GLUT1 or GLUT4, followed by an iodinated second antibody and autoradiography as described in Section 2.6.

The resulting autoradiographs show the changing level of GLUT1 and GLUT4 during differentiation of the mammary explants *in vitro*. The level of GLUT1 following 24hr intervals in explants cultured for a maximum of 72hr, either in the absence of hormones (NH), in the presence of insulin and cortisol (IC), or in the presence of insulin, cortisol and prolactin (ICP), is shown in Fig. 8.1(a). A significant increase in GLUT1 expression from time 0 was observed within 48hr of culture in the presence of insulin, cortisol and prolactin, compared to the explants cultured without prolactin. In mammary explant cultures from three different donor rats (two wells of explants / hormonal treatment / time point / rat) the presence of prolactin always resulted in an increase in detectable GLUT1 levels, which was not seen in the absence of prolactin. In the particular experiment shown in Fig. 8.1(a), the highest level of GLUT1 expression was seen after 48hr of culture, the levels of GLUT1 decreasing from this maximum following 72hr of culture. Individual experiments were found to vary in both the magnitude of GLUT1

Fig. 8.1 Levels of glucose transporters in mid-pregnant rat mammary gland explants



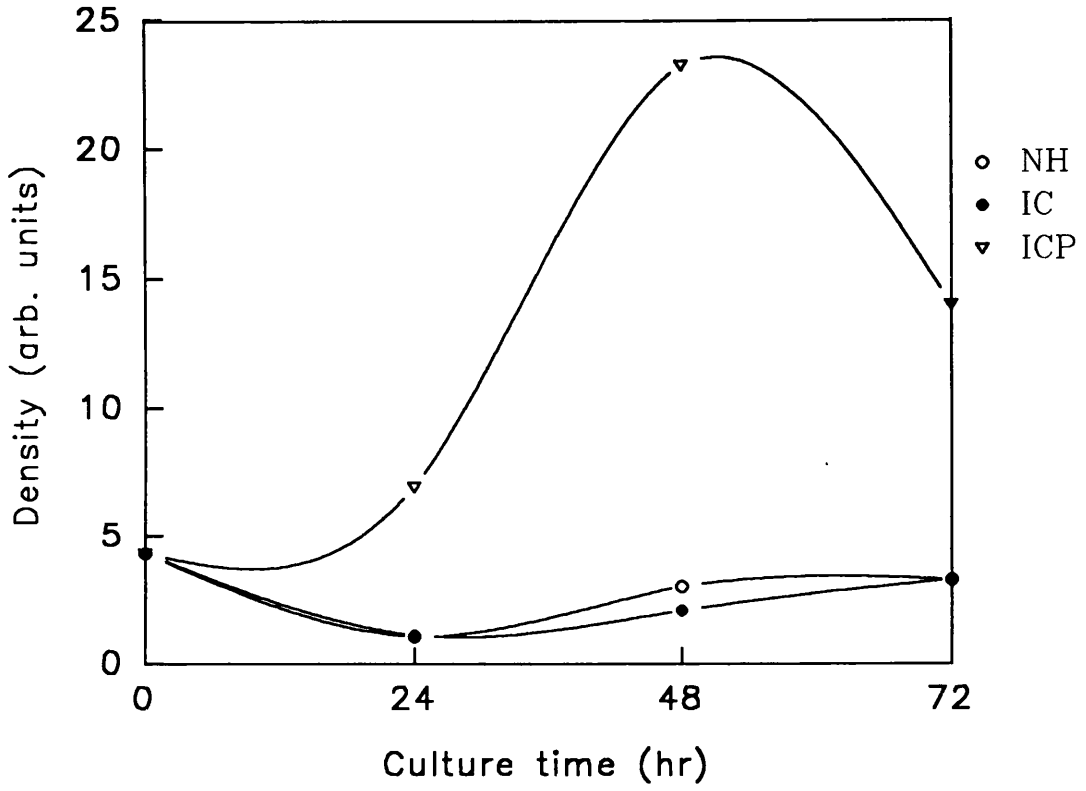
Mid-pregnant rat mammary gland explants were cultured in media containing no hormones (NH), insulin and cortisol (IC) or insulin, cortisol and prolactin (ICP). Glucose transporters were detected in mammary explant homogenate (which had been depleted in cytoplasm) equivalent to 5μg DNA, following electrophoresis on 10% SDS/polyacrylamide gels and Western blotting using antibodies raised against the *C*-terminal peptides of GLUT1(a) or GLUT4(b). On the two autoradiographs shown lane (0) shows glucose transporter expression at time 0, when the explant material was cut, equivalent to mid-pregnant mammary alveoli. The remainder of the blot shows glucose transporter expression following culture for 24hr, 48hr or 72hr as indicated.

expression and temporally, the time in culture required to reach the maximum expression being either 48hr or 72hr. This was thought to depend upon the mammary physiology of the donor rats, i.e. how well developed the tissue was before explanting.

A graphic representation of the density of the autoradiographic bands obtained from the single experiment, shown in Fig. 8.1(a), is shown in Fig. 8.2. In this particular experiment there was an approximately 5-fold increase in the level of GLUT1 detected in the mammary explants following a 48hr culture in the presence of insulin, cortisol and prolactin compared to time 0 expression, which then fell to about half this level following 72hr of culture despite the continued presence of prolactin. This fall in GLUT1 expression was not routinely found, and is discussed in the context of the mammary physiology of the donor animal in Section 8.7.

Fig. 8.1(b) shows the expression of GLUT4 observed in mid-pregnant mammary explants during 72hr of culture. The mammary explant homogenates used here to detect GLUT4 were the same as those used to for GLUT1 detection, shown in Fig. 8.1(a). GLUT4 was detectable in the mammary explant tissue, its expression remaining at the original level following culture in the presence of insulin, cortisol and prolactin for 48hr, but falling in the absence of prolactin, and after 72hr. As the explants were dissected from mid-pregnant rat mammary gland there was a substantial adipocyte population in the tissue. By restricting explants to just the alveoli wherever possible, adipocyte contamination was minimised, but impossible to eliminate. The normally high ratio of GLUT4 to GLUT1 in rat adipocytes however meant that it was unlikely that adipocyte GLUT1 was contributing substantially to the detection of mammary explant GLUT1 shown in Fig. 8.1(a). Receptors for prolactin have not been identified in adipocytes, therefore assuming

Fig. 8.2 Quantification of GLUT1 in mammary explants during culture



The intensity of the GLUT1 autoradiographic bands, on the Western blot shown in Fig. 8.1(a), were determined by densitometry, in order to quantify the relative levels. Explants were cultured in the presence of no hormones (NH), insulin + cortisol (IC) or insulin, cortisol + prolactin (ICP) for 0, 24, 48 or 72hr respectively. The magnitude and timing of the change in GLUT1 levels varied substantially between experiments, therefore, statistical analysis of the GLUT1 levels was not feasible.

that the explant GLUT4 was arising from adipocytes, it was interesting that its maintenance required prolactin.

8.3 *In vivo* effects of galactopoietic inhibitors on rat mammary GLUT1 levels

The dependence of the increase in GLUT1 levels upon prolactin, seen in mammary epithelial cells at lactogenesis, has been established from results in Section 8.2.2. To ascertain whether prolactin also influenced the continued high level of GLUT1 during galactopoiesis, plasma prolactin levels were artificially lowered *in vivo* and GLUT1 expression investigated. Treatment of mid-lactating rats with bromocryptine, together with the simultaneous employment of an antiserum raised against rat growth hormone (anti-rtGH), has been shown to inhibit galactopoiesis (Madon *et al.*, 1986), and to cause a 90% reduction in the level of GLUT1 detected in mammary plasma membranes (Fawcett *et al.*, 1991).

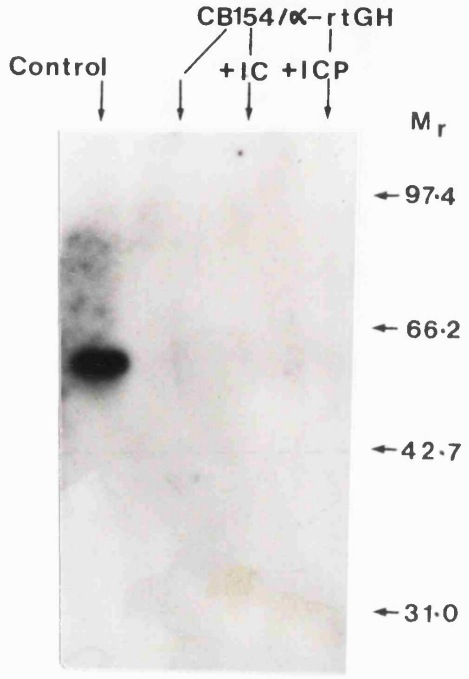
Rats between days 12-14 of lactation were treated with a sheep anti-rtGH antiserum (0.25ml 3x conc. γ-globulin fraction/100g body weight) and 0.5mg bromocryptine (Sandoz). The treatment was given subcutaneously at 9:30 a.m. and 4:30 p.m. for two days, by Miss. D. Panton. Following treatment, animals were killed by cervical dislocation and the inguinal and sub-abdominal mammary tissue taken. Epithelial cells were prepared by the method described in Section 2.2, using collagenase digestion, and the medium supplemented with 5μg/ml insulin and 10ng/ml cortisol. An aliquot of cells was pelleted at 14,000g, frozen in liquid N_2 and stored at -70°C. The remaining cells were split into two aliquots and resuspended in a modified Medium A, containing Earles salts in place of Hanks salts, 1x minimal essential amino-acids, 0.68mM glutamine, 50U/ml penicillin G, 50μg/ml streptomycin and 0.225% $NaHCO_3$, and supplemented with either insulin

(25ng/ml) and cortisol (10ng/ml) or insulin, cortisol and prolactin (1μg/ml). Less insulin and cortisol were required to maintain the cells from lactating rats than to proliferate and maintain explants from mid-pregnant rats, as physiologically insulin is present at a lower concentration during lactation, and less cortisol is required to maintain the short-term cultures. Cells were plated out into 6-well culture dishes and incubated at 37°C under 5%CO_2/95%O_2 for 2.5hr. The epithelial cells were then harvested by centrifugation in a microfuge, the pellets frozen in N_2 and stored at -70°C.

Homogenates of the cell pellets were prepared as described in Section 7.2, and samples of cell homogenate taken for protein and DNA assays (described in Section 2.9 and 2.8 respectively). Aliquots of homogenate (5μg DNA) were prepared as gel samples and electrophoresed on 10% SDS/polyacrylamide gels. Following transfer to nitrocellulose, GLUT1 was detected using antibodies raised against its' *C*-terminal peptide, an iodinated second antibody and autoradiography. Samples of homogenised epithelial cells isolated from mammary gland of control rats, used for the developmental studies in Section 7.2, were electrophoresed adjacent to the test samples.

The Western blots in Fig. 8.3 show GLUT1 expression in mammary epithelial cell samples from treated and control rats. Both contain the amount of protein equivalent to 5μg DNA (between 79-87μg protein). GLUT1 could not be detected in the mammary epithelial cells isolated from animals treated with bromocryptine and anti-rtGH, compared to the high levels of GLUT1 expression in the normal lactating rat mammary epithelial cells. Also shown in Fig. 8.3 is GLUT1 expression in epithelial cells from the bromocryptine and anti-growth hormone treated rats, which had been cultured in the presence of insulin and cortisol ± prolactin. In these

Fig. 8.3 Levels of GLUT1 in bromocryptine and anti-rtGH treated mid-lactating rat mammary gland epithelial cells



Epithelial cells were isolated from the mammary gland of peak-lactating rats treated for 48hr with bromocryptine and a sheep anti-rtGH τ-globulin fraction as detailed in Section 8.3. One aliquot of cells was harvested immediately following preparation (CB154/α-rtGH), while other cells were incubated *in vitro* for 2.5hr in the presence of insulin & cortisol (IC), or insulin, cortisol & prolactin (ICP). Cell homogenate protein, equivalent to 5μg DNA, together with cell homogenate from control, untreated rats (Cont.), was electrophoresed and GLUT1 detected by Western blotting as described in Section 2.6 using antibodies raised against the C-terminal peptide of GLUT1.

cells GLUT1 was also undetectable, demonstrating that prolactin was not able to induce synthesis of GLUT1 in short term cultures under the conditions used.

8.4 Expression of GLUT1 mRNA in rat mammary gland following treatment with inhibitors of galactopoiesis

A total RNA fraction had been prepared by Dr. M. Barber from the mammary glands of rats treated by Fawcett *et al.* (1991) with inhibitors of galactopoiesis. Samples of these RNA's were kindly donated and used to investigate the expression of GLUT1 mRNA in the mammary tissue.

RNA was available from animals treated for 48hr with the following agents:-

A) Normal sheep serum (0.25ml 3x conc. γ-globulin fraction / 100g body weight)

B) Sheep anti-rtGH antiserum (as above)

C) Bromocryptine (0.5mg)

D) Bromocryptine + anti-rtGH

E) Bromocryptine + anti-rtGH + bovine GH (0.5mg recombinant bovine GH (Monsanto) in 0.01M $NaHCO_3$)

F) Bromocryptine + anti-rtGH + human IGF-1 (0.5mg recombinant human IGF-1 (Ciba-Geigy) in 25% PVP)

Samples containing 40μg RNA were electrophoresed on 1.2% agarose/formaldehyde gels and Northern blotted onto Hybond N nylon then GLUT1 mRNA detected using a radiolabelled cDNA probe consisting of the entire BamH1 fragment of HepG2 cDNA, as detailed under Section 2.7. The Northern blots were

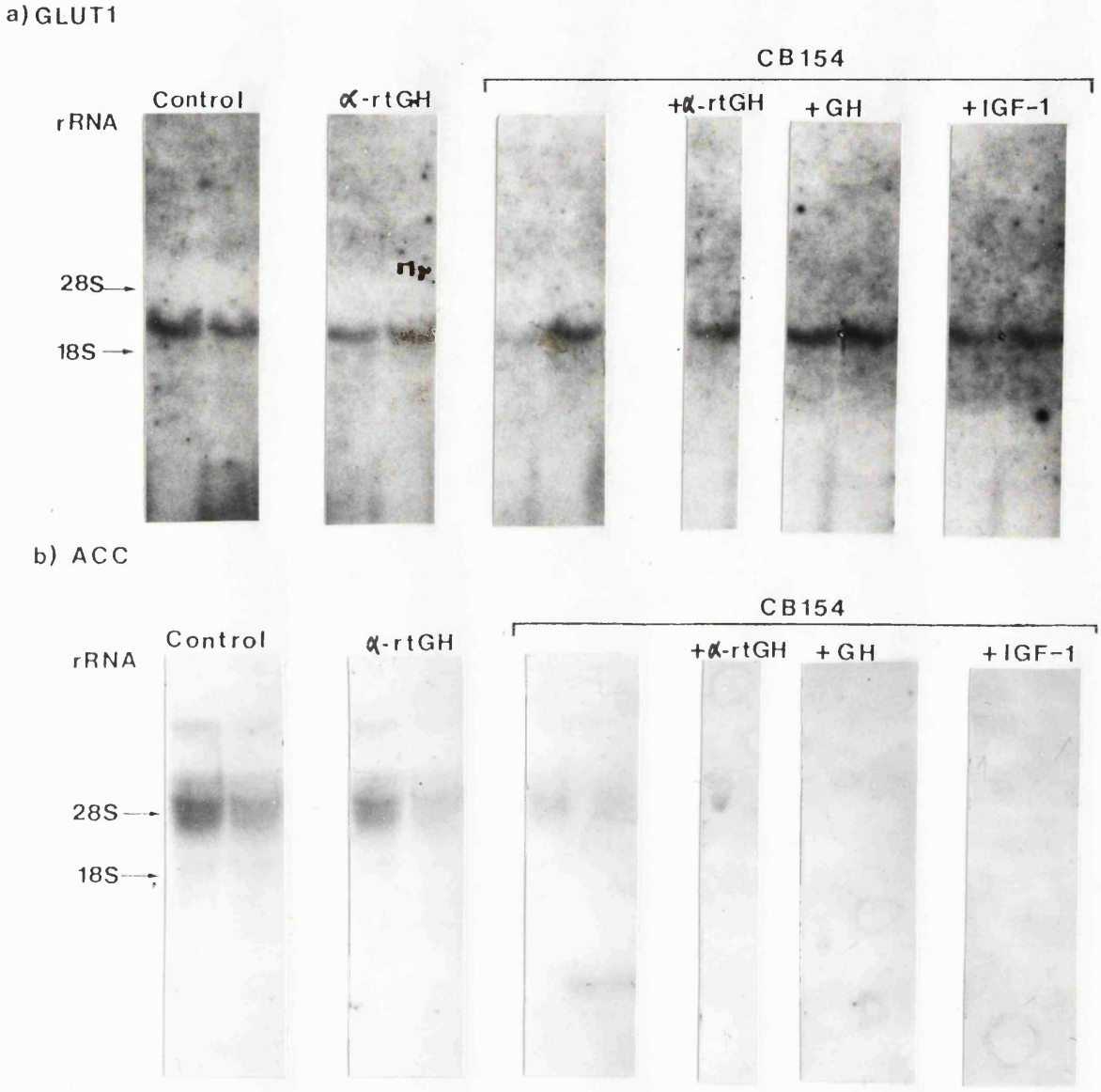
then stripped and re-probed using a [^{32}P] labelled cDNA probe to rat ACC, provided by Dr. M. Barber, and ACC mRNA detected by autoradiography.

The Northern blots in Fig. 8.4 show the expression of GLUT1 mRNA (a) and ACC mRNA (b) in mammary gland, following the treatments described above. The expression of GLUT1 mRNA appeared to be slightly decreased following treatment with bromocryptine ± anti-rtGH, and stabilised by the additional treatment with exogenous GH or IGF-1. However, any changes in GLUT1 mRNA were not large, and did not reflect the 90% loss of GLUT1 protein from the mammary plasma membrane described by Fawcett *et al.* (1991), or the apparent total loss of GLUT1 protein from the mammary epithelial cell homogenate, shown in Section 8.3. Unfortunately it was not possible to quantify the blots by densitometry, as the backgrounds were too high. However, the same pattern of expression was seen on Northern blots of RNA from four different animals under each treatment. The expression of ACC mRNA was found to change in the same manner as the results published by Barber *et al.* (1992). Following simultaneous treatment of rats with both bromocryptine and anti-rtGH, the ACC mRNA was lost from the mammary gland, and simultaneous treatment with either exogenous GH or IGF-1 failed to prevent this loss. The intensity of the RNA seen under U.V. irradiation indicated that there were no significant differences between the amount of RNA loaded onto the gels for each of the samples.

8.5 Levels of GLUT1 in isolated rat mammary acini incubated *in vitro*

In Section 8.3 it was shown that mammary acini, previously depleted in GLUT1 by treatment *in vivo* with inhibitors of galactopoiesis, did not have increased GLUT1 protein levels following incubation *in vitro* with insulin, cortisol and

Fig. 8.4 Expression of GLUT1 mRNA in total mammary RNA of bromocryptine and anti-rtGH treated mid-lactating rats



Rats were treated for 48hr with normal sheep serum (control), sheep anti-rtGH serum (α-rtGH), bromocryptine (CB154), CB154 + α-rtGH, CB154 + α-rtGH + bovineGH (GH), and CB154 + α-rtGH + humanIGF-1 (as described in Section 8.4), performed by Miss H.A.C. Fawcett, and the RNA prepared by Dr. M. Barber. Total mammary RNA (40μg) was electrophoresed on 1.2% agarose/formaldehyde gels and following transfer to Hybond N membranes, GLUT1 mRNA was detected using a [^{32}P]labelled HepG2 GLUT1 cDNA probe and autoradiography as described in Section 2.7. Probed blots were then stripped and reprobed for acetyl CoA carboxylase (ACC) mRNA expression using a [^{32}P]labelled cDNA probe for rat ACC provided by Dr. M. Barber. With the exception of CB154 + α-rtGH results of all treatments are shown for two different rats each.

prolactin. As mammary acini prepared from lactating tissue might lose their functional activity when cultured in suspension, it was possible that the cells incubated in the experiments in Section 8.3 were either unable to synthesize GLUT1 or unable to retain newly synthesized GLUT1 during the culture time. The half-life of GLUT1 in the mammary epithelial cells is likely to be short, as suggested by the almost total degradation of GLUT1 within 24hr of litter-removal (Section 7.5). Mammary epithelial cell plasma membrane can turn over in under 4hr, therefore the half-life of mammary plasma membrane GLUT1 could be much less than 24hr. The ability of normal lactating mammary epithelial cells to maintain GLUT1 levels during the isolation procedure and following incubation for up to 18hr with various hormone combinations was therefore investigated.

Epithelial cells from mid-lactating rat mammary glands were isolated according to the method in Section 2.2. The washed cells were resuspended in 26ml of modified Medium A described in Section 8.3, and 2x1ml aliquots pelleted at 14,000g and stored at -70^{o}C, for time 0 measurements. The remaining cells were divided into 3x8ml aliquots and each aliquot resuspended into 16ml of media supplemented with no hormones (NH), insulin (25ng/ml) and cortisol (10ng/ml) (IC), or insulin, cortisol and prolactin (1μg/ml) (IPC). Each 16ml aliquot was plated out as 8x2ml aliquots into 6-well culture dishes, and the cells incubated at 37^{o}C, in 5% CO_2/95%O_2. Two wells of cells were harvested at 2hr, 4hr, 7hr and 18hr, the cells pelleted at 14,000g and stored at -70^{o}C.

Epithelial cell pellets were thawed into 100μl PBS, pH7.2 containing the protease inhibitors, described in Section 7.2, and homogenised using a 500μl glass Dounce homogeniser, on ice. Aliquots of cell homogenate were used for DNA and protein assays as detailed in Sections 2.8 and 2.9 respectively. Samples of homogenate

corresponding to 7.5μg DNA or 150μg protein were used to prepare gel samples and electrophoresed on 10% SDS/polyacrylamide gels. Following Western blotting onto nitrocellulose, GLUT1 was detected using antibodies raised against the *C*-terminal peptide, an iodinated second antibody and autoradiography.

The Western blot shown in Fig. 8.5 demonstrates that expression of the GLUT1 protein is not significantly decreased in mammary epithelial cells following 18hr incubation *in vitro*, under any of the hormone treatments used. A similar result was obtained when a constant DNA loading was used, suggesting that either degradation of GLUT1 *in vivo* in the presence of prolactin antagonists is not due solely to the lower plasma prolactin concentration, or that the cells *in vitro* lacked a fully functional degradative pathway.

8.6 Detection of GLUT1 in mouse mammary gland

Much of the published information relating to differentiation of mammary tissue and its hormonal control *in vitro* has been obtained using mouse mammary gland primary epithelial cell culture. The presence of GLUT1 in mouse mammary epithelial cells was therefore investigated, using Western blotting.

A total membrane fraction was prepared from isolated lactating mouse mammary epithelial cells (gift from Mr. C. Bennett) using the method described in Section 2.3.1, but only isolating membranes to the stage identified as P3 in the nomenclature of Fig. 4.4(a). Pellets of mouse mammary epithelial cells from lactating or pregnant mouse mammary gland (2.5×10^6 cells) were also donated by Mr. C. Bennett and Ms. M. Rennison respectively. These cell pellets were dissolved directly into 100μl gel sample buffer containing 6M urea. The entire dissolved cell pellets, together with gel samples containing 50μg mouse mammary membrane

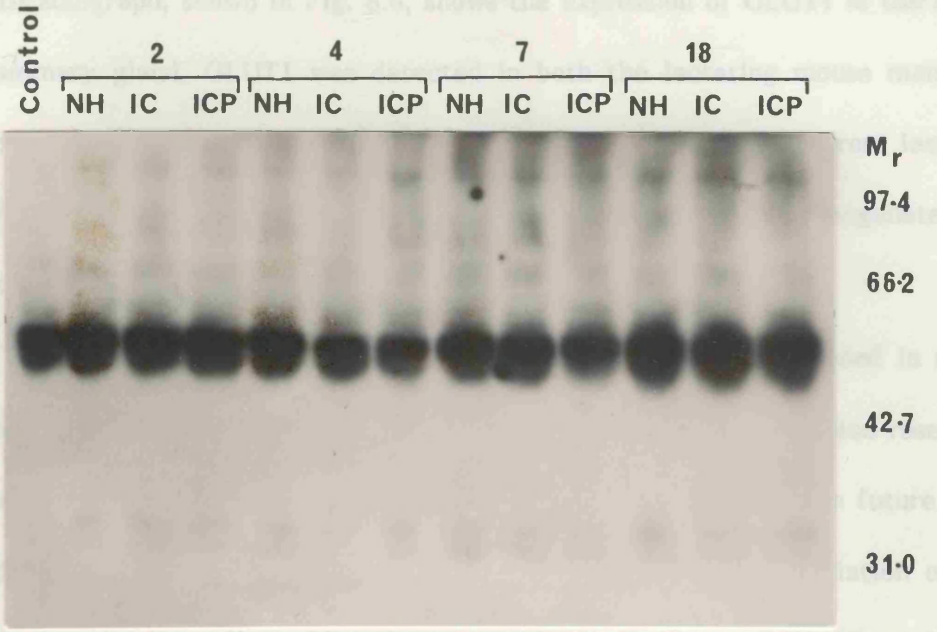
protein or 50μg rat mammary membrane (P3) protein, prepared as described in Section 2.3.1, were electrophoresed on 10% SDS/polyacrylamide gels. Following Western blotting onto nitrocellulose, GLUT1 was detected using anti *C*-terminal peptide antibodies, an iodinated second antibody and autoradiography. The resulting autoradiograph, shown in Fig. 8.6, shows the expression of GLUT1 in the mouse mammary gland. GLUT1 was detected in both the lactating mouse mammary membrane fraction and in mammary epithelial cell homogenate from lactating mice, however, it was not detected in mammary epithelial cell homogenate from the mid-pregnant mouse.

The fact that immunologically cross-reactive GLUT1 is expressed in mouse mammary epithelial cells and that its temporal pattern of expression resembles that of the rat in being restricted to the lactating gland, should in future allow more detailed studies to be performed on the expressional regulation of this protein, using the well-established primary culture of mid-pregnant mouse epithelial cells.

8.7 Discussion

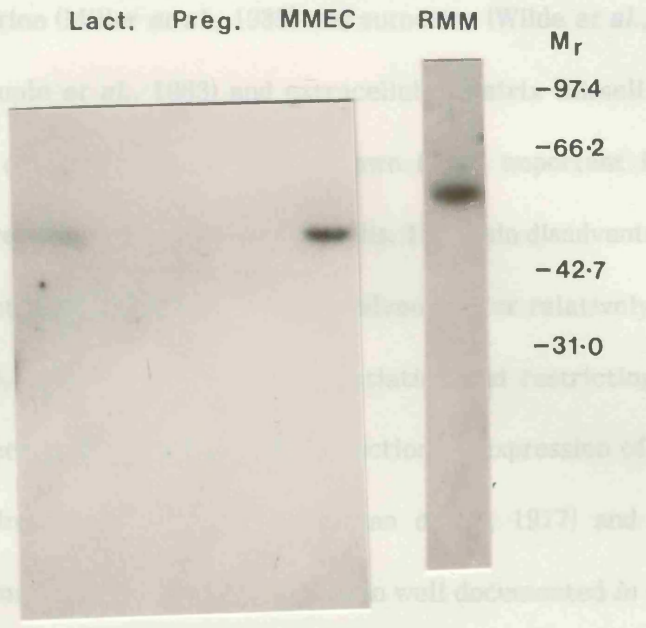
There are two *in vitro* techniques available through which to study lactogenesis under controlled conditions, primary epithelial cell culture on floating collagen rafts, or explant culture. In both cases cells/explants taken from mid-pregnant animals are cultured in suitable media and the synthesis of milk-specific proteins induced using appropriate hormone combinations. Both culture techniques require insulin in supraphysiological concentrations to maintain the culture viability, which is enhanced by the presence of glucocorticoids. However, synthesis of milk specific proteins is only induced when prolactin is also added to the media. Only a modest

Fig. 8.5 Retention of GLUT1 by mid-lactating rat mammary gland epithelial cells *in vitro*



Epithelial cells were isolated from mid-lactating rat mammary glands and incubated in insulin & cortisol (IC), insulin, cortisol & prolactin (ICP) or no hormones (NH) for 2, 4, 7 or 18hr as indicated. Samples of cell homogenates containing 150μg protein were electrophoresed on 10% SDS/polyacrylamide gels and GLUT1 detected following Western blotting using antibodies raised against the *C*-terminal peptide, as described in Section 2.6.

Fig. 8.6 GLUT1 in mouse mammary epithelial cells



The expression of GLUT1 in mouse mammary gland was investigated by Western blotting. Epithelial cells from peak-lactating (lact.) or mid-pregnant (preg.) mouse mammary gland and a membrane-enriched fraction of lactating mouse mammary epithelial cells (MMEC, 50μg) or rat mammary gland (RMM, 20μg), were prepared as gel samples and electrophoresed on 10% SDS/polyacrylamide gels. GLUT1 was detected by Western blotting using the method in Section 2.6 and antibodies raised against the GLUT1 *C*-terminal peptide.

degree of differentiation is obtained with mammary explants, however the advantage of explant culture is the maintenance of cell-cell contacts and the retention of mammary cells in their correct orientation and proportion, with an intact basal lamina. This is particularly important in the mammary gland where paracrine (Miller *et al.*, 1989) and autocrine (Wilde *et al.*, 1987) factors, cell shape (Haetuple *et al.*, 1983) and extracellular matrix (Bissell & Hall, 1987; Barcellos-Hoff *et al.*, 1989) have been shown to be important for the maintenance and differentiation of the epithelial cells. The main disadvantage of mammary explants is duct-sealing, which closes the alveoli after relatively short periods of culture (72hr), inhibiting further differentiation and restricting the useful life span to between 3 and 4 days. The induction of expression of a range of milk-specific proteins including casein (Emerman *et al.*, 1977) and α-lactalbumin (Oppat & Rillema, 1988) by prolactin has been well documented *in vitro*. While GLUT1 is not strictly a milk-specific or galactopoietic protein, the expression of GLUT1 by mammary epithelial cells does appear to be intrinsically linked to lactation and milk production, demonstrated by the results of Western blotting in Chapter 7. Increased levels of GLUT1 in mammary explants of mid-pregnant rats could be initiated by culture in the presence of insulin, cortisol and prolactin. Prolactin was found to be the absolute requirement, as culture in the presence of insulin and cortisol alone did not induce increased GLUT1 levels. The low level of GLUT4 in the mammary explants was sustained by the presence of insulin, cortisol and prolactin for 48hr, but did not increase. The relative levels of GLUT1 and GLUT4 (with a ratio of 1:9) usually expressed by adipocytes suggested that the increase in GLUT1 levels in the mammary explants did not originate solely from the mammary adipocytes. In these experiments, a change in glucose uptake by the cells, or in

lactose synthesis was not investigated in relation to the increase in explant GLUT1 levels. Preliminary investigations of explants cultured in the presence of [^{14}C]glucose indicated that [^{14}C]lactose was being synthesized and could be extracted from the media and the particle-free supernatant of explant homogenates according to the precipitation method of Kuhn & White, (1975) (results not shown), however the synchronous synthesis of lactose and increasing GLUT1 expression was not investigated. The relationship between lactose synthesis and GLUT1 expression *in vitro* remains to be investigated more thoroughly.

The GLUT1 detected in the mammary explants differed from that seen *in vivo* in that it migrated on SDS/polyacrylamide gels as a broader band than normally seen for mammary GLUT1, a finding which implies a more heterogeneous glycosylation. This could be due to a lack of the appropriate carbohydrates for correct glycosylation of GLUT1. It would be interesting to see how this apparently altered glycosylation state might affect the distribution of the mammary GLUT1 between intracellular membranes and plasma membranes.

The level of GLUT1 synthesized by the explants was found to vary between different experiments. As each experiment was performed using tissue from a single animal, it was assumed that the different level of response was due to the different physiological state of the mammary gland from the different donor animals. Variability in the magnitude of prolactin-stimulated casein accumulation in mammary epithelial cell culture (Emerman *et al.* 1977) has also been linked to the mammary physiology of the donor animal. Also, cells at different stages of development within the mammary tissue have different hormone sensitivities (Topper *et al.*, 1972).

In one explant experiment, the results of which are shown in Fig. 8.1(a) the level

of GLUT1 expression was seen to be lower at 72hr of culture than at 48hr, despite the continued presence of prolactin. This suggested that in this particular experiment, synthesis of GLUT1 was inhibited after 48hr, possibly due to duct-sealing and accumulation of milk proteins. This particular experiment gave the highest induction of GLUT1 expression within 48hr, which could suggest more rapid differentiation and feedback inhibition. Explant cultures were not continued for any longer than 72hr because of the problems of duct-sealing, however, in future experiments it would be interesting to see how duct sealing affected GLUT1 expression in more detail. Loss of GLUT1 from the mammary gland *in vivo* following a 24hr litter removal (shown in Section 7.5) could be due to lower plasma prolactin levels and/or due to feedback inhibition by a milk-specific protein, whereas in the explants, prolactin loss will not be a contributing factor. While prolactin is necessary for lactogenesis, the galactopoietic action of prolactin in maintaining lactation might not extend to maintaining mammary GLUT1 levels if the mammary gland is feed-back inhibited. Bromocryptine and an antiserum against growth hormone in combination inhibit lactation (Madon *et al.*, 1986) and cause a 90% decrease in the level of GLUT1 detected in plasma membranes of peak lactating rat mammary gland (Fawcett *et al.*, 1991) . However, it is not known whether GLUT1 is internalised following this treatment, as is seen with other hormonally controlled glucose transporters, for example GLUT4 in adipocytes. Treatment with bromocryptine alone has also been shown to significantly reduce GLUT1 levels in the mammary plasma membrane (Fawcett *et al.*, 1991). The manner in which growth hormone affects lactation and GLUT1 expression in mammary gland is not clear, as receptors for growth hormone have not been detected in mammary gland, however mRNA for the growth hormone receptor has

recently been identified in mammary tissue (Hauser *et al.*, 1990; Jammes *et al.*, 1990; Lincoln *et al.*, 1990). It was clear from the work of Fawcett *et al.*, (1991) that, with respect to GLUT1 expression, growth hormone is not acting through the IGF-1 receptors, as IGF-1 could not substitute for growth hormone in the treated, GLUT1-depleted animals. GLUT1 mRNA levels in these rat mammary glands did not reflect the changes in the GLUT1 protein seen in the plasma membrane or in the epithelial cells as a whole, or the changes in mRNA encoding another enzyme involved in lactation, ACC (Barber *et al.*, 1992). However, as the changing level of GLUT1 mRNA was, in these experiments, impossible to quantify, this subject requires more work.

While the results of these treatments indicate that both prolactin and growth hormone are required for the maintenance of glucose transporter expression in mammary gland, the mechanisms of prolactin, or growth hormone, action remain unclear. Incubation of mammary epithelial cells from bromocryptine and anti-growth hormone treated lactating rats with insulin, cortisol and prolactin for 2.5hr did not increase the level of detectable GLUT1 protein in the epithelial cells, although there was assumed to be GLUT1 mRNA present, as this had been observed in other animals following the same treatment (Fig. 8.4).

CHAPTER 9

CONCLUSION

9.1 Identification and characterisation of mammary glucose transporters during lactation

From the closing sections in the previous chapters a number of conclusions can be drawn from the work presented in this thesis. Two different isoforms of the mammalian facilitative D-glucose transport protein are present within the lactating rat mammary gland, predominantly the human erythrocyte/rat brain (GLUT1) isoform and also the insulin-sensitive (GLUT4) isoform. Sub-cellular fractionation of lactating rat mammary gland, immunocytochemistry and isolation of mammary epithelial cells have confirmed that the majority of the GLUT1 protein detected in mammary homogenate originates from the epithelial cells, and that the GLUT4 protein is derived from a different cell type within the gland, probably the mammary adipocytes. The protein detected by the anti-GLUT1 *C*-terminal peptide antibodies in mammary gland was shown to be very similar to the characterised GLUT1 proteins of human erythrocyte and rat brain in a number of ways. The glucose transport function of the protein was shown by the inhibition of cytochalasin B photolabelling by D-glucose and not L-glucose. Immunoprecipitation of the photolabelled protein demonstrated that the anti-*C*-terminal peptide antibodies were detecting this glucose transporter. The similarity of the antigenic epitopes, observed using site-specific anti-GLUT1 peptide antibodies, and by Northern blotting using a human erythrocyte GLUT1 cDNA probe, indicated that the mammary GLUT1 glucose transporter is very similar to the human erythrocyte GLUT1. Mammary GLUT1 is substantially glycosylated, although less

heterogeneously than the human erythrocyte GLUT1, resembling rather the glycosylation state of the rat brain GLUT1. However, in order to confirm the identity of the mammary GLUT1 as either the human erythrocyte, or the rat brain, transporter, the mammary protein needs to be sequenced.

9.2 Regulation of glucose transporter levels during mammary development

Early work described above had identified the major glucose transporter in the mammary gland during lactation as the GLUT1 protein. This isoform was therefore assumed to be the one isoform involved in the transport of glucose required by the epithelial cells to sustain lactation. The mammary gland shows significant development and differentiation during the gestational-lactational cycle. Epithelial cell numbers increase steadily during pregnancy, reaching a peak in early lactation (Shipman *et al.*, 1987). Around the time of parturition the epithelial cells undergo terminal differentiation, as lactation is initiated, becoming fully-functional during the final day of pregnancy. While the previous results had shown the presence of GLUT1 and GLUT4 in the mammary tissue during lactation, it was not known how the levels of these proteins might vary during tissue development. During the course of this work, Burnol *et al.* (1990) described the changing expression of both GLUT1 and GLUT4 in mammary gland homogenate, with the development of the tissue during pregnancy, and in differentiation during lactation. They observed an increased expression of GLUT1 and a concommitant decrease in the expression of GLUT4 as the gland develops. Similar results were obtained from my own work, described in Chapter 7. Glucose transporter levels over the period from a mature virgin rat through early- and late-pregnancy and in early- and mid-lactation were investigated. In agreement with the published results of Burnol *et al.* (1990), an

increase in mammary GLUT1 levels was found to be coupled to a decrease in mammary GLUT4 levels in whole homogenate. However, to investigate the relative contribution of the mammary epithelial cells and adipocyte populations to the total mammary gland GLUT1 and GLUT4 glucose transporter pools, isolated mammary epithelial cells were also used. Although these isolated mammary epithelial cells contained myoepithelium and connective tissue, they were not contaminated with mammary adipocytes. GLUT4 was not detected in the mammary epithelial cells at any developmental stage, suggesting that the decrease in mammary GLUT4 seen during pregnancy was due more to the displacement of mammary adipocytes by the epithelia than to down-regulation of GLUT4 expression. GLUT1 protein levels were very low in mammary epithelial cells during pregnancy, in contrast to very high levels of GLUT1 in early- and mid-lactation. This suggested that the expression of GLUT1 in the mammary epithelial cells was developmentally regulated, switching on during lactogenesis, around the time of parturition. This increase in GLUT1 expression by mammary epithelial cells at parturition is likely to reflect the increase in glucose uptake capacity by the epithelial cells, which increases 13-fold between mid-pregnancy and mid-lactation (Prosser & Topper, 1986), and the corresponding increase in lactose synthesis, for which glucose uptake is rate-limiting. Assuming that the difference between the low level of GLUT1 protein observed by myself in mammary glands of rats at day 20 of pregnancy, and the high levels observed by Burnol *et al.* (1990) in rats at day 21 of pregnancy is due to their rats already initiating lactogenesis, this switch in the expression of the GLUT1 protein must be very rapid.

The factors controlling the switch in GLUT1 expression were investigated *in vitro* using explants of mid-pregnant rat mammary gland, and inducing differentiation by

the use of prolactin, the most potent lactogenic hormone in rats. It was found that three day culture of mid-pregnant rat mammary explants in the presence of insulin, cortisol and prolactin always resulted in an increased level of the GLUT1 protein, which was not seen when prolactin was omitted from the culture medium. This result suggested that prolactin was the absolute requirement for the increased expression of the GLUT1 protein seen at lactogenesis *in vivo*. Prolactin is known to be necessary for the onset of milk protein (Emerman *et al.*, 1977; Razooki-Hasan *et al.*, 1982) and lactose (Oppat & Rillema, 1988) syntheses *in vitro*, and from the results in Section 8.2, it also appears to be required to initiate GLUT1 protein expression.

The explant work had established that prolactin was required for the initiation of GLUT1 expression by mammary gland, suggesting that the increase in plasma prolactin concentration seen in rats at parturition *in vivo* was the origin of the increased levels of GLUT1 seen in the mammary epithelial cells, shown in Section 7.5. However, it is known that in rats the galactopoietic role of growth hormone becomes more pronounced as lactation continues. Therefore, to see whether high levels of plasma prolactin were also necessary for continued expression of GLUT1 during lactation, bromocryptine was used *in vivo*. Bromocryptine, in conjunction with an antiserum raised against rat growth hormone, has been shown to abolish lactation (Madon *et al.*, 1986) and to significantly reduce the level of mammary plasma membrane GLUT1 (Fawcett *et al.*, 1991). The same treatment was shown in Section 8.3 to cause an almost total loss of GLUT1 from the mammary epithelial cells, within 48hr of treatment. It was therefore concluded that not only is prolactin required to increase GLUT1 levels at lactogenesis, but also to maintain the level of GLUT1 during galactopoiesis. The effects of suppressing prolactin and

growth hormone levels on the expression of mammary GLUT1 protein were not reflected to the same extent in the expression of GLUT1 mRNA, suggesting that the hormonal regulation could be post-transcriptional. It has been suggested that casein, and α-lactalbumin, mRNA in rats are both post-transcriptionally regulated (Qasba & Nakhasi, 1978).

The influence of prolactin on the mammary epithelial cell GLUT1 levels raises a number of questions regarding the influence of prolactin on the expression of glucose transporters by other cells in which prolactin receptors are expressed. The liver and the pancreas both express receptors for prolactin (Agius *et al.*, 1983), are involved in glucose homeostasis, and both tissues also express the GLUT1 protein, albeit at low levels under normal circumstances. Regarding the pancreas, plasma insulin concentrations are decreased during lactation (Flint *et al.*, 1979; Burnol *et al.*, 1986), possibly due to a lower glucose responsiveness of the pancreatic islets (Hubinot *et al.*, 1986). The secretion of insulin in response to glucose is believed to be related to the expression of certain glucose transport isoforms, specifically GLUT2 (Tiedge & Lenzen, 1991; Yasuda *et al.*, 1992; Inagaki *et al.*, 1992). It would be interesting to study the relative expression and sub-cellular distribution of GLUT1 and GLUT2 in the pancreas of pregnant compared to lactating rats, in order to ascertain whether altered insulin secretion is effected by prolactin. The expression of prolactin receptors by rat liver is inversely related to mammary epithelial cell prolactin receptors, suggesting that prolactin has opposing roles in the two tissues. Feeding rats extra glucose will decrease the hepatic prolactin receptors by 50% (Goodman *et al.*, 1990), suggesting that in liver, prolactin effects are less important when there is an excess of glucose, compared to a normal glucose diet. Prolactin might therefore play a part in the altered glucose

homeostasis that occurs during lactation, possibly by altering the relative expression of GLUT1 and GLUT2 in liver.

Litter-removal from peak-lactating rats for 24hr will inhibit milk synthesis (Agius *et al.*, 1983) and lead to an almost complete loss of GLUT1 from the epithelial cells. The inhibition of milk synthesis is presumably due to milk retention in the lumen of the mammary alveoli, withdrawal of the suckling stimulus, or the fact that litter-removal leads to lower plasma prolactin levels (Agius *et al.*, 1983). Milk retention has been shown to inhibit the secretion of milk components, and thus lactation through the action of milk-specific inhibitors. The action of these milk-specific inhibitors is unaffected by prolactin levels, shown by *in vitro* treatment of mammary epithelial cell, or explant cultures with a 10-30kD whey protein fraction from milk, that inhibits milk-protein production, partially by increasing the rate of milk-protein degradation (Razooki-Hasan *et al.*, 1982; Stewart *et al.*, 1988). Also, unilateral application of the inhibitor-containing whey protein fraction into lactating goat mammary glands *in vivo* will cause a transient reduction in the milk yield from the treated gland compared to the control gland (Wilde *et al.*, 1988), although plasma prolactin levels remain the same for both glands. Alternately, artificially prolonging suckling, by replacing an older litter with a younger litter, can extend lactation further than its natural time. The loss of mammary GLUT1 seen following litter-removal could therefore be an effect of the milk-specific inhibitor, by a mechanism distinct from the action of prolactin. Further evidence for the possibility of feed-back inhibition of GLUT1 levels came from the finding that in some explant experiments, the increase in the level of GLUT1 induced by prolactin was only transient and GLUT1 levels were lower after 72hr of culture than they were at 48hr. This finding suggests that as the explants become sealed,

and feed-back inhibited, the expression of the GLUT1 protein is switched off, despite the continued presence of prolactin in the culture medium. As these were only the results of preliminary investigations, a more detailed study of the effects of feed-back inhibition on mammary GLUT1 levels is required, as the loss of GLUT1 following inhibition of prolactin secretion is not associated with any milk-retention by the mammary glands of the treated rats. The expression of GLUT1 by the mouse mammary epithelial cells (Section 8.6) means that the well-established primary mouse mammary epithelial cell culture can be used to investigate in more detail differentiation *in vitro*, and to assess the effect of milk-specific inhibitors, specifically the 10-30kD whey protein fraction, on expression of the GLUT1 protein.

The requirement for prolactin to induce GLUT1 expression is possibly unique to the mammary epithelial cell, as any other tissues in which prolactin could increase GLUT1 expression would be in direct competition with the mammary gland for glucose. This could be the case for liver, where prolactin binding is inversely related to that in the mammary gland. Prolactin has been implicated in the maintenance of mammary tumours (Meites, 1972), and a number of human cancer cells have been shown to over-express GLUT1 and/or GLUT3 (Yamamoto *et al.*, 1990), therefore, there could be a relationship between GLUT1 expression in mammary tumours and their sensitivity to prolactin. The role of growth hormone in the expression of mammary GLUT1 is less well-defined to date. Fawcett *et al.* (1991) found that the anti-growth hormone antiserum would further reduce the expression of GLUT1 at the mammary epithelial cell surface in rats treated simultaneously with bromocryptine, compared to bromocryptine treatment alone. How this effect occurs is not known, as mammary epithelial cells do not possess

receptors for the rat growth hormone. It is possible that growth hormone acts through stimulating the local release of IGF-1, or directly through IGF-1 receptors. Infusion of IGF-1 into goats increases the rate of milk secretion (Prosser *et al.*, 1990). However, with respect to GLUT1 expression, IGF-1 was not found to substitute for growth hormone to increase the level of GLUT1 in the mammary plasma membrane of bromocryptine and anti-growth hormone treated rats, while growth hormone itself would partially restore the level of GLUT1. Therefore the role of the galactopoietic hormones requires further investigation.

In summary, expression of the mammary epithelial cell GLUT1 is regulated, by endocrine and/or autocrine factors, and GLUT1 mediates the very large increase in glucose transport required by the mammary epithelium during lactation. Mammary GLUT1 is therefore different from GLUT1 in other cell types where it is believed to act mainly to maintain the basal glucose transport.

9.3 Sub-cellular distribution of GLUT1

Following the sub-cellular fractionation of lactating rat mammary gland, the amount of GLUT1 present in plasma membrane and Golgi membrane-enriched fractions was assessed both by determination of the total pmol of cytochalasin B binding sites/mg membrane protein, which had been performed by Dr. R.J. Madon, and the pmol GLUT1/mg protein, determined by quantitative Western blotting. While there was no significant difference in the amount of GLUT1 protein in the mammary plasma membranes detected using both these methods, the detection of twice as many cytochalasin B binding sites in the mammary Golgi membranes, than immunologically detectable GLUT1 protein indicated the possible presence of a second class of cytochalasin B binding site in an intracellular membrane fraction.

This second cytochalasin B binding site is unlikely to be one of the five other characterised glucose transporters as Western blotting mammary homogenate had only identified GLUT1 and GLUT4 in the mammary gland, and GLUT4 was not present in the mammary Golgi membranes. The second population of cytochalasin B binding sites is at least as abundant as the GLUT1 protein, and so would be expected to be detectable on Western blots of mammary homogenate. As yet the nature of this second intracellular cytochalasin B binding site has not been established.

The Golgi-enriched membrane fraction was enriched in galactosyltransferase, and equivalent fractions would synthesize lactose (personal communication, H.A.C. Fawcett). However, it has not proved possible to inhibit the synthesis of lactose in these Golgi-membrane-enriched vesicles with cytochalasin B, and this fact, together with the substantial evidence for the presence of a non-specific proteinaceous pore in the mammary epithelial cell Golgi apparatus, would seem to rule out the involvement of the intracellular GLUT1 in the provision of glucose for lactose synthesis. Growing evidence for the dichotomy of Golgi membranes further suggests that in these Golgi membrane-enriched fractions, those vesicles containing GLUT1 could be separate from those vesicles involved in lactose synthesis. The function of the intracellular GLUT1 protein has not been established to date, however it is possible that it is involved in the regulation of GLUT1 levels by the mammary epithelial cells, a possibility which is discussed in more detail below. It is also possible that it is simply newly synthesized GLUT1 that is being translocated to the plasma membrane. Immuno-electron microscopy could be used in future to try and identify precisely the location of the intracellular GLUT1 protein, which would give more clues to its intracellular function.

Cellular membranes are continually regenerated by cells, and membrane proteins which turn over most rapidly can be very easily eliminated from the cell, as rapidly recycled proteins are more open to regulation through increased degradation of the internalised protein (Hare & Taylor, 1991). The plasma membranes of the mammary secretory epithelial cells are very rapidly turned over (Neville & Daniel, 1987), reportedly in under 4hr. This suggests that the half-life of GLUT1 within the mammary plasma membrane could be very short. This fact could account for the presence of the intracellular mammary GLUT1, as a pool for increasing or decreasing the plasma membrane GLUT1 as necessary. Under conditions where glucose uptake is not required, i.e. following litter-removal or lowering of plasma prolactin levels, glucose transporters could be preferentially degraded, rather than transported to the plasma membrane. The internal localisation of the GLUT1 protein needs to be identified, to determine whether it is associated with vesicles, or the *trans*-Golgi network, where protein sorting occurs (Griffiths & Simons, 1986), as well as determining whether it is associated with lactose synthesis. The presence of a large pool of internal glucose transporters is similar to the situation in insulin-regulatable cells, where translocation of the GLUT4 glucose transporter mediates, to a large extent, the stimulation of glucose transport. Glucose transport can therefore be controlled through the insertion and retrieval of glucose transporters into and from the plasma membrane (Lienhard, 1983). Tentative evidence for the presence of an intracellular pool of mammary glucose transporters was provided by the observed decrease in V_{max} for hexose uptake by mouse mammary epithelial cells following fasting, which suggested a decrease in the number of plasma membrane glucose transporters, possibly due to internalisation (Prosser, 1988). However, acute stimulation of glucose transport activity has not been observed in

mammary epithelial cells (Threadgold & Kuhn, 1984; Prosser & Topper, 1986) in response to insulin, or prolactin, suggesting that rapid translocation events similar to those in the adipocyte are not occurring. However, the increased metabolic clearance rate of glucose in lactating rats suggests that mammary glucose transport might be insulin-sensitive *in vivo* (Burnol *et al.*, 1983). Also, in the mammary gland of rats starved for 24hr there is a rapid increase in mammary glucose transport *in vivo*, from the low level observed during starvation up to the level normal for lactation, in response to refeeding or insulin treatment, which does not require protein synthesis (Williamson *et al.*, 1983; Threadgold & Kuhn, 1984; Page & Kuhn, 1984). This suggests that GLUT1 might be internalised during starvation, then redistributed to the plasma membrane upon refeeding, possibly in response to the restored blood glucose levels. The production of fatty-acids from glucose is insulin-responsive in mammary epithelial cells (Vernon & Flint, 1984). A sharp increase in plasma insulin levels, followed by the peak lipogenesis in mammary gland, are shown to occur in lactating, starved rats following refeeding (Mercer & Williamson, 1986). The mechanism behind these observations obtained following starvation is not yet known, and the distribution of GLUT1 within sub-cellular membrane fractions of starved rats compared to fed rats is yet to be determined, and would be an interesting subject to study. The appearance and loss of cell surface glucose transporters can be determined using the impermeant bis-mannose ligand, 2-N-[4-(1-azi-2,2,2-trifluoroethyl)benzoyl]-1,3-bis-(D-mannos-4-yloxy)-2-propylamine (ATB-BMPA) (Clarke *et al.*, 1991). If translocation is not the mechanism operating in the mammary gland to regulate glucose transport, it is possible that cell surface GLUT1 could be modified to modulate its' activity and kinetic studies on cells and membrane vesicles could be used to investigate this

possibility, however this would not explain the presence of the intracellular GLUT1 pool.

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Characterization of glucose transporters in lactating rat mammary glands

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Glucose is an essential substrate for the production of lactose in lactating rodent mammary glands and its uptake into the epithelial cells is rate-limiting for milk production [1]. Uptake across the plasma membrane occurs via a facilitated diffusion system similar in its characteristics to those of other mammalian cells [2]. However, there is some controversy about the mechanism by which glucose crosses the Golgi membrane to reach the site of lactose synthesis: some studies have yielded evidence for the presence of a non-stereospecific pore for sugar uptake [3], whereas others have yielded evidence for a transporter [4]. We previously reported that both plasma membrane- and Golgi membrane-enriched fractions from lactating rat mammary glands contain D-glucose-inhibitable cytochalasin B-binding sites, indicating the presence of glucose transporters in both [4]. Putative glucose transporters of apparent M_r 50 000 were detected in each fraction by Western blotting using an antibody raised against the C-terminal region of the human erythrocyte glucose transporter. Furthermore, using this antibody we were able to immunoprecipitate a radioactive protein of identical apparent M_r from membranes photoaffinity-labelled with [^{3}H]cytochalasin B [4]. The labelling of this protein was inhibitable by D- but not by L-glucose.

From the experiments described above, we concluded that both plasma membranes and Golgi membranes of lactating rat mammary gland epithelial cells contain D-glucose transport proteins related to those found in the human erythrocyte. However, recent cDNA cloning studies have revealed the existence of a family of at least four different but homologous glucose transporters which predominate in different

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mammalian tissues [5]. In the present study we therefore set out to determine whether the erythrocyte-type transporter constitutes all or only a part of the transporter content of epithelial cell plasma and Golgi membranes.

Subcellular membrane fractions were prepared from day 10–14 lactating rates and characterized as previously described [4]. The glucose transporter content of each fraction was then determined by quantitative Western blotting using affinity-purified antibodies raised against the C-terminal peptide (residues 477–492) of the human erythrocyte glucose transporter [6]. The sequence of this region of the erythrocyte/brain $t_{1/2}$ of glucose transporter is identical in human and rat, but completely different from the corresponding sequences present in other known members of the glucose transporter family [5, 7, 8]. The antibodies are therefore specific for this member of the transporter family. Samples of plasma and Golgi membrane fractions were electrophoresed on SDS/12% (w/v) polyacrylamide gels together with erythrocyte membrane standards containing known amounts (5–25 ng) of transporter, measured by cytochalasin B binding. After incubation of the blots with anti-transporter antibody and then ^{125}I-F(ab')$_2$ donkey anti-rabbit IgG, radioactive bands were located by autoradiography, excised and counted for γ-radioactivity. The relationship between the amount of transporter in the standards and radioactivity was linear over the range employed, and so the transporter content of mammary membrne samples could be determined from the standard curve by linear regression.

The transporter contents of plasma membrane and Golgi membrane fractions determined in this manner were 27.9 ± 5.4 (S.E.M., n = 9) and 22.8 ± 3.3 (S.E.M., n = 7) pmol/mg of membrane protein, respectively. Comparison with the corresponding value of 19 pmol/mg of plasma membrane protein, determined by measurement of cytochalasin B binding [4], indicates that the erythrocyte/brain type of transporter is the major species present in plasma membranes. However, the content of immunologically detectable transporters in Golgi membranes corresponded to only about half the value of 55 pmol/mg of protein previously determined by measurement of cytochalasin B-binding sites [4]. This finding suggests that there may be additional species of transporter present in the Golgi. If this is the case, their presence may contribute to the different K_d values previously measured for cytochalasin B binding to Golgi and plasma membranes [4].

Further evidence that at least a substantial fraction of the plasma membrane and Golgi vesicle transporters are of the erythrocyte/brain type was provided by Western blotting with a panel of anti peptide antibodies. These had been raised against several other regions of the human erythrocyte protein [9]. Cross-reactivity was observed beween the M_r 50 000 protein of both plasma membranes and Golgi membranes, and antibodies raised against peptides corresponding to residues 240–255, 450–467 and 460–477 of the human erythrocyte transporter. These findings indicate that the homology between the mammary gland epithelial cell glucose transporter and the human erythrocyte glucose transporter is not restricted to the C-terminal region of the molecules. It is therefore likely that the glucose transporters of rat mammary gland epithelial cell plasma membranes are encoded by the same gene as the rat brain transporter, previously shown to be almost identical in sequence to the human erythrocyte protein [8]. Golgi membranes also appear to contain this gene product, although the results outlined above suggest that they may in addition contain another type(s) of glucose transporter.

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