Identification of Reticulocyte Surface Specific Antigens: Their Induced Redistribution and Externalization by the Specific Antibodies in Sheep Reticulocytes.

by

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TO MY PARENTS

ABSTRACT

Sheep reticulocyte surface specific antigens have been isolated by anti-reticulocyte surface specific antibodies. Moreover, the major specific antigen has been identified as the transferrin receptor. Characterization of the receptor has shown it has a monomeric molecular weight of approximately 93,000 and that it probably exists as a dimer *in situ*. It has been shown that the antigenic sites differ from the transferrin binding sites and that the receptor spans the membrane. Moreover, it has been shown the transferrin receptors decrease and disappear when reticulocytes are cultured *in vitro*.

Incubation of reticulocytes at 37°C containing bound antibody results in a redistribution of the antibody-receptor complexes. Initially there is a diffuse distribution of the antibody on the cell surface which gradually changes into patches and/or caps on the cell surface. With time, antibody-receptor complexes are externalized and can be detected in the medium. The receptor antibody complex is externalized as a vesicle.

RÉSUMÉ

Des antigènes spécifiques de la surface membranaire de réticulocytes de mouton ont été isolés en utilisant des anticorps spécifiquement développés contre la surface cellulaire. Le récepteur de la transferrine a été identifié comme l'antigène spécifique majeur des membranes. La caractérisation du récepteur a permis de déterminer un poids moléculaire d'environ 93,000 pour sa forme monomérique et qu'il existe probablement *in situ* sous une forme dimérique. Il a été aussi établi que les sites antigéniques diffèrent de ceux impliqués pour la liaison avec la transferrine. De plus, mous avons montré que la quantité de récepteurs de la transferrine décroît pour éventuellement devenir nulle lorsque les réticulocytes sont cultivés *in vitro*.

L'incubation à 37°C des réticulocytes contenant des anticorps liés entraîne une redistribution des complexes récepteurs-anticorps. L'anticorps, qui est initialement distribué de façon diffuse à la surface des cellules, se regroupe graduellement en plaques à la surface cellulaire. Avec le temps, les complexes anticorps-récepteurs sont extériorisés et peuvent être détectés dans le milieu. Le complexe anticorps-récepteur est extériorisé sous une forme vésiculaire.

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NOTE

References at the end are references for Chapters 1 and 5, whereas Chapters 2, 3 and 4 have their own references.

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Chapter 1.

GENERAL INTRODUCTION

I-1 The Cell and the Plasma Membrane

A cell is distinguished from other forms of matter by its capacity for self regeneration. The process of self regeneration is protected from disturbances of the environment by maintenance of a selective entry of nutrients, excretion of products, and communication with the environment. The selectivity is controlled by the plasma membrane which forms the boundary of the cell. The unique function of the plasma membrane is determined by the composition and structure of the membrane.

I-2 Membrane Composition and Structure

I-2-1 Composition

The membrane contains proteins, lipids, and carbohydrates. The proteins and lipids are held together chiefly by noncovalent, principally hydrophobic, bonds to form extended sheets some 75Å thick on average. A typical membrane contains approximately equal amounts of proteins and lipids (by weight) with carbohydrates being minor constituents. Two kinds of lipids are included in the membrane: cholesterol and phospholipid, both are amphipathic. Carbohydrates are covalently linked to the lipids and proteins to form glycolipids and glycoproteins respectively.

I-2-2 Structure

The membrane constituents are arranged such that the membrane structure is very stable with unique properties. A currently accepted

view of membrane structure is the fluid mosaic model (Fig. 1) proposed by Singer and Nicolson (1). According to this model the amphipathic lipids are arranged to form a bilayer with the hydrophilic polar ends facing toward the two aqueous external environments and the hydrophobic portions facing the interior forming the hydrophobic core of the bilayer. This arrangement minimizes the interaction of hydrophobic portions of the lipids with water and provides a permeability barrier to hydrophilic substances. The experiments which support this bilayer structure date back to 1925 (2). Direct evidence for such a structure has come from X-ray diffraction studies of Caspar and Kirschner (3) on myelin and of Engelman (4,5) on mycoplasma membranes. The existence of the bilayer has been amply confirmed by other physical techniques (6).

There are two kinds of proteins associated with the membrane (1). The integral membrane proteins are intercalated into the lipid bilayer and interact with the hydrophobic portions of the lipids. Many integral membrane proteins span the full width of the bilayer and presumably have hydrophilic regions exposed on both sides of the membrane. The integral membrane proteins can be extracted from the membrane only after disruption of the lipid bilayer with agents such as detergents. These proteins are generally insoluble in water. There are many examples of integral membrane proteins which span the membrane once with the amino-terminal ends of the polypeptides exposed to the outside surface and the carboxyl-terminal ends to the cytoplasmic face, for example, glycophorin in erythrocytes (7,8), the heavy chain of the histocompatibility antigen (9,10), and the envelope glycoprotein of vesicular stomatitis virus (11). Also there are integral membrane proteins which span the membrane more than once with the

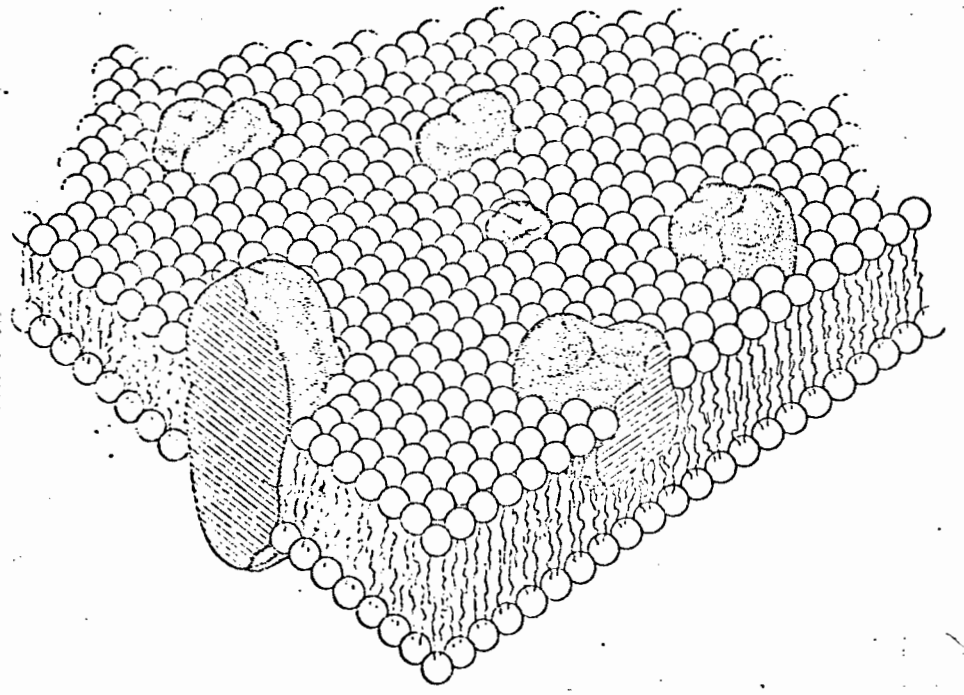

Fig.1-1 The lipid-globular protein mosaic model with a lipid matrix (the fluid mosaic model): Schematic three-dimensional and cross-sectional views. The solid bodies with stippled surfaces represent the globular integral proteins which are partially embedded in the lipid bilayer. (Reproduced from Singer, S.J., Science, 1972, 182, 720)

amino-terminal end at the cytoplasmic side of the membrane. For example, band 3 proteins of the erythrocyte membranes (12) have amino-terminal ends at the cytoplasmic side (13,14,15) and carboxyl-terminal portions span the membrane at least twice (14,16,17,18).

Peripheral membrane proteins are another class of membrane proteins which are not inserted into the lipid bilayer but reside on the surface of the membrane where they link noncovalently to the integral membrane proteins. The peripheral membrane proteins can be removed from the membrane without disrupting the lipid bilayer. They are generally soluble once they are removed. An example of peripheral membrane proteins is the cytoskeleton of erythrocytes (19,20,21). The cytoskeleton in erythrocytes consists of the spectrin-actin-band 4.1 network (22,23,24) which has been shown to attach to the cytoplasmic sites of band 3 proteins through other peripheral membrane proteins, ankyrins or syndeins (24,25,26,27,28,29,30,31).

The carbohydrate moieties of the glycolipids and glycoproteins are located exclusively on the outside surface of plasma membrane (32).

The stereospecifity of the interaction between the plasma membrane of the cell and the environment is most likely due to the protein contents of the membrane and to a lesser extent to the carbohydrate residues.

I-3 Membrane Fluidity

The fluid mosaic model of membranes (1) also proposes that the membrane is not static; but rather is a two dimensional fluid. The lipids and protein molecules are capable of moving laterally in the membrane plane.

I-3-1 Lipid Fluidity

Under physiological conditions, the fluidity of the membrane is to

a large extent determined by the fluidity of the bilayer lipid matrix (33).

That lipid molecules in a bilayer can undergo rapidly lateral diffusion in their own monolayer was originally demonstrated by Kornberg and McConnell (34) using spin labels. This lateral diffusion is due to the thermal motion of the lipid molecules. The bilayer is not always in a fluid state. Below a certain critical temperature, the bilayer lipids undergo phase transition from a fluid phase to a solid phase, (35,36). Under physiological conditions, the fluidity of the bilayer lipids is grossly determined by the following four factors (33): (a) The ratio of cholesterol to phospholipid (37,38). The cholesterol molecule consists of a rigid plane with specific residues which establish an alignment with the phospholipid chains (39,40). In most mammalian membranes under physiological conditions, the presence of cholesterol will decrease the fluidity. The cells seem to avoid the phase transition by the inclusion of cholesterol, which acts to eliminate the cooperativity of the phase change (35). However, in the solid state of lipid domains, cholesterol acts as a fluidizer by perturbing the structure regularity of the system (33). (b) The degree of unsaturation and length of the phospholipid acyl chain (41,42). Double bonds of natural fatty acids are virtually all of the cis configuration and their presence in phospholipid increases the disorder and fluidity of the bilayer. The fluidity of the bilayer decreases with increasing phospholipid acyl chain length. (c) The ratio of lecithin to sphingomyelin (43,44). These two phosphorylcholine phospholipids constitute more than 50% of the phospholipids in mammalian membrane (45). Because of inherent structural differences, their fluidity properties are markedly different. Natural lecithin bears highly unsaturated acyl chains

and therefore imparts high fluidity to lipid domains. Natural sphingomyelin, on the other hand, is highly saturated and forms a lipid domain of low fluidity. However, the rigidifying effect of sphingomyelin is also partially due to the inter- and intramolecular hydrogen bonds of its amide linkage and the free hydroxyl group, which condense the hydrocarbon-water interface region and presumably confer rigidity on the hydrocarbon region as well. (d) The ratio of lipid to protein. The fluidity of the membrane lipid bilayer is indirectly affected by the presence of protein molecules which decrease the fluidity of bilayer lipid. The effect of proteins is qualitatively similar to that of cholesterol (33).

Although the lipid molecules in the bilayer undergo rapid rotational and lateral diffusion in their own monolayer, the transmembrane diffusion or flip-flop diffusion (46) which has to force the hydrophilic polar head group of the phospholipid through the hydrophobic core of the bilayer is extremely slow (47,48). However, transmembrane movement has been demonstrated to proceed very rapidly, at least 10^5 times higher than the flip-flop diffusion, in growing bacterial membranes (49). This rapid transmembrane movement has been proposed to be catalyzed by proteins which either provide holes or act as carriers in the membrane.

Since the lipid molecules are assumed to undergo free lateral diffusion in the lipid matrix of the membrane bilayer, they are presumably homogeneously distributed in the monolayer (1). However, there is evidence which suggests the existence of lipid domains in the membrane in which lateral diffusion of certain lipids is restricted. For example, data suggesting the existence of diffusion barriers which separate the lipids into clusters using the sarcoplasmic reticulum was obtained using spin labels (50).

Furthermore, the existence of gel and fluid phase domains in the plasma membrane has been deduced from measurement of the fluorescence life time heterogeneity of diphenyl hexatriene lipid probes incorporated into these membranes (51), and from studies of the differential temperature dependent agglutinability of wheat germ agglutinin and concanavalin A (52).

The possible formation of lipid domains which may restrict the lateral movement of certain lipids may be due to protein lipid interactions, for example, the boundary lipid domains of certain integral membrane proteins such as Ca^{2+}-ATPase and cytochrome oxidase (53,54,55,56) or may be due to lateral phase separation (57,58) of lipids in the membrane.

I-3-2 Protein Mobility

Integral membrane proteins can be considered to be dissolved in a two dimensional lipid solvent (1,59). These proteins are relatively free to undergo lateral diffusion unless they are constrained by the membrane associated cytoskeletal systems (59,60,61) or through long range protein-protein interaction between the integral membrane proteins (59,60). For example, there is evidence which suggests the lateral diffusion of the integral membrane protein in the membrane plane of cells is controlled by the cytoskeletal system in erythrocytes (62,63,64,65,66) and other cells (67), and the existence of specialized membrane regions such as tight junction, gap junction, and neuromuscular junction may be due to long range protein-protein interactions (59,60).

The lateral diffusion of membrane proteins may be classified into two types which depend upon whether or not the lateral movement is under the direction of the cytoskeletal system and therefore requiring metabolic energy (33,60). One is the passive diffusion of integral membrane proteins

which is directly related to the lipid fluidity of membrane and occurs in a random way. This was first demonstrated by Frye and Edidin (68) who by fusing one fluorescently labelled cell with another non-fluorescent cell observed the fluorescein labelled protein markers diffuse rapidly from one cell into the other. This was one of the experiments which led to the proposal of the fluid mosaic model of membranes (1). More quantitative studies by Cone (69) showed that the passive lateral diffusion of rhodopsin in rod cells could be accounted for by the intrinsic viscosity of the lipid bilayer. Other experiments which also demonstrated passive lateral diffusion in the plane of the plasma membrane include the diffusion of fluorescently labelled Fab fragment, directed against muscle cell surface receptor (70), rhodopsin in the membrane plane of the rod cell (71,72), and band 3 in the Sendi virus fused human erythrocyte (73).

The other type of lateral diffusion of membrane proteins requires metabolic energy, may be directed by the cytoskeletal system, and is only indirectly related to the lipid fluidity of the membrane (33,60). For example, a multivalent ligand, such as antibody, binding to the surface receptor of B lymphocytes, can induce redistribution of the receptors first into clusters, then patches, and eventually to one pole of the cell to form a cap (74). The redistribution into patches is passive; however, the formation of cap requires metabolic energy and may be controlled by the cytoskeletal system of the cell (75).

The transmembrane diffusion of integral membrane proteins is almost impossible since it requires much more energy to force the larger hydrophilic portions of the protein molecules through the hydrophobic barrier of the bilayer.

I-4 Membrane Asymmetry

The difference of the internal environment of the living cell from the external milieu is mainly attributed to the asymmetrical structure of the plasma membrane which forms the boundary of the living cell. The asymmetrical structure of membrane is maintained to a large extent by the hydrophobic prohibition of the flip-flop diffusion of the amphipathic molecules of the membrane (76).

The asymmetry of carbohydrate and protein is complete because it is extremely difficult to force the large polar ends of the molecules through the hydrophobic core of the membrane after they are synthesized and inserted into the membrane. The asymmetry of carbohydrate and protein in membranes was first demonstrated by Bretscher (7,77) in studies of erythrocyte glycophorin which showed that the amino-terminal end of the protein is located outside the surface while the carboxyl-terminal end is located in the cytoplasmic side. That the carbohydrate is distributed exclusively on the external surface of plasma membrane was shown by Gahnberg and Hakamori (32). This principle also applies to intracellular membranes, Here carbohydrate is found only in the luminal face and not on the side of the membrane in contact with the cytoplasm (78). The lipid bilayer is also asymmetrical. Bretscher (79,80) first proposed that lipid composition in each of the monolayers of the bilayer is different. Subsequent studies (76,81) confirmed that the bilayer is asymmetrical in many cell membranes. For example, in erythrocyte membranes, the choline-containing phospholipids are located mainly in the outer layer and the amino phospholipids preferentially in the inner layer (76,81). However, the lipid asymmetry is not as complete as carbohydrate and protein and also the details of the lipid asymmetry can vary from one membrane to another (76). The

incompleteness and variety of lipid asymmetry may result from the slow but not completely inhibited flip-flop diffusion of lipid molecules and the probable existence of a facilitated mechanism which catalyzes a rapid transmembrane movement of lipid molecules (76).

In addition, cell asymmetry also exists. For example, apical (mucosal) and basolateral (serosal)surfaces of epithelial cells exhibit different membrane morphology, ionic permeability, distribution of enzymes and sensitivity to hormones and drugs (82,83). This asymmetry may be maintained by the tight junction which may act as a barrier to lateral diffusion of membrane components between the apical and basolateral surfaces (83,84,85).

I-5 Membrane Assembly

Two overall principles seem to be clear in membrane biogenesis (86,87,88) especially in higher cells. One is that membranes are not synthesized de novo. Membranes can only grow by the insertion of newly synthesized lipids and proteins into the preexisting membrane in a vectorial manner. The other is that membrane components are synthesized and inserted into sites which are distinct from their ultimate destinations. Therefore, a transport process of membrane components to their destinations is involved. For example, plasma membrane components of eukaryotic cells are probably synthesized in the endoplasmic reticulum in which they are sorted out and then transported to the plasma membrane. That is, there are two distinct stages in plasma membrane assembly: (a) synthesis and insertion, and (b) intracellular transport.

I-5-1 Lipid Assembly

Lipids are synthesized within the matrix of the membrane itself by enzymes which are themselves integral membrane proteins (89). In order to study the sideness of the assembly of lipid, Kennedy and Rothman (49)

used Bacillus megatherium, which has only a single cellular membrane whose major phospholipid is phosphatidylethanolamine, as a model. They pulsed the cell with radioactive precursors for phospholipid synthesis. The inside and outside distribution of the radioactive phosphatidylethanolamine was then determined by using the impermeable reagent trinitrobenzene sulfonic acid (TNBS), which labelled the amino group of the phosphatidylethanolamine (90). The results showed that newly synthesized lipids are found first on the monolayer at the cytoplasmic side and later become equilibrated at both sides. This study was interpreted to reflect the simple way in which the bilayer is assembled (49,76). Phospholipid synthesis occurs only at the cytoplasmic side. Newly synthesized lipids then are released into the cytoplasmic monolayer of the membrane. Since the free flip-flop diffusion is slow, newly synthesized lipids mix with the old lipids in the cytoplasmic monolayer by lateral diffusion. To permit the growth of the membrane as a bilayer, a facilitated process must be used which allows rapid transmembrane translocation, at a rate around 10^5 times greater than the free flip-flop diffusion (49,76), of the newly synthesized lipids into the outside monolayer. This proposed facilitated process of lipid transmembrane movement was suggested to exist only where membrane grows actively (76). The probable existence of a facilitated process to permit rapid transmembrane movement makes the asymmetry of lipids in the membrane fundamentally different from the asymmetry of protein molecule. The protein asymmetry is a non-equilibrium state established at the time of synthesis and insertion of the proteins. With the phospholipids, the asymmetry may reflect a thermodynamic equilibrium between the two monolayers (88).

Subsequent studies by Coleman and Bell (91) and Zilversmit and Hughes (92), when taken together, also lead to a similar picture of bilayer assembly in the endoplasmic reticulum, the principal site of lipid biosynthesis in higher cells (93,94,95).

I-5-2 Membrane Protein Cotranslational Insertion

In eukaryotic cells,the integral membrane proteins are primarily synthesized and co-translationally inserted into the membrane of endoplasmic reticulum by membrane bound ribosomes in a way similar to the vectorially cotranslational insertion and discharge of secretory proteins (87,88). The biogenesis of the glycoprotein of vesicular stomatitis virus has been well studied and may provide an example for the cotranslational, vectorial, insertion of integral membrane protein into the membrane. Vesicular stomatitis virus contains a single envelope glycoprotein (G) which forms spikes on the surface of the virion. This 70,000K glycoprotein contains two asparagine-linked complex oligosaccharrides (96,97) and is positioned in the virion such that the amino-terminal end is exposed at the surface of the virion and a region near the carboxyl-terminal end spans the envelope membrane (98) with the carboxyl-terminal end exposed inside the virion. The G proteins are synthesized in the endoplasmic reticulum of infected cells using the host cell's genetic machinery and viral mRNA. The synthesized G proteins are then transported from the endoplasmic reticulum to the plasma membrane of the host cell where the viral genome, which is also synthesized inside the host cell, buds to form the virion. In this way, the G protein is incorporated into the envelope of the budded virion (88).

The synthesis of G protein starts in free ribosomes in the rough

endoplasmic reticulum of infected cells (88). When a leader sequence at the amino-terminal portion is finished and exposed of the ribosome, the free ribosome then binds to the membrane of the rough endoplasmic reticulum and the nascent G polypeptide chain is cotranslationally inserted into the lumen of the rough endoplasmic reticulum. This leader sequence is characterized by a high proportion of hydrophobic amino acid residues and may act as an insertional signal (102) to initiate the association of the nascent polypeptide chain-mRNA-ribosome complex with the membrane and facilitate the insertion and passage of the nascent G polypeptide chain into the lumen of the rough endoplasmic reticulum (87,88). This leader sequence is then removed in the lumen (103,104). The nascent polypeptide chain is then folded and glycosylated by en bloc transfer of a preformed oligosaccharide from lipid linked oligosaccharide donors in the lumen (105). The cotranslational discharge of the nascent G polypeptide chain into the lumen continues until it reaches a region near the carboxyl-terminal end in which a hydrophobic segment is followed immediately in the carboxyl-terminal portion by a highly charged segment (106). This region may act as the stop transfer signal (87) for the cotranslational discharge of the nascent G polypeptide chain: after the hydrophobic segment is embedded into the membrane, the highly charged amino acid residues which immediately follow the hydrophobic segment may then prevent the remaining carboxyl-terminal portion of the nascent polypeptide chain from entering the membrane and therefore the remaining carboxyl-terminal portion is exposed at the cytoplasmic side (87). The final picture of this cotranslational insertion of G polypeptide is the G polypeptide positioned with amino-terminal end exposed in the lumen and the carboxyl-terminal end at the cytoplasmic side of the rough endoplasmic reticulum. The G polypeptide

completes its glycosylation after being transported to the Golgi apparatus (88) and obtains the right sideness as in the budded virion, after being further transported to and fusing with the plasma membrane. Other examples of the cotranslational insertion of integral membrane proteins include the heavy chain of major histocompatibility antigen (107,108), glycophorin (107), and band 3 protein (109.110). However, the insertional signal in band 3 protein is not in the amino-terminal end but near the middle region of the protein and is permanent (87,110). Fig. 2 is a scheme for the synthesis, insertion, and glycosylation of the G protein (88).

It is important to point out that cotranslational insertion is not the only mechanism for protein to be incorporated or transferred through membrane. In semiautonomous organelles like mitochondria and chloroplasts, there is evidence to show that many proteins are incorporated or transferred through the membrane post-translationally (87).

I-5-3 Sorting Out and Transport

In the eukaryocytic cell, once the proteins and lipids have been synthesized in the endoplasmic reticulum, they have to be transported to their destinations. To account for intracellular transport between subcellular compartments, Palade has described a membrane flow mechanism (111). It was proposed that the membrane is transported from the Rough Endoplasmic Reticulum to the Golgi Apparatus and then to the Plasma membrane by budding of small vesicles containing proteins and lipids from the membranes of one organelle and fusing with the membrane of the next (86). However, since the newly synthesized membrane components have different destinations, a sorting-out and directing mechanism must also be involved to account for the selective recognition and transport of newly

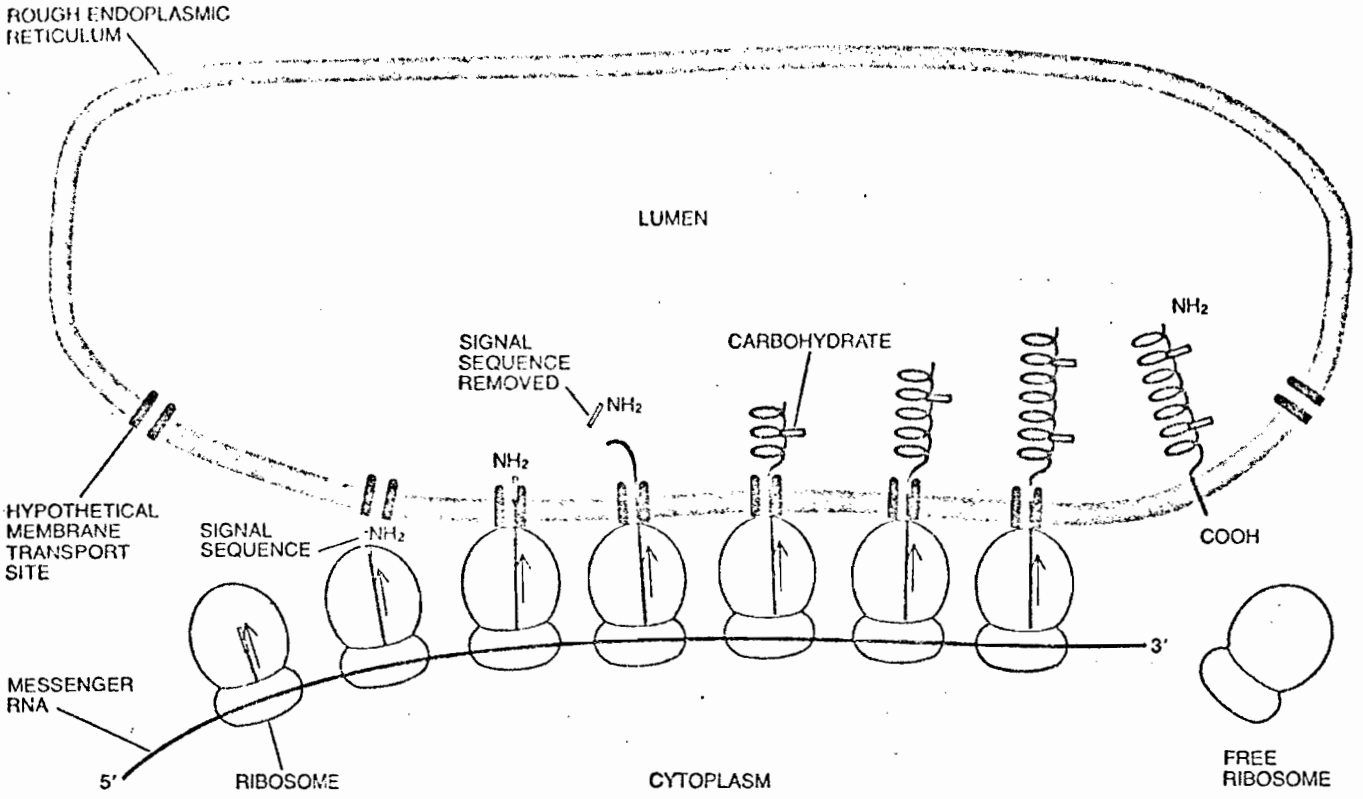

Fig.1-2 A diagrammatic summary of synthesis, insertion and glycosylation of the G protein. (Reproduced from Lodish and Rothman, Sci. Am., 1979, 240, 48)

synthesized membrane components to their respective destinations (87,112). Although the sorting-out process and directing mechanism are obscure, a possible mechanism (87,112) analogous to receptor mediated endocytosis (113,114,115) has recently been suggested. It was proposed that the newly synthesized membrane components of the same destination may be specifically recognized and segregated into specific domains in the endoplasmic reticulum from which they bud out and are directed to their destinations.

I-6 Ligand-Receptor Interaction

I-6-1 Receptor Concept

All cell functions can be reduced to specific molecular interactions within the cell and between the cell and its environment. To accomplish these specific molecular interactions, stereospecific molecules must exist in the cell which can recognize and interact with the complementary molecules. For specific interaction between the cell and its environment, the plasma membrane is no doubt the primary organelle. It is the stereospecific interactions of receptor molecules in the plasma membrane of the cell with the complementary ligand molecules from the environment that lead to the transfer of information from the environment into the cell. This is the central theme of the receptor concept which was originally proposed by Paul Ehrlich (116) as the side chain theory in the immune response and elaborated later by a number of other workers in the field of pharmacology, endocrinology, and immunology (117). It is now widely appreciated that specific ligand and surface receptor interactions play a crucial role in biological functions such as fertilization, embryonic development, nervous system actions, hormonal regulation of growth and development, the transport of required substances e.g. cholesterol, the interaction of virus and

toxin with the target cells, and the immune response to the nonself antigens (113, 114, 115, 117).

I-6-2 Ligand Induced Redistribution and Internalization

Among the specific ligand and surface receptor interactions, there exists a category of ligands, which are peptide in nature, are able to bind specifically to receptors on the cell surface and induce the receptors to undergo redistribution and internalization (endocytosis) (75, 113, 114, 115).

For discussion purposes, ligand induced redistribution and internalization is separated into two categories, the receptor-mediated endocytosis (113, 114, 115) and antibody induced redistribution and internalization (75).

a. Receptor-mediated Endocytosis

Receptor-mediated endocytosis has been used to describe those ligand-receptor interactions which result in redistribution of the ligand-receptor complexes, from an originally random distribution on the cell surface into clusters in the coated pits (118) of the plasma membrane, and ultimate internalization of the complexes through the coated pits. Fig. 3 is a scheme for the receptor mediated endocytosis.

Usually the ligand-receptor complexes are diffusely distributed on the cell surface at $4^{o}C$. However, with time at $37^{o}C$, they redistribute into the coated pits, the specialized membrane domains which are underlaid on the cytoplasmic side by proteins, principally clathrin (119). From the coated pits the ligand-receptor complexes are internalized to form receptosomes (114, 115) which are uncoated endocytic vesicles. The formation of receptosomes from the coated pits has been proposed to occur

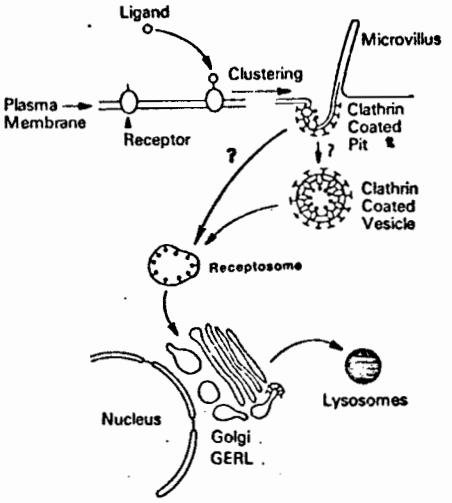

Fig.1-3 A diagrammatic summary of a proposed scheme of receptor-mediated endocytosis in cultured fibroblasts. (Reproduced from Pastan and Willingham, 1981, Ann. Rev. Physiol., 43, 239)

either indirectly following the formation of coated vesicles (113) and the dissociation of the coated protein clathrin from the coated vesicles or directly from the coated pits without the coated vesicle stage (114,115). The receptosomes then are directed to the Golgi region where they presumably fuse with certain domains of the Golgi Endoplasmic Reticulum Lysosome Complex (GERL) system. The ligands may traverse the GERL en route to the newly formed lysosome (114,115) where the ligands are degraded (113,114,115). However, the fate of the receptors which are internalized together with ligand is not clear, although it is known that the receptors are strongly down regulated in some cases, and only moderately or not down regulated in other cases. For example, epidermal growth factor (120) and insulin receptor (121,122) are strongly or moderately down regulated; however, receptors for low density lipoprotein (123) and α_2-macroglobulin (124) were shown to recycle back to the surface intact.

The ligands which have been shown directly or indirectly to trigger the receptor-mediated endocytosis include peptide hormones, e.g. epidermal growth factor, insulin, and 3,3',5-triiodo-L-thyronine (125,126,127,128); transport protein, e.g. low density lipoprotein (113,129, 130) and transferrin (131); other proteins, e.g. lysosomal enzyme (132) and α_2-macroglobulin (125,126,128); virus, e.g. Semliki Forest virus (133,134) and influenza virus (135); toxin, e.g. pseudomonas toxin (136).

Although the molecular mechanism which is involved in receptor-mediated endocytosis is not known, certain observations about receptor-mediated endocytosis have been made: (a) It is an energy dependent process (137); (b) The redistribution into coated pits and hence the internalization, is inhibited by transglutaminase inhibitors such as methylamine and dansylcadasverine which act as competitive inhibitors for

transglutaminase. This suggests that the transglutaminase, which may function to cross-link the amino group of lysine of one protein to the carboxyl group of an adjacent protein, may play a role in the induced redistribution (126,136,138, 139,140); (c) Microtubules seem to act to direct the receptosome to the Golgi region, since colchicine arrests the receptosome motion inside the cell (115).

Receptor-mediated endocytosis has generally been considered to be the specific cellular route to direct large molecules, such as proteins, to enter the cell (113,114,115). It may have a wide variety of significances. For many peptide hormones which trigger their physiological responses by acting at the cell surface, the internalization and degradation of the hormones may thus terminate their action. The internalization and degradation of the receptors may result in the decrease of receptor numbers on the cell surface (down regulation) thereby regulating hormone function. For a peptide hormone such as triiodotyronine,which has a nuclear action,the receptosome may function to transfer the hormone to the nucleus to exert its function. For a transport protein such as low density lipoprotein and transferrin which carry cholesterol and iron respectively, endocytosis may lead to an elevated concentration of these substances in the cell.

b. Antibody Induced Redistribution and Internalization

It has been known for a decade that antibody to a surface receptor can induce cap and internalization of the receptor in round cells such as lymphocytes (75). For example, anti-surface immunoglobulin (Ig) and anti-surface immunoglobulin M (IgM) antibodies can induce cap and internalization of surface Ig and surface IgM in B lymphocytes (74,75) and cultured B lymphoblastoid cells (141) respectively. The receptors are originally

diffusely distributed on the cell surfaces. After binding of antibodies, the antibody-receptor complexes start at 37°C to redistribute on the cell surfaces. The antibody-receptor complexes are redistributed first into clusters, then patches, and finally cap at one pole of the cell. The antibody-receptor complexes are internalized into the cell to form endocytic vesicles during and after the cap formation (75,141,142,143). The internalized vesicles then are directed to the Golgi region and finally appear in the lysosome where the antibody then is likely to be degraded (75,144). Cap formation from patches is an energy dependent process (74,145).

In flat cells like the fibroblasts (146,147,148), however, it has been shown that when antibody to a surface receptor, e.g. anti-histocompatibility antibody, binds to the cell, the induced patches do not continue to redistribute into cap as in round cells. Instead, the patches line up directly over the actin, myosin, and α-actinin containing stress fibers (149) inside the cell. But the antibody-receptor complexes are also internalized to form endocytic vesicles which are ultimately directed to the lysosome.

It has also been shown that antibodies to the receptors whose functions and physiological ligands are known such as the insulin receptor, low density lipoprotein receptor, and epidermal growth factor receptor may mimic the physiological functions of the ligands or mimic the induced redistribution and internalization by the ligands. For example, anti-insulin receptor antibody has been demonstrated to mimic the physiological function of insulin (150,151,152,153,154) and may also mimic the induced redistribution by insulin (155). Anti-low density lipoprotein receptor antibody has also been shown (156) to mimic the receptor-mediated endocytosis induced by low density lipoprotein, although it is unlikely that

the antibody functions as a cholesterol carrier. Anti-epidermal growth factor receptor antibody provides another example where the antibody mimics the epidermal growth factor in both the induced and physiological function (157).

It has been proposed, by extrapolating from the study of anti-histocompatibility antibody induced redistribution and internalization in fibroblasts (147), that antibody as well as lectin induced redistribution and internalization may represent a different type of ligand induced redistribution and internalization from the receptor-mediated endocytosis (113,114,115). This proposal is based on the observation that the antibody-receptor complexes do not use coated pits to enter the cells. However, since the direct demonstration (141) that anti-IgM-surface IgM complexes in cultured B lymphoblastoid cells also use the coated pits to enter the cells, this proposal may not be valid for all cases of antibody induced redistribution and internalization. Whether coated pits are used or not, there are many cases of antibody induced redistribution and internalization which suggest that actin, myosin, and α-actinin systems may be involved in the antibody induced redistribution and internalization both in round and flat cells (146,147,158,159,160).

The significance of antibody induced redistribution and internalization is not clear. It may have implications in the immune response and autoimmune disease,as for example,antibody production in B lymphocytes (75) and Myasthenia gravis. Myasthenia gravis is a neuromuscular disorder resulting from a deficiency of acetylcholine receptors in the neuromuscular junction (161). The autoantibodies to the acetylcholine receptor were found to be present in the circulation of myasthenic patients (162,163,164) and were shown to be able to accelerate the degradation of

acetylcholine receptor (165,166,167,168) in cultured skeletal muscle. The accelerated degradation of receptors may result from the antibody induced internalization and degradation of the acetylcholine receptor.

I-6-3 Antibody Induced Redistribution and Externalization

Complementary to the phenomenon of ligand induced redistribution and internalization is the ligand-induced redistribution and externalization, i.e. after ligand binding the ligand-receptor complexes undergo redistribution at 37°C and are then externalized (shed) into the environment. In tumor cells it has been demonstrated that anti-tumor specific surface antigen antibody can induce redistribution of the tumor specific surface antigen and then shedding of the antibody-antigen complexes into the environment (169,170,171,172). Moreover, in normal cells, it has also been suggested that anti-histocompatibility antibody may induce redistribution and shedding of the anti-histocompatibility antigen-antibody complexes from the B lymphocyte (173,174).

Antibody induced redistribution and externalization (shedding) may play an important role in the escape of the tumor cell from destruction by the host immune system. It has been proposed that the antibody-antigen complexes or antigens shed by induction or spontaneously from the tumor cell surfaces may act as competitors of the effector process in the immune response thereby preventing the tumor cell from being killed (171,175,176). However, the significance of the antibody induced redistribution and externalization in normal cells, such as B lymphocyte, is not known.

I-7 Reticulocytes and Erythrocytes

I-7-1 General Description

The reticulocyte is an immature red blood cell in its last stage

of differentiation and can be identified by the presence of reticulum after staining with a supravital dye (177). The mammalian reticulocyte is formed in the bone marrow by the expulsion of the nucleus from the late erythroblast (178) and at the last stages matures into the erythrocyte in the peripheral circulation. Although without a nucleus, the reticulocyte still possesses some of the organelles of the earlier precursors, including ribosomes and mitochondria. Reticulocytes therefore still maintain the ability to synthesize proteins (mainly hemoglobin); (179,180,181); bind transferrin and take up iron (182,183,184,185); and transport amino acids (186,187,188,189,190,191,192). Reticulocytes possess mitochondria and derive their energy from both a mitochondrial oxidative phosphorylation as well as glycolysis (181). The ribosomes (193,194,195) and mitochondria (196,197) disappear when the reticulocytes mature into erythrocytes with the concomitant loss of the functions associated with these organelles (198,199,200,201). Oxygen consumption in reticulocytes may be up to 30 times higher than in erythrocytes (181, 200). ATP levels decline with maturation and are lower still in aged cells (190,202,203). The rate of glycolysis is also much higher in reticulocytes than erythrocytes (201,202). However, the enzymes needed to carry out glycolysis and pentose phosphate pathway persist and are functional in the erythrocyte (204,205).

Accompanying the loss of intracellular structure and activity during maturation, the reticulocyte also loses some of its plasma membrane associated activity. These include the transport of amino acids (186,187, 188,189,190,191,192), iron uptake (182,183,184,185), and transferrin binding activity (183,206,207). However, some membrane associated activities

are only partially lost e.g. β-adrenergic binding activity (208,209,210), Na^+, K^+-ATPase and Ca^{2+}ATPase activities (211).

During maturation of reticulocytes, membrane surface and cell volume are considerably reduced (181,212). The decrease of cell surface area has been suggested to be due to the membrane surface remodelling or fragmentation (212,213,214). One proposed mechanism by which membranes are remodelled is the internalization of a part of the cell membrane or the shedding of membrane fragments (215).

In a normal animal, the circulating reticulocyte level is very low. During conditions of reduced hematocrit such as induced anemia by massive bleeding or administration of phenylhydrazine, the presence of reticulocytes in the circulation increases. These reticulocytes are believed to be larger than normal and shorter lived than those entering circulation under normal conditions (216,217,218,219,220,221).

I-7-2 Erythrocyte Membrane

Because of their abundance, availability, and ease of preparation and absence of nucleus and other intracellular organelles,the erythrocytes have long been used as a model to study the composition, structure, and function of biological membranes (22,24,222,223). The standard procedure for isolating erythrocyte membranes (224) produces a hemoglobin free ghost that is approximately the same size and shape as the intact erythrocyte. The human erythrocyte membrane contains many lipids and about ten major discernible polypeptides, some of which are glycosylated. The membrane polypeptides of human erythrocyte have been named by Fairbank et al (225), according to their migration on a SDS polyacrylamide gel and staining with Commassie brilliant blue and peroxidic acid-Schiff's (PAS) reagent (Fig.4). Band

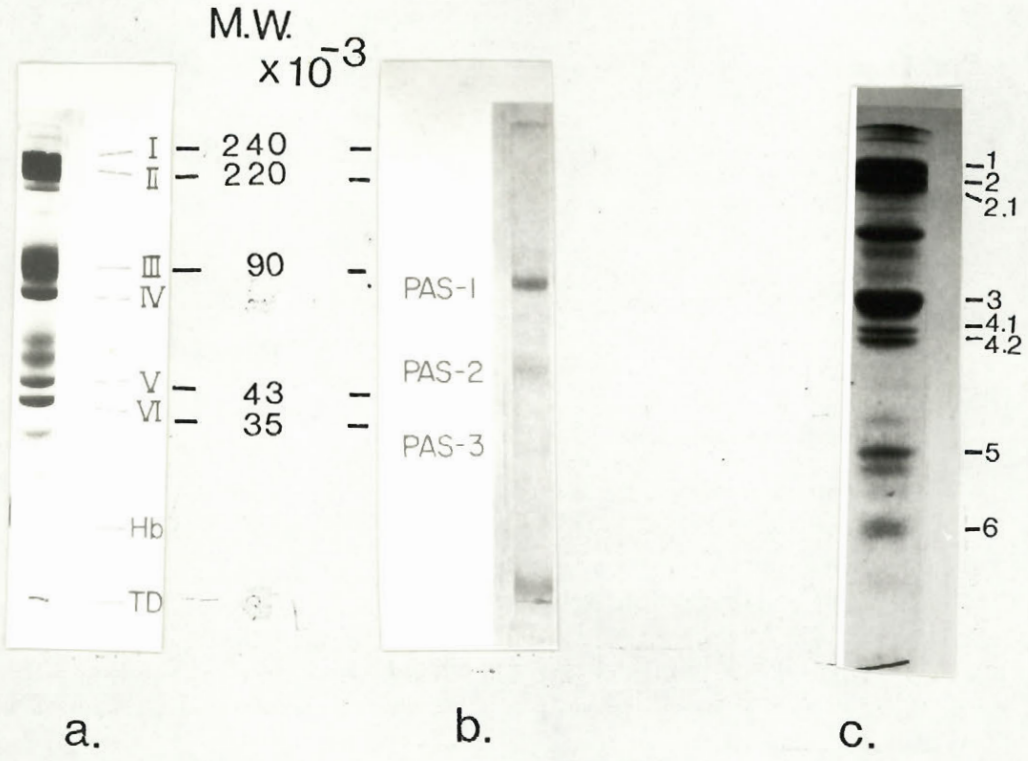

Fig.1-4 SDS-polyacrylamide gels of polypeptides of human and sheep erythrocyte membranes. a. b. human erythrocyte, c sheep erythrocyte. a. c. stained with Coomassie brilliant blue, b stained with PAS. (Reproduced a. b. from Fairbank et al, 1971, Biochemistry, 10, 2606; c. from Wiedmer and Lauf, 1981, Membrane Biochem., 4, 31)

3, PAS 1, PAS 2, PAS 3, PAS 4, and band 7 are integral membrane proteins, of which band 3 and PAS 1 are the most prominent species. Band 1, band 2, band 2.1, 2.2, 2.3, band 4.1, band 4.2, band 5, and band 6 are peripheral membrane proteins which are located in the cytoplasmic side of the membrane (222,223). Band 3 comprises the anion transport system (12,226,227). PAS 1 and PAS 2 are interconvertible and PAS 1 may be a dimeric form of PAS 2 (223,228). A sialoglycoprotein designated as glycophorin A has also been shown to be the basic subunit of the PAS 1 dimer (223,229). Band 3 (12) and glycophorin (7,8) have been shown to be transmembrane. Band 1 and band 2 consist of spectrin, the major cytoskeletal protein in red cells (222,230,231). Band 2.1 and the sequence related group of polypeptides designated as band 2.2, 2.3 contain the high affinity binding sites that attach spectrin to the membrane (25,26,27,28). This group of peripheral membrane proteins has also been called syndeins (28) or ankyrins (27), with ankyrin being more widely used. Ankyrin is attached to the membrane by binding at the cytoplasmic sites of band 3 (29,30,31). Band 5 is actin (232). Spectrin, actin, and band 4.1 interact together to complete a spectrin-actin-band 4.1 network which forms the principal cytoskeleton of the red cell (24). Band 6 is glyceraldehyde 3 - phosphate dehydrogenase (233,234). A representative scheme for erythrocyte membrane organization is given in Fig. 5. The spectrin-actin-band 4.1 network forms a continuous cytoskeleton which underlies the bilayer membrane and is attached to the membrane by ankyrins or other peripheral proteins to the cytoplasmic sites of band 3 or other transmembrane proteins. It is important to note that erythrocyte cytoskeletal system, unlike many other cells, contains no microtubules and intermediate filaments (24).

Reticulocytes synthesize only a few membrane proteins (235,236).

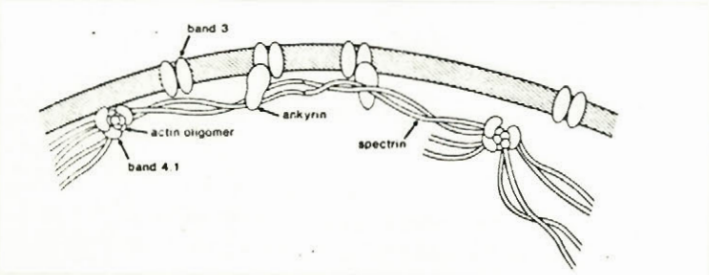

Fig.1-5 A possible arrangement for the major polypeptides of the erythrocyte cytoskeleton and its linkage with the integral membrane proteins. (Reproduced from Branton et al, 1981, Cell, 24, 24)

Similarly, very few membrane proteins appear to be lost during its maturation into erythrocyte (237). Presumably therefore the membrane proteins and their organization do not differ in a marked way from those of the erythrocyte membrane.

In sheep, the membrane proteins are in principal similar to those of the human erythrocyte. However, there are polypeptides which only appear in the sheep erythrocyte but not in human erythrocyte membrane. For example, there is a major peptide, positioned between band 2 and band 3, that appears only in sheep erythrocyte membranes (238) (Fig. 4).

I-8 Iron, Transferrin, Transferrin receptor, and Iron Transport

I-8-1 Iron and transferrin

Iron is required for many metabolic processes in cells such as the oxygen storage and transport, electron transport in the bioenergetic pathway, as well as many oxidation and reduction reactions. As a result, iron is essential for almost all living species (239). In order to carry and transport iron to mammalian cells a plasma protein transferrin with a very high affinity for iron has evolved. Transferrin is a single glycopeptide with a reported molecular weight between 76,000 - 81,000 (240,241). Transferrin has two binding sites for iron and for each iron molecule bound to the specific site, one bicarbonate molecule is concomitantly bound (240,241). Without bicarbonate or another anion substitute such as oxalate, glycolate, nitrilotriacetate(242,243) to satisfy the anion binding site, specific binding of iron to transferrin does not occur (244). No prosthetic group is involved in iron binding to transferrin. The two iron binding sites are in their own structural domains (245) and the two domains are virtually independent (246,247). The amino acid

sequence around the two binding sites is very similar in human transferrin (248). The iron which is associated with transferrin is ferric iron (240, 241). Ferrous iron is bound weakly if at all by transferrin (249). However, when iron is presented to transferrin as a simple ferric salt, hydrolysis, complex formation, and nonspecific binding may make the specific binding variable and unpredictable (250). Therefore, to load iron to the specific sites of transferrin the preferable procedure is to provide ferric chelates or freshly prepared ferrous salt (251). The reason for the latter is probably because ferrous ion is only slowly hydrolysed but is oxidized to ferric state on binding to transferrin before hydrolysis occurs. The apparent stability constant for the binding of iron to the two iron binding sites at pH 7.4 and atmospheric pCO_2 is $4.7 \times 10^{20} M^{-1}$ and $2.4 \times 10^{19} M^{-1}$ respectively (252).

As an iron carrier, transferrin is required for cell growth (253, 254,255,256,257). Transferrin also has a bacteriostatic effect (258) since it limits availability of iron to the bacteria.

I-8-2 Transferrin receptor

The transport of iron from the transferrin to the cell first involves the binding of Fe-transferrin to the cell surface receptor as originally proposed by Jandle and Katz (183). The binding sites or transferrin receptors probably have a ubiquitous distribution on animal cells. The transferrin receptor has been shown to be present on the surface of the reticulocytes and other erythroid cells (131,183,185,206,207,259,260, 261,262,263), human placentae (264,265,266,267), mitogen activated lymphocytes (268), and other cells (269,270,271,272,273,274,275,276,277).

The concentration of transferrin receptors on the cell surface

appears to be related to the iron requirement of the particular cell. Cells which require iron for the synthesis of hemoglobin like the reticulocytes and their precursor erythroid cells, or rapidly growing normal or malignant cells (268,272,274,275,276,277,278) have abundant receptors on their surfaces. However, cells which may have less requirement for iron such as erythrocytes and other nongrowing cells (268,275,279) show a relative lack of the receptor.

The transferrin receptor is an integral membrane protein. The monomeric subunit has an apparent molecular weight in the range 90,000 - 100,000 (260,262,265,272,274,277,280,281,282), and it may exist as a disulfide bonded dimer in its active form (260,272,275,280,281,282), with two binding sites for transferrin (282).

I-8-3 Anti-transferrin receptor antibody

Monoclonal antibody raised against the transferrin receptor of malignant cells (272,274,275) and conventional anti-transferrin receptor antibody (283) have been obtained. It has been suggested that the antibody binding site in the transferrin receptor may be different from the sites of transferrin binding (274). Also, the antibodies against the receptor may influence the physiological function of the receptor since it has been reported that anti-transferrin receptor antibodies inhibit the growth of human melanoma cells in nude mice (284). Moreover, it has been demonstrated that the antibody may be used as a carrier of toxin to direct the antibody coupled toxin to the growing malignant cells and specifically kill them (284).

I-8-4 Iron transport

In reticulocyte and erythroid cells, there is general agreement that the initial stage for iron transport is the binding of iron-

transferrin to the transferrin receptor on the cell surface (183,240,241). However, how the iron is then transferred from the iron-transferrin-receptor complexes on the cell surface into the cell is not clear. Two concepts (240,285) prevail about the possible mechanism. One proposal corresponds to the suggestion that after the binding of iron-transferrin to the receptor, iron is transferred by an unknown mechanism from the cell surface into the cell without internalizing the iron-transferrin-receptor complexes (183,259,286, 287). The other proposal is that the iron-transferrin-receptor complex is internalized into the cell, and the iron then is released from the complex into the intracellular site with the transferrin eventually released from the cell (131,288,289,290).

A scheme proposed by Morgan (241) to describe the steps in the iron transport involving internalization of the iron-transferrin-receptor complexes is shown in Fig. 6.

Iron transport by internalization of the iron-transferrin-receptor complexes has also been proposed in other cells (271,291).

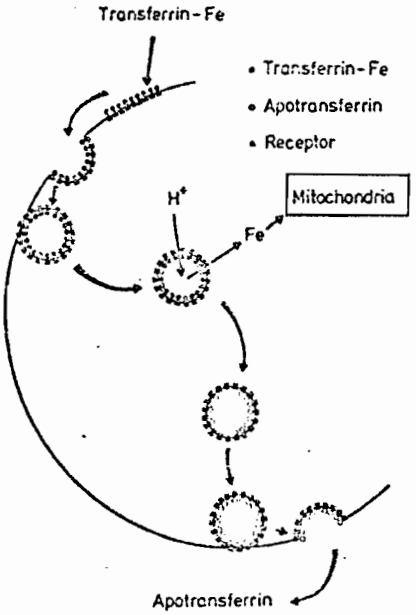

Fig.1-6 Diagrammatic representation of the steps involved in the uptake of transferrin-bound iron by immature erythroid cells. (Reproduced from Morgan, E.H., 1981, Mole. Aspect. Med., 4, 1)

PURPOSE OF WORK

Reticulocytes lose some of their membrane associated functions, e.g. Na^+-depedent amino acid transport, iron uptake and transferrin binding activity, during maturation. The loss of these functions may reflect the physical loss of the entities which are responsible for these functions. To try to identify and isolate the physical entities (i.e. proteins) responsible for these lost functions, the first approach would be to determine whether specific protein losses can be followed during reticulocyte maturation. I was originally interested in the purification of a Na^+-dependent amino acid transporter whose function disappears when sheep reticulocytes mature (190,191). In order to obtain a basis for the purification work, the membrane proteins between sheep reticulocyte and erythrocyte were compared using an immunological approach. Rabbit antiserum against sheep reticulocytes was prepared. After absorption with sheep erythrocytes, the antiserum was found to agglutinate sheep reticulocytes but not erythrocytes. The results suggested that there are surface antigens which exist only in reticulocytes but not erythrocytes. The results then provided an approch to isolate and determine the nature of reticulocyte surface specific antigens.

The purpose of this work was to isolate these reticulocyte surface specific antibodies and use them to isolate and identify reticulocyte surface specific antigens, and to study the behaviour of the antigens on the sheep reticulocytes using the purified antibody as probes.

In order to detect reticulocyte membrane specific proteins, immunoprecipitation using Staphylococcus aureus (Cowan Strain) and sheep erythrocyte-preabsorbed rabbit anti-sheep reticulocyte antiserum was carried out. The same approach was also used to follow the change of these specific antigens during sheep reticulocyte maturation in vitro.

CHAPTER 2

DISAPPEARANCE OF RETICULOCYTE SURFACE SPECIFIC MEMBRANE PROTEINS DURING SHEEP RETICULOCYTE MATURATION IN VITRO

ABSTRACT

Reticulocyte-specific antiserum has been used to isolate reticulocyte specific plasma membrane proteins (antigens) from sheep reticulocytes. A specific precipitation of ^{125}I-labelled plasma membrane proteins was obtained when detergent extracts of ^{125}I-labelled plasma membranes were incubated with this antiserum and Staphylococcus aureus (Cowan strain). Similar treatment of extracts of mature cell plasma membranes did not yield this specific ^{125}I-labelled precipitate. During in vitro maturation of reticulocytes, there is a decrease in the immuno-precipitable material. The observations suggest that this approach can be used to follow changes in membrane proteins during reticulocyte maturation in vitro.

INTRODUCTION

Circulating mammalian reticulocytes retain a number of synthetic and enzymatic activities which gradually disappear with cell maturation. These activities include: (1) protein synthesis (1,2); (2) mitochondrial function (2-5); (3) transferrin binding and iron uptake (6-10) and Na^+-dependent amino acid transport (11-16). The changes in iron uptake and amino acid transport activity involve alterations in the plasma membrane per se although structural changes in the membrane have not yet been shown directly. Changes in activity may be associated with losses of specific membrane proteins or alterations in the activity of the proteins without an actual loss of the particular proteins.

It was reasoned that antibodies prepared against reticulocytes and used in conjunction with Staphylococcus A protein might provide a means of identifying reticulocyte specific plasma membrane proteins which disappear on maturation. The validity of this approach is confirmed by the present report which shows that antiserum prepared against reticulocytes can be used to identify reticulocyte specific membrane proteins (antigens) and to follow the disposition of the proteins (antigens) during in vitro maturation.

Since maturation of sheep reticulocytes can be followed in vitro (15,16), such a system should provide a convenient way to study the mechanism of maturation or ageing of the plasma membrane at the molecular level.

MATERIALS AND METHODS

1. Isolation of Reticulocytes and Incubation Procedures

Reticulocyte production in sheep was induced by phlebotomy as described previously (15,16). Whole blood was washed 3 times with isotonic saline and the washed cells were suspended to approximately 80% hematocrit and centrifuged at 3,500 rpm in a Sorvall HB4 rotor for 1 hr. Following centrifugation, a layer containing the top 25% of the cells was removed, transferred to 15 ml centrifuge tubes and recentrifuged at 3,500 rpm in an HB4 rotor for 1 hr. The top 10% layer which usually contains over 80% reticulocytes, was carefully collected. At each stage of centrifugation, the buffy layer of white cells was removed. A reticulocyte count of about 80% was used in most experiments. Reticulocytes (1% hematocrit) were incubated *in vitro* in a medium of the same composition as used previously by Benderoff *et al.* (15,16). Mature red cells were obtained from the bottom 25% layer after the first centrifugation or from normal, unbled sheep.

2. Immunological Procedures

One ml of a 1% suspension of sheep reticulocytes or mature red cells was injected subcutaneously into rabbits with complete Freund's adjuvant, followed by 5-6 successive intravenous injections at weekly intervals using the same number of cells. Seven to eight weeks after the first injection, antiserum was collected and inactivated at 56°C for 30 minutes. The rabbit antiserum against sheep reticulocytes was

exhaustively absorbed with mature sheep red cells, until there was no agglutination reaction against mature sheep red cells. The antiserum so obtained is called reticulocyte specific antiserum. The non immune serum used was obtained from rabbit blood drawn prior to immunization.

3. Iodination of Plasma Membranes

The reticulocyte and mature cell plasma membranes were isolated following the procedure of Dodge et al. (17). The isolated membranes were iodinated using ^{125}I and lactoperoxidase as described by Reichstein and Blostein (18,19). The membranes were then dissolved in 1% Triton, 1% aprotinin, 154 mM NaCl, 20 mM Tris, pH 7.4 at 0°C and centrifuged at 16,000 rpm for 2 hrs using a SS 34 rotor or at 100,000 g for 1 hr. The pellet obtained was discarded and the supernatant was subjected to immunoprecipitation. To follow the changes in the plasma membranes during maturation of the cell in vitro, aliquots containing equal numbers of cells were taken at the intervals given in the figures. Membranes were prepared from the cells on the day the sample was taken and kept frozen until all samples in the series had been taken. Then the membranes from all the samples were iodinated as described above. Determinations of the total ^{125}I incorporated showed that all samples prepared on a particular day contained the same amount of radioactivity within ~5%. The possibility exists that the labelled proteins are derived from intracellular membranes such as mitochondria rather than the plasma membrane itself. To address this question, samples of intact reticulocytes and mature cells were also subjected to iodination as described (18,19)

prior to isolating the plasma membranes and the specific membrane proteins. It is known that only the external surface of the plasma membranes becomes labelled when this procedure is applied to intact cells (18,19).

4. Immunoprecipitation of Membrane Proteins

A 100-200 ul sample of the supernatant (30-40 ug protein/100 ul) obtained from the iodinated, dissolved membranes was incubated for 1 hr at 37°C with reticulocyte specific antiserum or with non immune serum. Subsequently 200 ul of a 10% suspension of formaldehyde-fixed Staphylococcus aureus (Cowan strain) was added and the incubation continued for another hour at 37°C. The immune complex was centrifuged and washed three times with 0.25% Triton X-100, 10mM Tris HCl pH 7.4, 5 mM EDTA in 0.154 NaCl (20). To measure total ^{125}I-protein precipitated, the washed pellet was counted. To estimate the radioactivity associated with specific membrane proteins, the antigen-antibody complex was dissociated from the bacterial cell surface by heating the washed pellet at 100°C for 3 min in 2% SDS, 10% mercaptoethanol, 154 mM NaCl and 20 mM Tris HCl, pH 7.4. The Staphylococci were centrifuged down and an aliquot of the supernatant was subjected to SDS gel electrophoresis using SDS polyacrylamide gels according to the Laemmli procedure (21). After electrophoresis the gels were sliced into 2 mM sections and counted in a Packard γ-counter.

To ascertain that sufficient antiserum was added to precipitate all the labelled immunoreactive proteins, the dissolved membranes were treated as above using variable amounts of antiserum. Usually 6 μl of preabsorbed reticulocyte specific immune serum was sufficient to precipitate

the maximum amount of ^{125}I-labelled proteins from 33 µg membrane protein contained in 100 µl solubilizing medium. A volume of 200 µl of a 10% suspension of Staphylococcus aureus was found sufficient to precipitate out the maximum amount of ^{125}I-labelled proteins contained in the 100 ul of membrane extract. Using (1) mature cell membranes with reticulocyte specific antiserum or (2) reticulocyte membranes with non immune serum or (3) reticulocyte membranes with reticulocyte preabsorbed immune serum, identical levels of ^{125}I-labelled proteins were found associated with the Staphylococcus aureus pellets. The latter level of radioactivity was 25% of that found with reticulocyte membranes and reticulocyte specific antiserum. This "background" radioactivity was not observed after subjection of the dissociated proteins to SDS gel electrophoresis.

5. Indirect Immunofluorescence and Methylene Blue Staining

100 µl of a washed 1% reticulocyte or mature cell suspension in phosphate-buffered saline at pH 7.4 was incubated with 10 µl of immune or non-immune serum at 25°C for 60 minutes. The cells were washed twice with phosphate buffered saline pH 7.4 and then incubated for 60 minutes at 25°C with 50 µl (1 mg/ml) of FITC-labelled-protein A. After washing, the cell pellet was resuspended with phosphate-buffered saline to give a 1% cell suspension and a drop of the suspension was placed on a polylysine coated glass coverslip. The cells were allowed to sediment under gravity for a few minutes and the excess fluid in the drop was removed carefully. A drop of new methylene blue solution was then added to the cover slip. After 5 minutes, the excess methylene blue solution

was removed and the cover slip rinsed gently with phosphate buffer-saline at pH 7.4. The cover glass then was mounted on the slide. Fluorescence of the stained cells were viewed under a Zeiss microscope using visible and fluorescent illumination. Kodacolor 400 negative film was used for the photography.

6. ATP and K^+ Analyses

The cellular ATP levels were analyzed using the luciferin luciferase procedure described by Stanley and Williams (22). Cellular K^+ was analyzed by flame photometry using an internal Li^+ standard. Hemoglobin content was determined by the Drabkin method (23).

7. Materials

Na^{125} was purchased from Frosst Co., Montreal, Quebec. Firefly lantern extracts for ATP determination, lactoperoxidase and glucose oxidase and aprotinin were purchased from Sigma Co., St.Louis, Mo, USA. Fluorescein conjugated protein A was a product of Pharmacia Fine Chemicals, Pharmacia, Dorval, Quebec. Tissue culture media and calf serum were obtained from Grand Island Biological Co., Burlington, Ontario. The culture of Staphylococcus aureus (Cowan strain) was obtained from Dr. D. Thomson, Montreal General Hospital.

RESULTS

Immunization of rabbits with sheep reticulocytes results in the production of an antiserum directed against the reticulocyte cell surface. After absorption of the rabbit antiserum with mature sheep red cells until no agglutination of mature cells is obtained with undiluted serum, the absorbed serum will agglutinate sheep reticulocytes at 64-fold or greater dilution (Fig.1) suggesting that the antiserum is directed against reticulocyte-specific surface antigens. Unless otherwise stated, antisera used in all experiments was pre-adsorbed with mature cells. Over a dozen rabbits have been shown to produce anti-sheep reticulocyte antiserum which after absorption with sheep erythrocyte cause reticulocyte agglutination.

To substantiate the conclusion that the antiserum is directed against the reticulocyte membrane, two types of approaches were used: (i) indirect immunofluorescence and (ii) immunoprecipitation studies.

Indirect Immunofluorescence

Reticulocytes and mature red cells were incubated with anti-reticulocyte serum, washed and incubated with FITC-labelled protein A. The results show (Fig.2) that immunofluorescence is observed only with cells which have a stainable reticulum. No fluorescence is seen when reticulocytes are incubated with (1) non immune rabbit serum, (2) when mature cells are incubated with anti-reticulocyte serum or (3) when reticulocytes are incubated with reticulocyte preabsorbed antiserum.

1. 3. Reticulocyte

2. 4. Erythrocyte

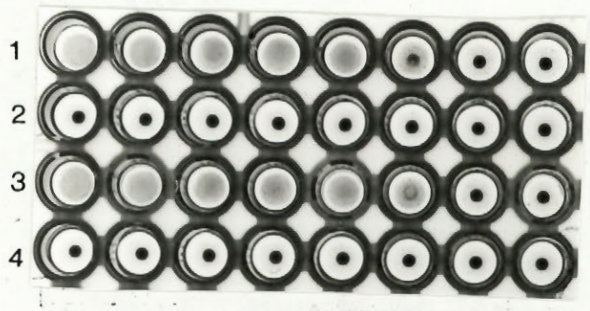

Figure 2-1. Agglutination Test

An aliquot (0.1 ml) of a 2% suspension of reticulocytes or mature red blood cells was added to equal volumes of serial two fold diluted complement inactivated-antiserum, preabsorbed with mature cells. The titer plates incubated at room temperature to allow agglutination to occur. A duplicate assay is shown with reticulocytes in the first and third tiers and with mature cells in the second and fourth tiers. It is evident that serum diluted up to 64-fold still causes agglutination of reticulocytes.

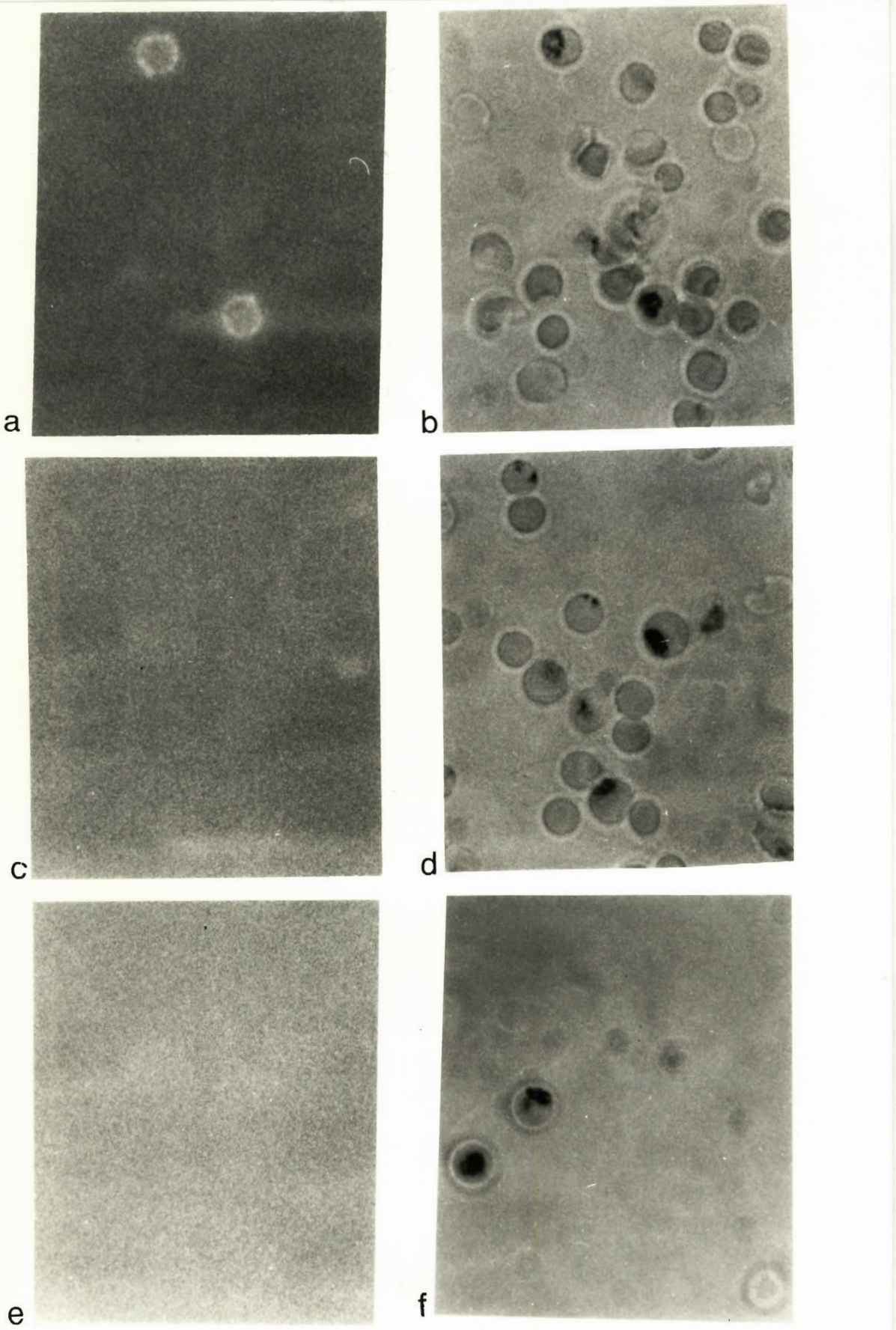

Fig. 2-2

Figure 2-2. Binding of FITC-labelled Protein A to Reticulocytes in Presence of Reticulocyte Specific Antiserum.

Indirect immunofluorescence and methylene staining were carried out with a mixture of reticulocytes and erythrocytes as described in methods. Fluorescence (a,c,e): (a) immune serum, (c) non immune serum, (e) immune serum preabsorbed with reticulocytes; (b,d,f)visible light - same fields as a, c, and e respectively. (magnification 1,000X)

Immunoprecipitation

After immunoprecipitation of the solubilized iodinated plasma membrane proteins with antiserum and Staphylococcus aureus, the immune-complex was dissociated from Staphylococcus aureus as described in Methods. An aliquot of the eluate was subjected to SDS gel electrophoresis using the Laemmli procedure (21). The gels were sliced and counted following the procedure described by Reichstein and Blostein (18,19). The data show that two clear iodinated components are detected on the gels in the 85-95K region (Fig.3). An additional broad peak is frequently also detected in the 200K region but its level of radioactivity is generally much lower than the activity in the 95K region. No labelled components were obtained from mature red cell membranes after incubation with reticulocyte specific antiserum, nor from reticulocyte membranes treated with either non immune serum or immune serum preabsorbed with reticulocytes. The latter observations suggest that the reticulocyte specific marker is on the plasma membrane.

To ascertain that the ^{125}I-labelled peptides detected originate from the plasma membrane and not from cytosolic components which adhere to the membranes during isolation, the labelling with ^{125}I and lactoperoxidase was conducted with intact reticulocytes and mature cells followed by isolation of membranes. This procedure results in labelling of surface components only (18,19). Prior to immunoprecipitation, the total radioactivity in the ^{125}I-labelled sample used was similar ($\pm$ 5%) with both reticulocytes and mature cells. Isolation of the Staphylococcus A precipitable material in a typical experiment gave 820 cpm in the

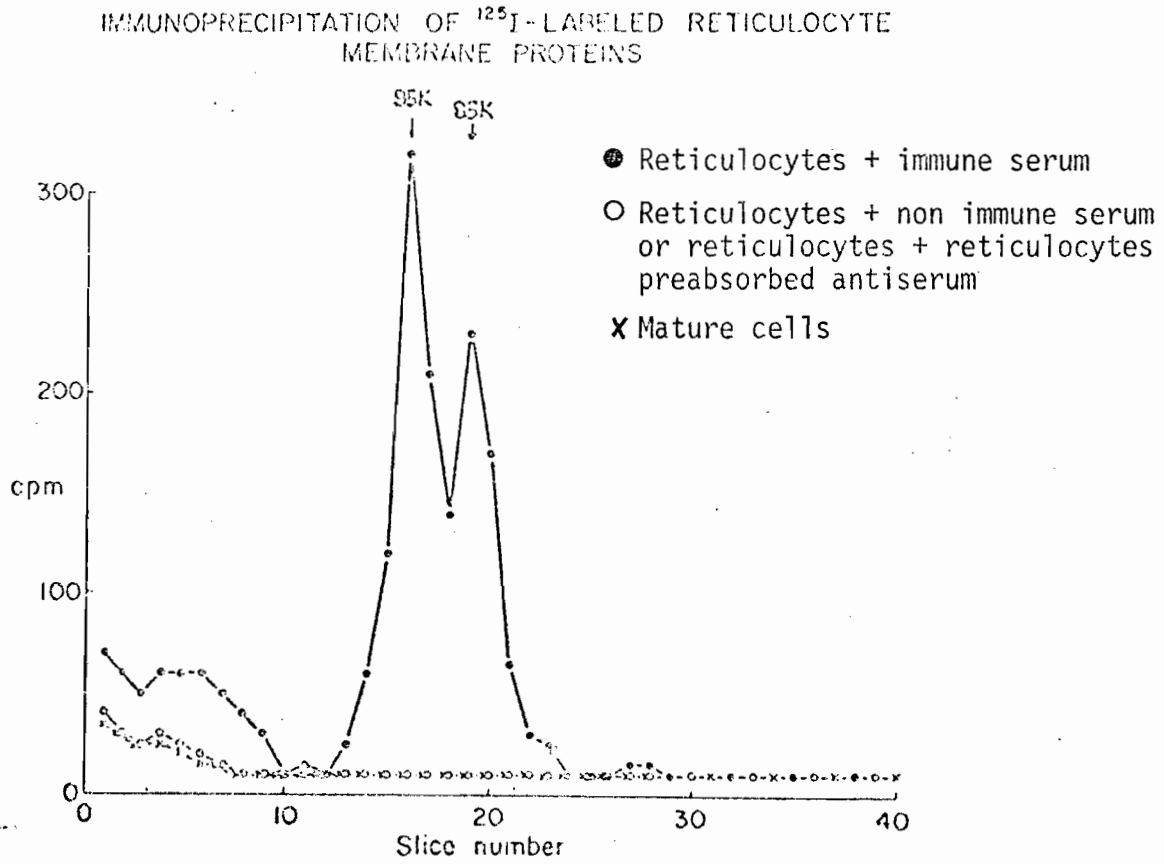
IMMUNOPRECIPITATION OF ^{125}I-LABELED RETICULOCYTE MEMBRANE PROTEINS
95K
85K
300
200
100
0
cpm
0
10
20
30
40
Slice number
Reticulocytes + immune serum
Reticulocytes + non immune serum or reticulocytes + reticulocytes preabsorbed antiserum
Mature cells

Fig.2-3.

Figure 2-3. Immunoprecipitation of Iodinated Membrane Proteins.

Isolated membranes from reticylocytes and mature cells were iodinated, dissolved, and treated with serum and Staph. aureus as described in Methods. Prior to the addition of serum the dissolved reticulocyte membrane sample contained 33 μg protein and a total radioactivity of 2.5×10^5 cpm in 100 μl. After treatment with immune serum and Staphylococci, the immuno-complex was centrifuged, washed, then dissociated with 2% SDS in 0.154 M NaCl (see Methods) at 100°C. The eluate containing 3,500 cpm, was applied to an SDS gel, electrophoresed, sliced into 0.2 cm sections and counted. A total of 1,800 cpm was recovered after counting all gel fractions. This represents a recovery of 75% of the specifically labelled protein in the SDS eluate. A second aliquot of labelled reticulocyte membrane proteins was treated with non-immune serum or with reticulocyte preabsorbed antiserum. After dissociation with 2% SDS in saline, the total radioactivity recovered (1,130 cpm and 1,100 cpm respectively) was applied to the gel (non-specific labelling). No radioactive peaks above background were obtained with the latter two samples after electrophoresis.

Using an aliquot of labelled membrane proteins (37 μg and 1.8×10^5 cpm in 100 μl) from mature cells, the treatment with immune serum precipitated out a similar amount of radioactivity (970 cpm). The SDS eluates obtained from this immuno-complex showed no detectable radio labelled peaks above background after electrophoresis. The source of this high radioactivity in the non immune precipitate is not known. The data in the above figure are expressed as cpm per slice.

immune complex derived from reticulocytes whereas only 240 cpm were obtained from mature cells. Moreover, it is evident from the immunofluorescent data (Fig.2) that a cell surface component must react with antibody and FITC labelled protein A, since the fluorescence is concentrated at the periphery of the cell, and this peripheral fluorescence disappears if reticulocyte-preabsorbed antiserum is used instead of antiserum preabsorbed with mature red blood cells.

These data show that the reticulocyte specific antiserum reacts specifically with components on the reticulocyte cell surface but not with those on the surface of the mature sheep red cell.

Disappearance of Reticulocyte Membrane Proteins During Maturation In Vitro

If the peptides identified above are associated with proteins whose function will be lost on reticulocyte maturation, it may be possible to demonstrate that there is a reduction in these peptides during incubation in vitro. Reticulocyte maturation was followed in vitro as described before (15,16). At the intervals given, samples were removed, and plasma membranes isolated and stored as indicated in Methods.

The results (Fig.4) show that the total amount of ^{125}I-labelled immuno-precipitable material decreases with time in culture. After 96 hrs the level of radioactivity isolated from reticulocyte membranes is no different from that obtained from mature cell membranes. If SDS eluates from the Staphylococcus A complex are subjected to SDS PAGE, it may be seen that the labelled peaks disappear with time in culture (Fig.5)

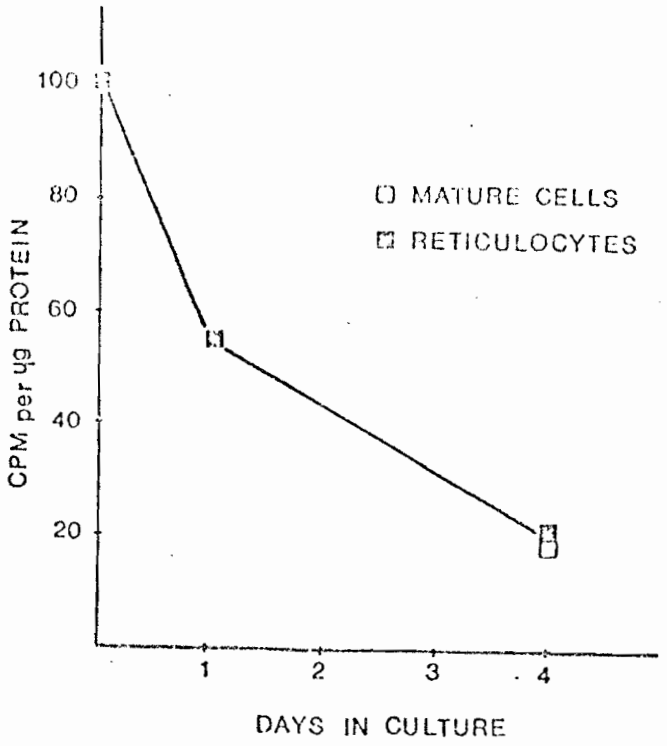

Figure 2-4. Diminished Labelling of the Reticulocyte Surface Specific Proteins with Time in Culture.

Equal numbers of cells were incubated in a series of culture flasks. At intervals, the flasks were removed and the cells centrifuged, washed and the plasma membranes isolated and stored frozen. When the last sample was taken, all the membrane samples were iodinated with ^{125}I. The iodinated membranes were washed and then dissaggregated as described in Methods. To 600 µl of dissolved membranes (containing 100-120 µg protein) antiserum was added following by a 10% suspension of Staph. aureus (see Methods). The pellet containing the immuno-complex was washed three times and then counted. The counts are expressed as cpm per ug protein contained in the original sample of dissolved membranes. After labelling the membranes and prior to precipitation with antiserum, the total cpm in each sample was 0 time - 5×10^5, 1 day - 4.3×10^5, 4 days - 4.4×10^5, mature cells 5.3×10^5. A representative experiment is shown from 3 similar experiments.

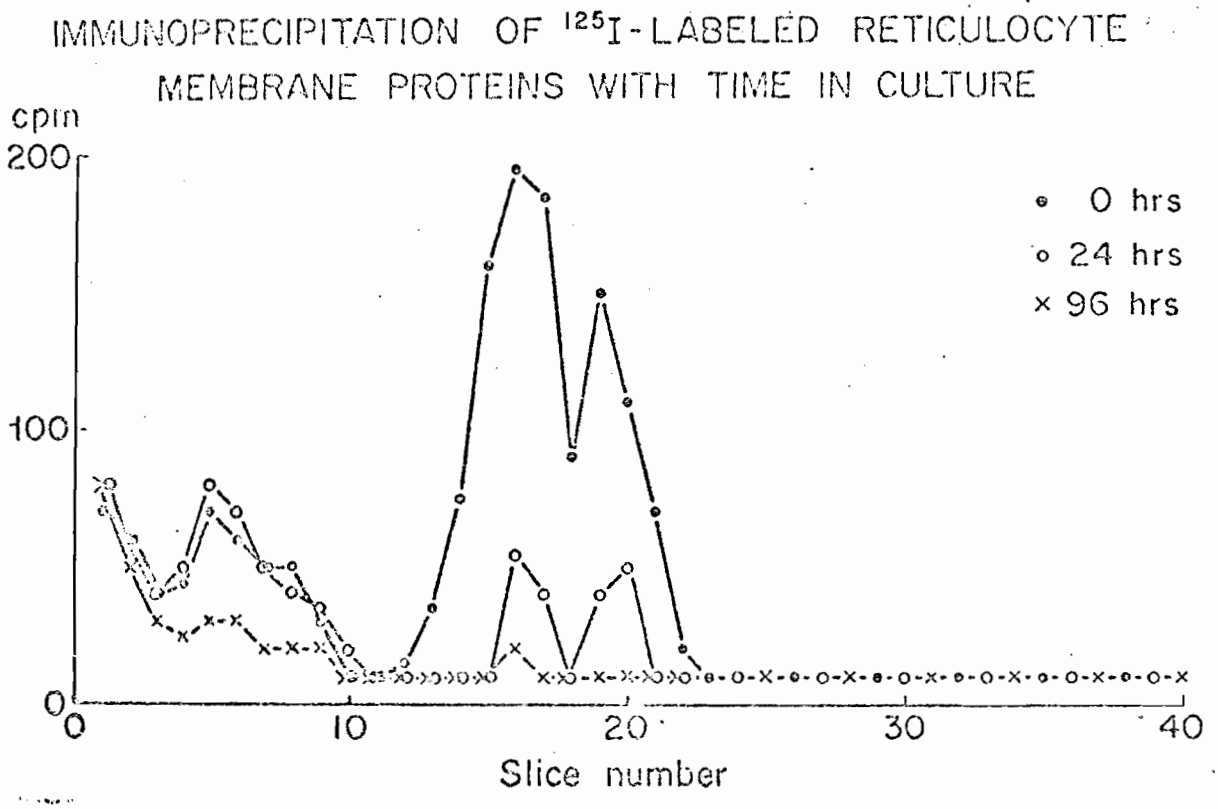

Figure 2-5. Decrease of the Reticulocyte Surface Specific Proteins with Time in Culture.

The procedures are identical to those described in Figure 3, but samples for electrophoresis were from Figure 4.

and by 96 hours the level of radioactivity approaches that seen with the mature cells.

Several lines of evidence argue against the possibility that the loss of iodinated peptides is due to either loss of cells (cell lysis) or non-specific changes in the sites available for iodination. Firstly, there is no significant increase in hemoglobin detected in cell-free supernatants of the medium indicating the absence of cell lysis during incubation. Secondly, the total ^{125}I incorporated into the plasma membrane proteins prior to precipitation with antiserum stays constant during the culture periods employed. These studies support the conclusion that the proteins identified with reticulocyte specific antiserum are characteristic of the reticulocyte and become undetectable or lost upon maturation.

To assess whether other metabolic parameters are maintained while the specific membrane components of the reticulocytes are lost, cellular K^+ and ATP levels were assessed with time in culture. After 48 hrs, the cellular ATP level of the reticulocytes has dropped to about 25% of the original level and is close to that reported for mature human red cells (23), (Table 1). The cellular K^+ level remains relatively constant. These data indicate that cellular integrity is maintained. Similar results were reported earlier by Benderoff et al. (15,16).

TABLE 2-1.

Days in Culture	0	1	2	3
Cellular ATP level (mM)	1.96	1.03	0.82	0.58
$(K)^+$ mM	104	117	117	104

Samples of cells were taken at the intervals stated. One aliquot of washed cells was lyzed immediately and cellular K^+ determined by flame photometry using a Li standard. A second aliquot of cells was frozen in buffered medium until all samples were taken. Then the cells were thawed and an aliquot taken for ATP estimation by the method of Stanley and Williams (22).

DISCUSSION

Earlier studies (24,25) have already shown that an antiserum against reticulocytes, which does not agglutinate mature cells, can be prepared by standard immunological procedures. The present report shows that this antiserum can be used to isolate plasma membrane components (antigens) which are characteristic of the reticulocytes. With culture time in vitro during which cellular integrity is maintained, there is a diminished capacity of reticulocyte plasma membrane components to react with reticulocyte specific antiserum. The present work also shows that the disappearance of the reticulocyte specific membrane proteins follows a time course similar to that seen for the loss of Na^+-dependent glycine transport and Na^+-dependent histidine exchange (15,16). Since an earlier study by Schulman and Nelson (24) showed that rabbit transferrin does not inhibit the agglutination of rabbit reticulocytes by the rabbit reticulocyte specific antiserum raised in guinea pig, it seemed that the specific antiserum was not against the transferrin binding proteins whose activity disappears during reticulocyte maturation. The possibility exists that the reticulocyte specific membrane proteins may have a relation with the amino acid transport activity.

The possibility must be considered that the peptides detected in this work are derived from cytosolic components, such as mitochondria, which adhere to the plasma membranes during isolation. Since these components are also lost during maturation, their absence from mature

cell membranes would not be surprising. The evidence obtained, however, strongly suggests that the peptides detected originate in the plasma membrane. Thus (i) peripheral immunofluorescence with FITC labelled protein A is observed with reticulocytes incubated with reticulocyte specific antiserum whereas there is no immunofluorescence if the antiserum is preabsorbed with reticulocytes. (ii) Although most of the studies reported here were carried out by labelling isolated plasma membranes (and hence adhering cytosolic components may be labelled), control experiments with intact cells radioiodinated with lactoperoxidase were also carried out. Starting with ^{125}I-labelled cells relatively little immuno-precipitate is obtained from mature cells compared to that from reticulocytes. Moreover, this difference does not arise from a lesser overall labelling of mature cells membrane proteins since the starting material was labelled to an equivalent extent with both mature cell and reticulocyte preparations. As with the immunofluorescence studies, preabsorption of the antiserum with reticulocytes results in a loss of its specific capacity to preciptate out labelled components from the iodinated reticulocyte plasma membranes.

Although there is an earlier claim that a reticulocyte specific plasma membrane protein is lost on maturation of the reticulocyte (26) those data did not completely eliminate the possibility of contamination with membranes originating from other cellular components e.g. mitochondria. In the present work, the possibility of contamination is unlikely and the protein is identified as a plasma membrane component which is lost on maturation or which loses immunoreactivity on maturation of the cells.

The molecular weights of the peptides detected earlier (26) are different from those reported here.

In conclusion, the present approaches provide possibilities to study the physiological functions of the reticulocytes specific membrane proteins (antigens) and the mechanism involved in their disappearance.

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In order to study the sheep reticulocyte surface specific antigens (proteins), the antibodies against the sheep reticulocyte surface specific antigens were purified and used to isolate and characterize the specific antigens.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF SHEEP RETICULOCYTE SURFACE SPECIFIC ANTIGENS: AN IMMUNOLOGICAL APPROACH

ABSTRACT

A simple method to obtain antitransferrin receptor antibody is described. Rabbits were immunized with washed, whole sheep reticulocytes and the antiserum obtained was preabsorbed with sheep, mature, red blood cells to remove all cross reacting antibodies against surface components. The remaining antiserum was incubated with sheep reticulocytes to absorb antibodies against reticulocyte surface components. These bound antibodies, eluted from the cell surface, were purified by Protein A chromatography. The antibodies thus obtained, directed against reticulocyte surface components, were coupled to Sepharose-4B and used to isolated specific plasma membrane components from reticulocytes. The studies show that the antibodies are directed against the transferrin receptor. No reactivity with transferrin is obtained and antibodies against other surface components are not detectable.

The receptor isolated with the antibody has been shown to have a subunit molecular weight of 93,000. The present data are consistent with reported molecular weights of this receptor and that the receptor may exist as a dimer since ^{125}I-tyrosyl peptide maps of the 93,000 and 186,000 components isolated are shown to be identical. Data are presented for the transmembrane nature of the receptor and for the presence of different binding sites for transferrin and the antibody on the receptor.

INTRODUCTION

Previous studies by Schulman and Nelson (1) have shown that guinea pig antiserum, raised against rabbit reticulocytes and followed by exhaustive absorption with rabbit erythrocytes, do not cross-react with rabbit erythrocytes. In their report, the nature of the reticulocyte specific surface antigens was not identified. Furthermore, since transferrin did not prevent reticulocyte agglutination by the antiserum, the possibility that the antiserum was directed against the transferrin receptor was not considered. We have reported that rabbit antiserum against sheep reticulocyte surface specific antigens can be used to isolate reticulocyte specific membrane proteins (antigens) (2). In the present work, we report that the specific antibodies in the antiserum can be isolated and routinely identified as anti-transferrin receptor antibody with antisera from over a dozen of rabbits. The studies identify the transferrin receptor as a major antigenic component on the sheep reticulocyte surface and show that the transferrin receptor can be isolated and purified with these antibodies.

Reticulocytes are simple cells and synthesize only a few membrane proteins (3, 4, 5). Moreover, reticulocytes maintain almost all of their membrane proteins during maturation (6). The observations that transferrin receptors disappear during reticulocyte maturation (2) and anti-reticulocyte surface specific antibodies are mainly anti-transferrin receptor antibodies, therefore, provide an experimental system to study the possible mechanism for the selective modulation of membrane proteins in cells.

MATERIALS AND METHODS

1. Isolation of Reticulocytes

Reticulocyte production in sheep was induced by phlebotomy as described (7,8). Whole blood was washed 3 times with isotonic saline and the washed cells were suspended to approximately 80% cytocrit and centrifuged at 3,500 rpm in a Sorvall HB4 rotor for 1 hr. Following centrifugation, a layer containing the top 25% of the cells was removed, transferred to 15 ml centrifuge tubes and recentrifuged at 3,500 rpm in an HB4 rotor for 1 hr. The top 10% layer which usually contains 80-100% reticulocytes, was carefully collected. At each stage of centrifugation, buffy coat of white cells was removed before using the red cell layer. A reticulocyte count of about 80% was used in most experiments. Reticulocytes were incubated in vitro in a medium of the same composition as used previously by Benderoff et al. (7,8). Mature red cells were obtained from the bottom 25% layer after the first centrifugation or from normal, unbled sheep.

2. Immunological Procedures

One ml of a washed 1% suspension of sheep reticulocytes prepared as described above or mature red cells was injected subcutaneously into rabbits with complete Freund's adjuvant, followed by 5-6 successive intravenous injections at weekly intervals using the same number of cells. Seven to eight weeks after the first injection, antiserum was collected and inactivated at 56°C for 30 minutes. The rabbit antiserum against sheep reticulocytes was exhaustively absorbed with mature sheep red cells.

The antiserum thus obtained is designated antiserum I. To purify anti-reticulocyte surface specific antibodies, antiserum I was incubated for 24 hrs at 0°C with reticulocytes which had been washed 3-4 times with phosphate-buffered saline. The cells were then pelleted, washed 4 times with isotonic saline, and resuspended to obtain a 10% suspension in isotonic saline. To the suspension at 0°C was added an equal volume of 0.2 M glycine, pH 2.3, in isotonic saline. After 5 minutes, the suspension was neutralized with phosphate-buffered saline pH 7.4, and immediately centrifuged at 12,000 X g for 10 min. The supernatant (antiserum II) was passed through a protein A-Sepharose-4B affinity column. The column was washed extensively with phosphate-buffered saline at pH 7.4 to remove unadsorbed materials. IgG bound to the protein A-Sepharose-4B column was then eluted with 0.1 M glycine-buffered saline at pH 3.0 (9). The IgG fraction eluted with glycine buffer represents a purified IgG fraction which agglutinates only reticulocytes. Rabbit antiserum against mature red blood cells was used directly after heat inactivation at 56°C without further purification. Rabbit anti-bovine albumin and goat anti-rabbit-γ-globulin antiserum were purchased from Grand Island Biologicals (New York, N.Y.).

3. Transferrin Purification

Transferrin was purified from sheep plasma as described (10,11). Briefly, to 75 ml of sheep plasma was added 25 ml of 1 M Tris HCl pH 7.4, 750 ug of ferrous ammonium sulfate and 10 μCi of ^{59}ferrous citrate. The mixture was incubated at 4°C overnight. The Fe treated plasma was applied to a Sephadex G-150 column (5 x 90 cm) which had been equilibrated

with 0.1 M Tris buffer, pH 7.4 and the column was eluted with the same buffer. The ^{59}Fe-containing fractions were collected, concentrated with an Amicon ultrafiltration apparatus and dialyzed against 0.05 M Tris HCl, pH 8.2 at 4°C for 48 hrs with 3 changes of buffer. After dialysis, the ^{59}Fe-containing sample was chromatographed on a DEAE-52 column using a linear buffer gradient from 0.05 M Tris HCl, to 0.5 M Tris HCl at pH 8.2. The ^{59}Fe-containing fractions were collected and dialyzed against phosphate-buffered saline pH 7.4 prior to use. Alternatively, before gel filtration through Sephadex G-150, the ^{59}Fe-labelled proteins of plasma which precipitated between 50-75% saturated ammonium sulfate were isolated and dialyzed against the buffer used for gel filtration. The remaining procedure was identical to that described above.

4. Purification of Membrane Protein with Transferrin and Immunoaffinity Columns

The transferrin and the immunoaffinity columns were prepared by coupling 2-5 mg of the respective proteins (purified IgG or transferrin) to 1 gm of CNBr activated Sepharose-4B (12). Reticulocyte or mature red cell membranes were prepared using the procedure described by Dodge et al. (13). The membrane proteins (1 mg/ml) were solubilized with a solution containing 1% Triton X-100, 10 mM Tris HCl, pH 7.4, and 1% Aprotonin (Sigma) using constant stirring at 0°C for 1 hr. The solution was diluted with phosphate-buffered saline (pH 7.4) to give 0.25% Triton then centrifuged either at 16,000 rpm for 2 hrs using a Sorvall SS-34 rotor or at 100,000 X g for 1 hr. The solubilized, diluted membrane protein solution was applied to the immunoaffinity column and the column was washed with 30 bed volumes

of phosphate-buffered saline containing 0.25% Triton (pH 7.4), followed by elution with 0.25% Triton X-100 in 0.1 M glycine-buffered saline at pH 2.3 (9).

To isolate the transferrin receptor with the transferrin affinity column, the diluted TX-100 solubilized membrane protein solution was adjusted to pH 5 as suggested by Ecarot-Charrier et al. (14) with 0.1 M sodium citrate-buffered saline containing 0.25% Triton X-100 and applied to the immobilized transferrin Sepharose-4B column. The column was washed extensively (30 bed volumes) with the same Triton-citrate-buffered-saline and then eluted with 0.1 M glycine buffered saline pH 2.3, containing 0.25% Triton X-100. The eluates from the immunoaffinity column or the transferrin column were neutralized and dialyzed at 4°C against distilled water for at least 24 hrs with 2 or 3 changes of water. The samples then were lyophilized and used for electrophoresis.

5. Electrophoresis

The Laemmli (15) method was used for electrophoresis. The lyophilized proteins were dissolved in 2% SDS, 10% glycerol, 10% mercaptoethanol, and 20 mM Tris HCl, pH 7.4 and heated 5 minutes at 100°C, prior to electrophoresis.

6. Immunoprecipitation of Transferrin

To 0.2 ml of antiserum I or anti-mature red blood cell antiserum was added 0.01 ml of Fe-transferrin (0.5 mg/ml) and incubated at 4°C for 24 hrs. Then 0.2 ml of goat antiserum against rabbit IgG was added and incubation continued for another 48 hrs at 4°C. The aggregates formed

were centrifuged down at 16,000 rpm in Sorvall SS-34 for 10 min and counted.

7. Immunofluorescence and Double Staining of Reticulocytes

Fluorescent antibody was prepared as described (16). For the double staining methods (methylene blue and FITC-antibody) 100 µl of a 1% reticulocyte suspension in phosphate-buffered saline at pH 7.4 was incubated with 10 µl of FITC-antibody (500 µg/ml) at 0°C for 90 minutes. The cells were washed twice with phosphate buffered saline pH 7.4. The cell pellet was resuspended with phosphate-buffered saline to give a 1% cell suspension and a drop of the suspension was placed on a polylysine coated glass coverslip. The cells were allowed to sediment under gravity for a few minutes and the excess fluid in the drop was removed carefully. A drop of new methylene blue solution was then added to the cover slip. After 5 minutes, the excess methylene blue solution was removed and the cover slip rinsed gently with phosphate buffer-saline at pH 7.4. The cover glass then was mounted on the slide. Fluorescence stained cells were viewed under a Zeiss microscope. Kodacolor 400 negative film was used for the photography.

8. Membrane Surface Iodination

The iodination of the outside surface of the intact cell and the cytoplasmic surface of the ghost were carried out as described (17,18).

9. Peptide Mapping

The method described (19) was used except that the electrophoresis buffer was (butanol : pyridine : acetic acid : water = 100 : 50 : 50 : 1,800) as used by Julien and Mushynski (Private Communication).

Materials

^{59}Ferrous citrate and Na ^{125}I were purchased from Frosst Company (Montreal, Canada); rabbit anti-bovine albumin and goat anti-rabbit globulin antiserum were purchased from Grand Island Biologicals (New York, U.S.A.); aprotinin (protease inhibitor), fluorescein isothiocyanate (isomer I) and polylysine were purchased from Sigma Company (St. Louis, U.S.A.); CNBR-Sepharose 4B, protein A-Sepharose 4B and Sephadex G-150 were purchased from Pharmacia (Sweden); and DEAE-52 was purchased from Whatman Company (England).

RESULTS

Preparation of an IgG Fraction Specific for the Reticulocyte Surface

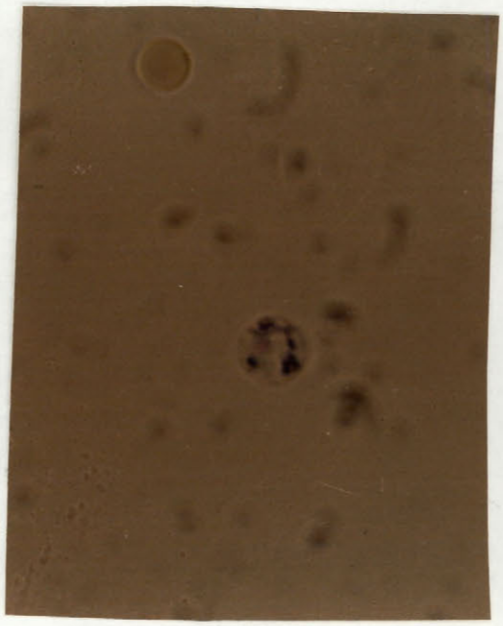
To verify that the protein A purified IgG fraction reacts with reticulocyte and not mature red cell membranes, two experimental tests were applied, (1) a double staining technique and (2) an affinity column technique.

(1) Using the double label procedure (FITC-antibody and methylene blue staining) the data in Figure 1 show that cells stainable with methylene blue also bind FITC-labeled purified IgG. Cells devoid of reticulum do not show fluorescence. Moreover, if fluorescein-labelled rabbit anti-bovine albumin antibody is used, no fluorescence is seen with either mature cells or reticulocytes. These data suggest that only reticulocytes react with the purified IgG.

(2) An immunoaffinity column prepared from the purified IgG fraction retains reticulocyte plasma membrane proteins but not mature cell components. The data in Fig. 2 show that three molecular weight species from a TX-100 extract of reticulocyte plasma membranes are retained by the immobilized IgG column. In contrast, no detectable retention of membrane proteins is observed with similar extracts from the mature cell membranes. To test the specificity of the protein retention, an immunoaffinity column of rabbit anti-bovine albumin was used in place of the purified IgG. No detectable proteins from the reticulocyte membranes were retained by the anti-bovine albumin affinity column (not shown). Thus, the data show that a specific IgG fraction against reticulocyte membrane proteins can be obtained by immunizing

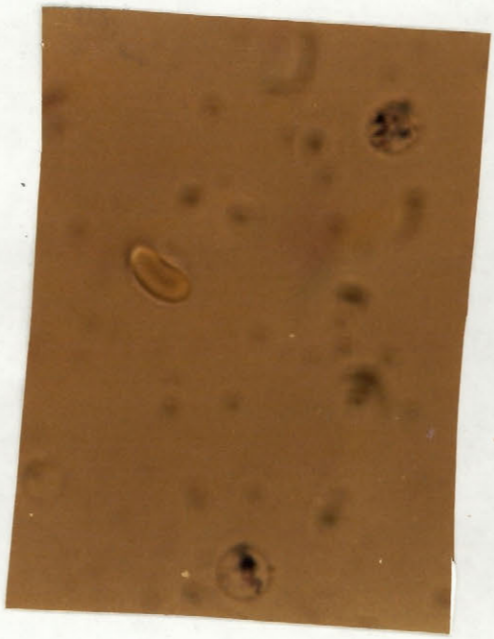
Fig.3-1.

a.



b.

c.



d.

Fig.3-1. Binding of FITC-IgG to the reticulocyte

A mixture of reticulocytes and erythrocytes were labelled with antibody followed by staining with methylene blue. Fluorescence is only present in the methylene blue stainable reticulocyte when FITC-purified IgG is used. Fluorescence micrographs (a) staining with FITC-purified IgG (b) staining with FITC-anti-bovine albumin antibody; c and d correspond to a and b respectively under visible light illumination. (Magnified X 1,000). The purified IgG used for FITC labelling was prepared as described in Methods prior to attachment to FITC.

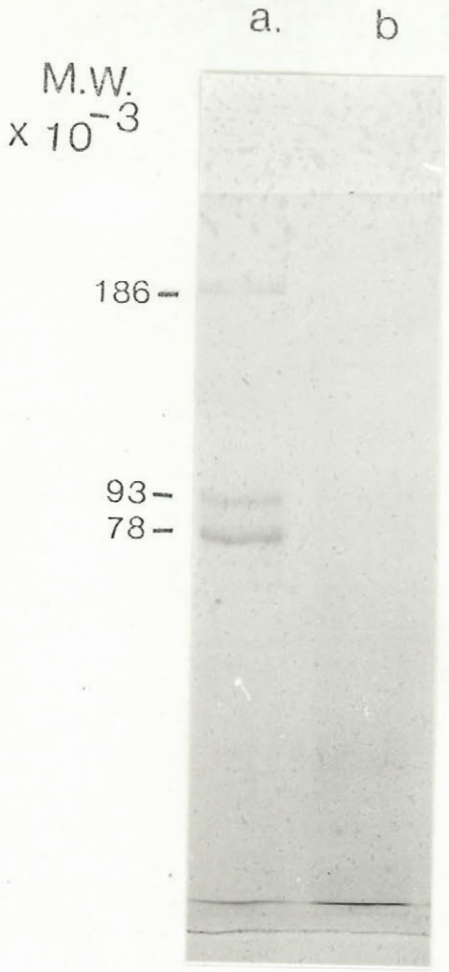


Fig. 3-2. Reticulocyte membrane proteins isolated by the purified IgG-Sepharose-4B Column. Solubilized membrane proteins from mature RBC or from reticulocytes were applied to the column. After washing with 0.25% Triton X-100 in phosphate buffered saline, pH 7.4, the column was eluted with glycine buffer pH 2.3 as described in Methods. The eluates were dialyzed, lyophilized and redissolved in 2% SDS, 10% mercaptoethanol, followed by electrophoresis in 6% SDS-polyacrylamide gel. Staining was with Coomassie blue. (a) Solubilized reticulocyte membrane proteins; (b) solubilized mature red cell membrane proteins.

rabbits with intact sheep reticulocytes. Rabbit antiserum with the properties described above has been obtained from over a dozen different rabbits.

Identification of the Membrane Proteins Retained by the Immobilized IgG Column

The peptides isolated by the immunoaffinity column have molecular weights of about 186K, 93K and 78K (Fig.2). It is known that the reticulocyte surface contains transferrin receptors in high concentration (20,21,22). The 93K and 186K molecular weight species of the peptides retained by the purified IgG-Sepharose-4B column are similar to those reported for the transferrin receptor in other systems (14,22-29). The 78K species corresponds to the molecular weights reported for transferrin (30) from various sources. The comparable molecular weights suggest that the peptides isolated by the immunoaffinity technique may be the transferrin receptor and transferrin. To determine whether such is the case several experiments were carried out:

(i) ^{125}I-Iodotyrosyl Peptide Mapping

After SDS gel electrophoresis of the peptides retained by the immunoaffinity absorbent, the three peptides were cut from the gel and subjected to ^{125}I-iodotyrosyl peptide mapping. The results, shown in Fig. 3a, b, indicate that the 186K and 93K species have very similar peptide maps, suggesting that the 186K species is a dimer of the 93K component. The 78K is entirely different. It appears, however, that the 78K peptide has a map similar to sheep transferrin (Fig. 3c, d) thus identifying it as transferrin itself.

Fig.3-3

a.

b.

c.

d.

Fig. 3-3. ^{125}I-tyrosyl Peptide Mapping. The areas corresponding to 186K, 93K,78K and sheep transferrin were cut from Coomassie blue stained gels and were iodinated with Na^{125}I. The iodinated peptides were treated with trypsin, the digests eluted from the gels, and electrophoresed and chromatographed on thin layer cellulose plates using Polygram CEL 300 (Macherig-Nagel Co., Germany). Radioautography on Kodak XR 1 film was used to visualize the radiolabelled areas. (a) 186K, (b) 93K, (c) 78K, (d) sheep transferrin.

(ii) Studies with Immunoaffinity and Transferrin Columns

Transferrin was coupled to Sepharose-4B and the solubilized reticulocyte membrane proteins were passed through the transferrin affinity column. The peptides isolated after passage through a transferrin affinity column are shown in Figure 4a. It may be seen that the same protein profile is obtained as that with immunoaffinity column (Fig.4b). In conjunction with the data on the peptide maps, the data suggest that both transferrin and the receptor are isolated by the purified IgG-Sepharose-4B columns.

(iii) Verification that Immobilized Transferrin and the Immunoaffinity Procedure Isolate the Same Peptides

If the two affinity procedures retain the same peptides, prepassage of the solubilized membrane proteins through one column should remove all protein species capable of binding to the other column. The data in Figure 4 (c and d) verify this prediction. No detectable membrane proteins are retained by the transferrin column after prepassage through the immunoaffinity column and vice versa. These observations confirm that the proteins retained by the immunoaffinity column are identical to those retained by the transferrin column and that the proteins retained are transferrin and the transferrin receptor.

Identification of the IgG Purified by Reticulocyte Absorption as Anti-Transferrin Receptor Antibody

Since transferrin has a high affinity for its receptor (29), coisolation of transferrin and transferrin receptor by the purified IgG immunoaffinity column could result from anti-transferrin or

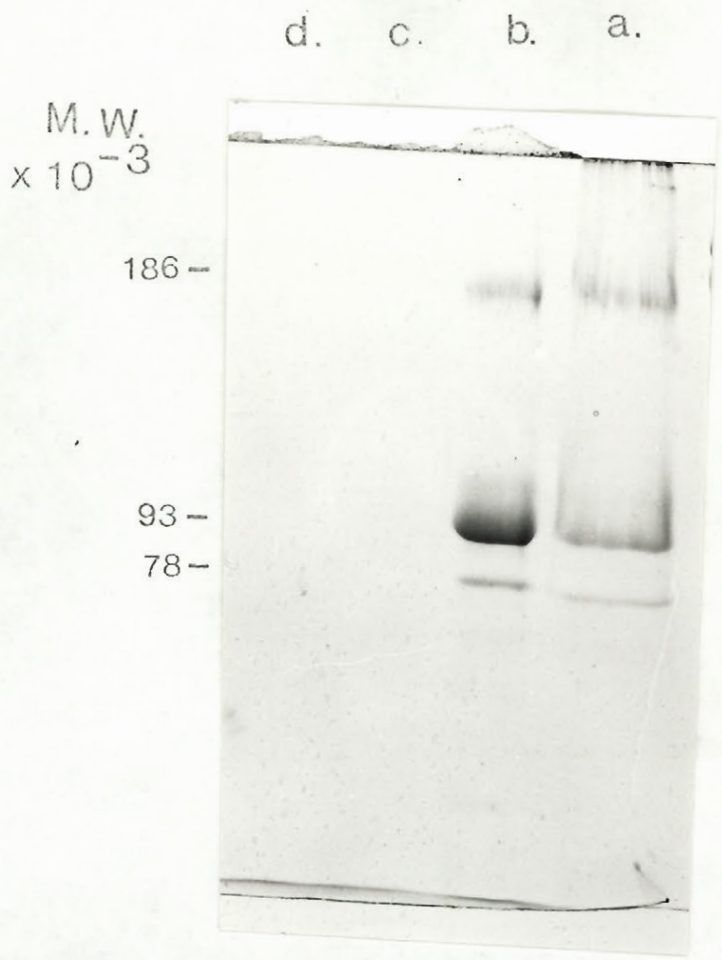


Fig. 3-4. Identity of Reticulocyte Membrane Proteins Isolated by the Purified IgG-Sepharose-4B and Transferrin Sepharose-4B Columns.

(a-b) Solubilized membrane proteins each from equivalent numbers of reticulocytes were applied to both the purified-IgG-Sepharose-4B and transferrin Sepharose-4B columns. The proteins retained were eluted with glycine buffer and electrophoresed on SDS-polyacrylamide gel followed by staining with Coomassie blue. (a) Eluates from the purified IgG immunoaffinity column; (b) eluates from the transferrin affinity column. (c) Same as (a) except the solubilized reticulocyte membrane proteins were passed through the transferrin column before being applied to the purified IgG column. (d) Same as (b) except the solubilized reticulocyte membrane proteins were passed through the purified IgG column before being applied to the transferrin column.

anti-transferrin receptor antibody or both.

To determine if the antibody isolated is directed against transferrin or its receptor two experiments were carried out:

(a) Studies with ^{59}Fe-Transferrin: ^{59}Fe-transferrin was incubated with antiserum I or rabbit antiserum against the mature red cell, followed by the addition of goat antiserum against rabbit IgG. The immunoprecipitate formed was isolated and counted. The data in Table 1 show that the anti--reticulocyte antiserum (antiserum I) and the anti-mature cell antiserum precipitate equal and small amounts (2%) of ^{59}Fe-transferrin. Hence, the antibody against reticulocytes does not react preferentially with transferrin suggesting that the antireticulocyte antibody is not directed against transferrin itself.

(b) Studies with Immunoaffinity Columns. If the IgG fraction contains antibodies against transferrin itself, transferrin should be retained by the immunoaffinity column. Moreover, purified IgG should be retained by the immobilized transferrin column. The results show (Fig. 5b and e) that transferrin is not retained by the immunoaffinity column nor is the IgG fraction retained by the transferrin column. These data show that the IgG fraction purified as described is not directed against transferrin itself and support the conclusion that the antibody obtained is directed against the transferrin receptor.

Lack of Identity of the Transferrin Binding Sites and the Antibody Binding Sites

The data above showed that the transferrin column does not retain the purified IgG fraction (and vice versa). However, if detergent

TABLE 3-1

ABSENCE OF SPECIFIC ANTIBODIES REACTING WITH TRANSFERRIN IN THE ANTISERUM AGAINST RETICULOCYTE

	cpm
Added transferrin (^{59}Fe)	4000
Transferrin precipitated with Antiserum I	99
Transferrin precipitated with anti-mature cell serum	55

To 0.2 ml of antiserum I or anti-mature red cell antiserum was added 0.01 ml of ^{59}Fe transferrin (0.5 mg/ml) and the mixture was incubated at 4°C for 24 hrs. Then 0.2 ml of goat anti-rabbit-γ-globulin antiserum was added and incubation continued for another 48 hrs at 4°C. The aggregates formed were centrifuged down at 16,000 rpm for 10 min and the pellets counted using a Packard γ-counter.

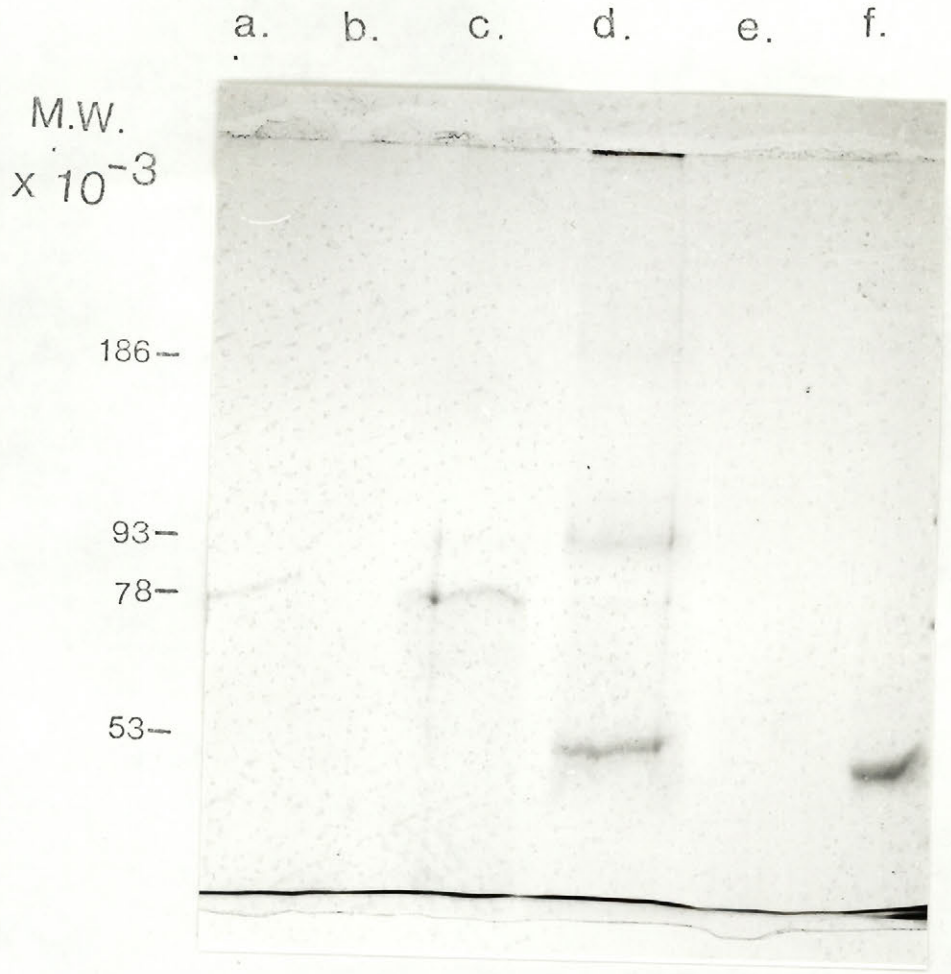


Fig. 3-5.

Fig.3-5 The Purified IgG is not Anti-Transferrin Antibody. 0.1 ml of transferrin (~1 mg/ml) and purified IgG(~1 mg/ml) were passed through the purified IgG immunoaffinity and transferrin affinity columns respectively with and without the presence of detergent extracts of the reticulocyte membrane. The pH 2.3 glycine eluates were electrophoresed in 6% SDS polyacrylamide gels followed by staining with coomassie brilliant blue. (a) Transferrin marker; (b) eluate from an IgG column following passage of transferrin; (c) as in (b) except a detergent extract of reticulocyte membranes was prepassed through the column; (d) eluate from a transferrin column through which a detergent extract of membranes had been prepassed followed by IgG; (e) as in (d) above without the membrane extract; and (f) IgG standard.

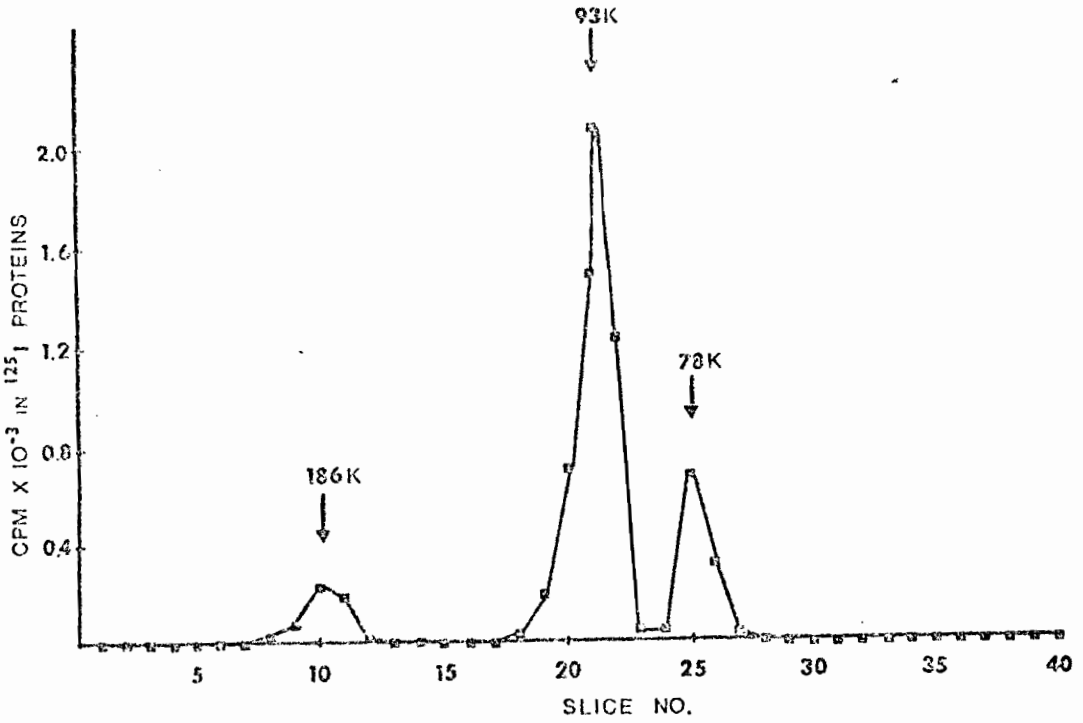
extracts of reticulocyte membranes are first applied to the transferrin column, the purified IgG can be retained by the column together with transferrin and transferrin receptor. The reciprocal experiment with the immunoaffinity column shows that transferrin can be retained by the column only when extracts of the reticulocyte membrane proteins have been prepassed through the column (Fig. 5c and d). These results suggest that the binding sites for the antibody are distinct from those for transferrin. Recently, Sutherland et al. (26) showed that in human leukemic cells, transferrin is bound to an immobilized monoclonal antibody against the transferrin receptor only in the presence of membrane extracts and that 100-fold excess of transferrin does not affect binding of the receptor to the immunoaffinity column. The present data are consistent with their observations and with the conclusion that the antibody binding sites are distinct from the transferrin binding sites on the receptor.

Transmembrane Orientation of the Putative Transferrin Receptor

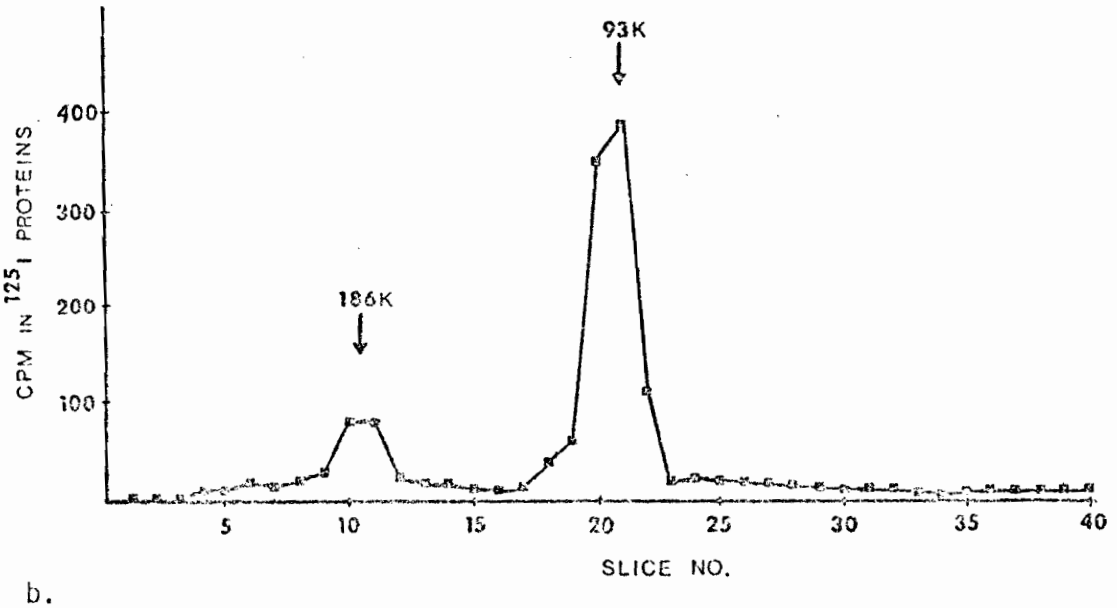
To determine whether the putative receptor spans the membrane or is accessible only at the external surface, techniques were applied that have been used to determine the disposition of other red cell membrane proteins. Inner and outer membrane surfaces were iodinated with lactoperoxidase following the procedures described previously (17,18) for the external surface of the intact reticulocyte and the internal surface of resealed ghosts prepared from reticulocytes.

The labelled membranes were extracted with TX-100 and the solubilized material chromatographed on an immunoaffinity column. The eluate obtained with glycine buffer was electrophoresed in

cylindrical 6% SDS-polyacrylamide gels, and 0.2 cm slices of the gel were counted. Labelled peptides corresponding to molecular weights of 186K, 93K and 78K were obtained from the membranes derived from externally iodinated cells (Fig.6a). Peptides of 93K plus a weakly labelled species of 186K were obtained with membranes from ghosts labelled at the cytosolic surface (Fig. 6b). The fact that the 186K and 93K peptides retained by the immunoaffinity column can be labelled from both surfaces suggests a transmembrane orientation for the transferrin receptor. The relatively weak labelling at the cytosolic surface may be due to the fact that more membrane proteins are accessible for labelling at the cytosolic surface than at the external surface and thus ^{125}I may become limiting. Alternatively, the transferrin receptor may have fewer tyrosines accessible to iodination at the cytosolic surface. It is noteworthy that the 78K peptide (transferrin) is not measurably labelled by iodination of the internal surface. This observation supports the conclusion that the receptor spans the membrane since externally bound transferrin is unlikely to be accessible for iodination at the internal surface.


93K
78K
186K
CPM X 10^{-3} IN ^{125}I PROTEINS
2.0
1.6
1.2
0.8
0.4
5
10
15
20
25
30
35
40
SLICE NO.

a.


93K
186K
CPM IN ^{125}I PROTEINS
400
300
200
100
5
10
15
20
25
30
35
40
SLICE NO.

b.

Fig.3-6.

Fig. 36. Accessibility of the Transferrin Receptor at the External and Cytoplasmic Surface to Lactoperoxidase Catalyzed Iodination.

Reticulocytes were labelled with $Na^{125}I$ and lactoperoxidase to tag the external surface membrane proteins. The cytoplasmic surface membrane proteins were labelled with lactoperoxidase incorporated into resealed ghosts. Membranes were solubilized and passed through the anti-transferrin receptor immunoaffinity column. The proteins retained were eluted and electrophoresed in 6% SDS-polyacrylamide disc gel. Gels were sliced into 0.2 cm and counted. (a) External surface iodination; (b) cytoplasmic surface iodination.

DISCUSSION

Using a relatively simple procedure to isolate reticulocyte-specific antibodies, antibodies have been used to probe the nature of the reticulocyte specific membrane proteins and to identify them as the transferrin receptor and transferrin.

A comparison of the membrane proteins retained from reticulocyte membranes by the immunoaffinity column and the immobilized transferrin column shows the proteins are identical. This conclusion is based on the observations that the molecular weights of the components retained are the same and prepassage of the membrane extracts through one column removes all material capable of binding to the other column.

Since the two molecular weight species isolated (186K and 93K) have identical peptide maps and the molecular weights of the peptides have a simple arithmetic relationship suggest that the 186K peptide is a dimer of the 93K species. The molecular weights and structure of the receptor in situ have not yet been established although several investigators have concluded that it is maybe dimeric (14,22,24,25,26,29) with a molecular weight in the range reported here. If the 186K peptide is indeed a dimer of the 93K component, the data would suggest that even heating at 100°C with reducing agents may be insufficient to disaggregate the receptor completely.

Peptide mapping and molecular weight estimation of the third component (78K) suggest that it is transferrin itself. That transferrin is co-isolated with its receptor is not surprising since transferrin is tightly bound to the receptor (29). Control experiments show that the antibody produced is not directed against transferrin.

That transferrin is co-isolated with the immunoaffinity column as well as the transferrin column suggests that (1) the sites for antibody binding and transferrin binding are different and (2) that the receptor has more than one site for transferrin binding. Similar conclusions have been reached by others. Sutherland et al. (26) have presented experimental evidence that the binding sites for antibody and transferrin are different in human leukemic cells. Enns and Sussman (29) have concluded that there are two binding sites for transferrin on the receptor in human placenta. These observations are in accord with our conclusions on the sheep reticulocyte system.

Using the antibody against reticulocytes our earlier reports (2,31) and the accompanying report show that the membrane components, now identified as transferrin and the transferrin receptor, disappear during maturation of reticulocytes in vitro. Therefore, the disappearance of the transferrin receptor can be followed in vitro as has already been shown for the amino acid transport systems (7,8). The mechanisms whereby membrane components disappear during maturation of the reticulocyte are not yet established.

In conclusion, it is significant to note that the transferrin receptor has recently been recognized by identifying it as the antigen to a monoclonal antibody made against conspicuous cell surface glycoproteins (25,26). In the present communication the transferrin receptor was identified as the antigenic component when rabbit antibodies are produced against sheep reticulocytes. To our knowledge this is the first report that the major surface antigenic marker characteristic of the sheep reticulocyte is the receptor for transferrin. All these data are consistent

with the conclusion that the receptor is prominent on surfaces of growing cells and highly antigenic in heterologous species. In the absence of evidence to the contrary, it is tempting to speculate that if there is polymorphism of the receptor in a given species, the transferrin receptor may be a member of a class of histocompatability antigens expressed on cell surfaces.

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Sheep reticulocyte surface specific antigens (mainly, transferrin receptors) disappear during maturation. In order to study the possible mechanism for the disappearance, the purified anti-transferrin receptor antibodies were used as probes.

CHAPTER 4

IN VITRO REDISTRIBUTION AND EXTERNALIZATION OF ANTI-TRANSFERRIN RECEPTOR ANTIBODY-TRANSFERRIN RECEPTOR COMPLEX IN SHEEP RETICULOCYTES

ABSTRACT

A class of anti-sheep reticulocyte surface specific antibodies has been isolated and identified as anti-transferrin receptor antibodies. Studies of the behaviour of transferrin receptor in sheep reticulocytes are reported here which involve the use of FITC- and ^{125}I-labelled anti-transferrin receptor antibodies. The FITC-antibody was bound to sheep reticulocytes at 0°C. After warming to 37°C, the FITC-antibody-receptor complex started to redistribute from an original diffuse state into clusters, patches, and eventually into caps (usually bipolar or tripolar) on the cell surface. The redistribution was followed by externalization of the antibody-receptor complex as shown by FITC-and ^{125}I-labelled antibodies. Capping did not necessarily precede externalization. Both the redistribution and externalization were shown to require metabolic activity.

The externalized vesicles have been isolated by Sepharose 6B gel filtration or with protein A affinity column. Under the electron microscope, the vesicles had uniform size and knob - like structures on their surface which presumably were antibodies. After electrophoresis on SDS-polyacrylamide gel, specific peptides appeared which coelectrophoresed with the transferrin receptor (molecular weight of monomer 93K), transferrin (78K), and antibody. The relative absence of other plasma membrane proteins derived from sheep reticulocytes indicated that the antibody-receptor complex was selectively externalized.

The kinetics of the externalization of antibody-receptor complexes were studied by using ^{125}I-antibody. The half-time for the

externalization was about 6 hours under the experimental conditions.

INTRODUCTION

It is known that antibody to the surface receptor of lymphoid cells can induce capping and internalization of the surface receptor (for review see Schreiner and Unanne. 1976; Singer et al., 1978). Studies of this type have been conducted with antibodies to the surface immunoglobulin (Ig) of B lymphocytes (Taylor et al., 1971), and antibodies to the surface immunoglobulin M (IgM) of cultured B lymphblastoid cells (Salisbury, Condeelis, and Satir, 1980). Prior to antibody binding, the surface Ig and IgM are diffusely distributed on the cell surface. Subsequent to antibody binding and at 37°C, the antibody-receptor complex starts to redistribute into clusters, patches, and finally cap is formed at one pole of the cell. The antibody - receptor complex is internalized to form endocytic vesicles during and after cap formation(Taylor et al., 1971; Linthicum and Sell, 1974; Ault, Karnovsky and Unanue, 1973; Salisbury, Condeelis and Satir, 1980). Ultimately, the vesicles are directed to the Golgi region and presumably fuse with the lysosomes where the antibodies become degraded (Ault, Karnovsky, and Unanue, 1973; Engers and Unanue, 1973).

Complementary to the phenomenon of antibody induced redistribution and internalization is antibody induced redistribution and externalization. That antibodies to surface antigens can induce redistribution and externalization (shedding) of the surface antigen has been shown with many tumor cells (Leonard, 1973; Leong et al., 1979; Nordquist, Anglin and Lerner, 1977; Calafat et al ., 1976). It has been proposed that the externalized antibody- antigen complex may compete with the tumor cell for processing by the immune system, thereby allowing the tumor cell to escape destruction (Alexander, 1974;Nordquist, Anglin and Lerner, 1977).

With non malignant cells, the evidence for antibody induced redistribution and externalization is less well established. For example, it has been proposed that with lymphocytes, the anti-histocompatibility antibody may induce the redistribution and externalization (shedding) of the histocompatibility antigen (Miyajima, Hirata and Terasaki, 1972; Cullen et al.,1973). In the present work, evidence is presented that redistribution and externalization of the transferrin receptor of sheep reticulocytes can be observed in the presence of polyclonal antibodies directed against the transferrin receptor.

The transferrin receptor is known to play a central role in the transport of iron from the iron-transferrin complex into the cell (for review see Morgan, 1981). It has a ubiquitous distribution on growing animal cells (Trowbridge and Omary, 1981; Sutherland et al., 1981; Hamilton, Wada and Sussman, 1979; Larrick and Gresswell, 1979; Galbraith et al., 1980), owing presumably to the iron requirement for proliferation. The transferrin receptor is abundant on reticulocytes, the enucleated erythroid cell which still retains the ability to synthesize hemoglobin and therefore requires iron (Lowenstein, 1959). The transferrin receptor concentration gradually decreases and eventually disappears during reticulocyte maturation (Van Bockxmeer and Morgan, 1979; Pan, Blostein and Johnstone, 1982, submitted; Frazier et al., 1982).

We have previously purified a class of antibodies against the reticulocyte plasma membrane and identified them mainly as anti-transferrin receptor antibodies (Pan, Blostein and Johnstone, 1982, submitted). Here evidence is presented that the anti-transferrin receptor antibody can induce the transferrin receptor to cluster, patch and/or cap on the reticulocyte surface. Moreover, vesicles containing the antibody-receptor

complex are externalized into the medium.

The reticulocyte is a simple cell and, like the erythrocyte, has lost most intracellular organelles. The mechanism for redistribution and externalization in reticulocytes may therefore occur via a route which is not dependent on these organelles. Since the reticulocyte synthesizes few new membrane proteins, (Lodish and Small, 1975) the present system may provide a relatively simple system to study the molecular events involved in modification during reticulocyte maturation.

MATERIALS AND METHODS

Isolation of Sheep Reticulocytes, Plasma Transferrin and Transferrin Receptor

Reticulocyte production in sheep was induced by phlebotomy as described (Benderoff, Blostein and Johnstone, 1978; Benderoff, Johnstone and Blostein, 1978). For the isolation of reticulocytes, a modified differential centrifugation method was used (Benderoff, Blostein and Johnstone, 1978: Pan, Blostein and Johnstone, 1982). Plasma transferrin was isolated by methods described by Morgan et al. (Morgan,1964; Morgan, Huebers and Finch, 1978), with the modification that the transferrin was first fractionated in 50% - 75% saturated ammonium sulfate before gel filtration.

For isolation of the transferrin receptor either a transferrin affinity column or an immunoaffinity column using the purified anti-transferrin receptor antibody (see later) coupled to CNBR Sepharose 4B (Axen, Porath and Ernback, 1967) was used. The reticulocyte membrane was isolated as described by Dodge et al. (Dodge, Mitchell and Hanahan, 1968) and dissolved in 1% Triton X-100, 10mM Tris HCl, pH 7.4 and 1% aprotronin (Sigma). The solution was diluted with phosphate buffered saline (pH 7.4) to give 0.25% Triton X-100, and centrifuged at 100K x g for 1 hour.

Transferrin receptor was then isolated from the supernatant fraction by either of the affinity column procedures. Following loading of the immunoaffinity column, the column was washed with 30 bed volumes of phosphate buffered saline containing 0.25% Triton X-100 pH 7.4 and the bound protein fraction eluted with 0.1 M glycine buffer pH 2.3.

Purification using the transferrin affinity column was accomplished by initially adjusting the pH of the solubilized supernatant fraction to 5 in order to stabilize the transferrin-receptor complex (Ecarot -Charrier et al., 1980). The material was then applied to the affinity column and, following a washing step in 0.1 M citrate buffered saline (pH 5.0), retained protein was eluted with 0.1 M glycine buffer (pH 2.3). The eluates were collected, neutralized, dialyzed, lyophilized and subjected to electrophoresis.

Antibody Preparation

Anti-transferrin receptor antibody was isolated as previously described (Pan, Blostein and Johnstone, 1982). Briefly, rabbits were immunized with sheep reticulocytes and the antiserum obtained exhaustively absorbed with sheep erythrocytes. The remaining antiserum was then absorbed with sheep reticulocytes. The reticulocytes were washed several times with phosphate buffered saline (pH 7.4). The cells were incubated with 0.1 M glycine buffered saline pH 2.3 at 0°C for 10 minutes and neutralized with phosphate buffered saline (pH 7.4). After neutralization the cells were immediately centrifuged at 16,000 rpm for 10 minutes using a Sorvall SS-34 rotor. The supernatant obtained was applied to a protein A affinity column. The column was washed with phosphate buffered saline pH 7.4 at room temperature, eluted with 0.1 M glycine buffered saline pH 3.0 (Eye, Prowse and Jenkin, 1978) and the eluate was neutralized. The antibodies obtained, which are directed against sheep reticulocyte surface specific antigens, have been identified mainly as anti-transferrin receptor antibodies (Pan, Blostein and Johnstone, 1982).

Iodination of the Purified Antibodies

Iodination of the purified antibodies was carried out with chloramine T (Hunter and Greenwood, 1962). To 1 mg of antibody in 0.2 ml of 0.5 M phosphate buffer pH 7.4 was added 1 mCi of $Na^{125}I$ and 1 μg of KI each in 25 μl. To initiate the reaction, 50 μl of chloramine T (10 mg/ml) was added and the mixture was stirred at room temperature for 1 minute. The mixture then was transferred to 0°C and 250 μl of sodium metabisulfite (10 mg/ml) and 10 μl of KI (10mg/ml) were added. The iodinated protein was separated from free ^{125}I by filtration on Sephadex G-25.

Iodinated Antibody binding and Separation

^{125}I-antibody binding was carried out by incubation of a 2% suspension of reticulocytes in phosphate buffered saline pH 7.4 with ^{125}I-antibody ($\sim$0.25-$\sim$1μg/ml, specific activity $\sim$1.5 -$\sim$4.5 x 10^5 cpm/μg) at 0°C for 90 minutes. The separation of ^{125}I-antibody labelled reticulocytes from the free ^{125}I-antibody was achieved by filtration of the sample through a Sepharose 6B column (1.5 x 7 cm) at 4°C. The cells were eluted in the void volume and were well separated from free ^{125}I-antibody.

Determination of ^{125}I-Materials Released into the Culture Medium

^{125}I-antibody labelled reticulocytes were obtained as described above and resuspended to give a 1% cell suspension in ice cold culture medium supplemented with 2% fetal calf serum, flushed with 95% O_2 and 5% CO_2 and incubated at 37°C. At various intervals 0.4 ml samples was taken and centrifuged at 12,000 xg for 1 minute in an Eppendorff centrifuge. The supernatants and the pellets were counted. To determine if the supernatants contained degraded protein, 40 ul of

an albumin solution (1 mg/ml) was added to the supernatants followed by 60 µl of 40% TCA. The TCA treated solution was centrifuged and both the supernatant and pellet were counted. To identify and isolate the radioactivity released into the culture medium, cell free supernatants (20-50 ml) from 6 hour cultures were applied to a 2.5 ml column of protein A Sepharose 4B (Pharmacia). The affinity column was washed with phosphate buffered saline pH 7.4 until no further radioactivity was eluted with the buffered saline (3-4 bed volumes). The elution medium was then changed to 0.1 M glycine buffered saline pH 3.0. The eluate was collected, neutralized with phosphate buffer and counted. About 60-80% of the radioactivity applied was retained by the column. For electrophoresis, the eluate was dialyzed against H_2O for 24 hours with 2 to 3 changes, lyophilized, dissolved and then subjected to SDS-polyacrylamide gel electrophoresis(PAGE). For the negative staining, the eluate was first pelleted at 100K x g for 1 hour .

Immunofluoresence Technique

FITC-antibody was prepared as previously described (The and Felkemp, 1970). To label the reticulocytes, a 2% suspension of reticulocytes in phosphate buffered saline (pH 7.4) was incubated with about 5 ug/ml of FITC-antibody at 0°C for 90 minutes. This suspension was centrifuged and washed twice at 4°C with phosphate buffered saline (pH 7.4) to remove free FITC-antibody. The washed FITC-antibody labelled reticulocytes were resuspended to 1% cell suspension with ice cold culture medium supplemented with 2% fetal calf serum, flushed with an air containing 95% O_2 and 5% CO_2, and incubated at 37°C. Samples taken

before transferring to 37°C and during incubation at 37°C were mounted on slides and examined under a Zeiss fluorescence microscope. A control suspension was fixed before treatment with FITC-antibody. These cells were fixed with 2% formaldehyde at room temperature for 5 minutes, centrifuged, washed several times with 0.1 M glycine buffered saline (pH 8.0) and stained with FITC-antibody at 37°C for 30 minutes. The distribution of immunofluorescence on the prefixed reticulocytes was uniform and similar to that of FITC-antibody labelled reticulocytes which were maintained at 0°C. The film used for photography was Kodacolor ASA 400 and the exposure time was 5 minutes.

Electron Microscopic Studies

(a) Negative Staining. The cell free supernatant derived from incubation of reticulocytes labelled with ^{125}I-antibody or FITC-antibody was centrifuged at 100K x g for 1 hour. The pellet obtained was stained with 1% ammonium molybdate and examined under a Philips model 300 electron microscope. In experiment where the cell free supernatant was passed through a protein A affinity column, the acid eluate from the protein A column was immediately neutralized and then treated as above.

(b) Thin section. 2% reticulocytes in phosphate buffered saline pH 7.4 were incubated with purified anti-transferrin receptor antibody ($\sim$ 25 μg/ml) at 0°C for 90 minutes. The sample was filtered through Sepharose 6B column to remove the free antibody and the cells were resuspended to 1% cell suspension with ice cold culture medium containing 2% fetal calf serum. An aliquot was taken from the cell suspension. The cell suspension was then flushed with 95% O_2 and 5% CO_2 and transferred to

37°C. After 30 minutes, another aliquot was taken. Both aliquots were centrifuged in an Eppendorff centrifuge for 1 minute immediately after withdrawing from the cell suspension. To the pellets 1% glutaraldehyde in phosphate buffered saline (pH 7.4) was added, resuspended and centrifuged after 3 minutes at room temperature. The pellets were washed several time with 0.1 M glycine buffered saline (pH 8.0). After washing and centrifugation, the pellets were resuspended into 2% suspension with phosphate buffered saline pH 7.4 and incubated with ferritin-labelled protein A (~50 µg/ml) at 37°C for 30 minutes. The free ferritin-labelled protein A was removed by filtration through Sepharose 6B column. The cells were collected and centrifuged. The pellet was resuspended with 2.5% glutaraldehyde in phosphate buffered saline (pH 7.4). This suspension was left for 5 minutes at room temperature, centrifuged in an Eppendorff centrifuge for 5 minutes and then left at 4°C. After 1 hour the fixative was removed and replaced with 0.1 M phosphate buffer pH 7.4 containing 6% sucrose. The pellets were postfixed for 1 hours in 1% osmium tetroxide in Palade buffer; dehydrated; and embedded in Epon 812. Thin sections were prepared, stained with Reynolds' lead and uranyl acetate and examined under electron microscope. Ferritin-protein A was prepared as described (Templeton, Douglas and Vail, 1978).

Kinetic studies for externalization

To study the kinetics of ^{125}I-antibody release from ^{125}I-labelled reticulocytes, ^{125}I-antibody (~0.4 µg/ml, specific activity ~4.5×10^5 cpm/µg)

was bound to reticulocytes at 0°C for 90 minutes and the labelled reticulocytes were resuspended to give a 1% cell suspension in culture medium containing 2% fetal calf serum and incubated at 37°C. At intervals, 0.4 ml samples were taken and centrifuged at 12,000 x g for 1 minute. The cell free supernatants then were passed through a Sepharose 6B column (1.5x7 cm), eluted with phosphate buffered saline pH 7.4; 20 drop fractions were collected and counted. The radioactivity associated with the void volume was considered vesicle bound. At a particular interval, the radioactivity associated with the released vesicle is expressed as a percentage of the total radioactivity in the vesicle and cell of that time interval.

To study the metabolic requirements for externalization phosphate buffered saline pH 7.4 or serum free culture medium was used to replace the serum containing culture medium.

ATP and K^+ Determination

ATP was determined using the luciferin luciferase procedure (Stanley and Williams, 1969). Cellular K^+ was analyzed by flame photometry using an internal Li^+ standard.

Protein Determination

The Lowry method (Lowry et al., 1959) was used.

Electrophoresis

The Laemmli system (Laemmli, 1970) was used.

Materials

$Na^{125}I$ was purchased from Frosst Company (Montreal, Canada);

Aprotinin (protease inhibitor), ferritin, fluorescein isothiocyanate (isomer I), and firefly luciferase were purchased from Sigma (St. Louis, U.S.A.); CNBR-Sepharose 4B, protein A, Protein A-Sepharose 4B, Sephadex G-25, G-150 and Sepharose 6B were purchased from Pharmacia (Sweden); cellulose nitrate tube was purchased from Beckman (U.S.A.); culture medium and fetal calf serum were purchased from Grand Island Biologicals (New York, U.S.A.).

RESULTS

Time Course of ^{125}I-Antibody Binding to Sheep Reticulocytes and Erythrocytes

A 2% suspension of reticulocytes or mature red cells was incubated with ^{125}I-anti-transferrin receptor antibodies ($\sim$0.25 µg/ml) at 0°C. At the intervals given, 0.4 ml samples were chromatographed through Sepharose 6B columns and fractions of twenty drops each were collected and counted for ^{125}I. A typical result after incubation of reticulocytes with antibodies is given in Fig. 1a. Two radioactive peaks were resolved. The first peak was associated with cell fractions and the second peak corresponded to free ^{125}I- antibodies. The radioactivity associated with the cells was deemed cell bound. This result shows that Sepharose 6B filtration provides an excellent separation of free and cell associated ^{125}I-antibodies. The time course of ^{125}I-antibody binding to the cells is given in Fig. 1b. It may be seen that the binding reached a steady state after 60 minute incubation at 0°C. At the concentration used, about 35% of the total ^{125}I-antibodies added were cell-associated at steady state. Non-specific binding is small as shown by the fact that little cell associated radioactivity is obtained with erythrocytes. This observation is consistent with the fact that erythrocytes do not have (or have few) surface receptors for transferrin.

Release of ^{125}I-labelled Protein into the Culture Medium

To follow the fate of the cell bound ^{125}I-antibody, the ^{125}I-antibody was bound to reticulocytes at 0°C, the cells were filtered, resuspended in fresh culture medium to give a 1% cell suspension and

Separation of Cell Associated IgG from Free IgG

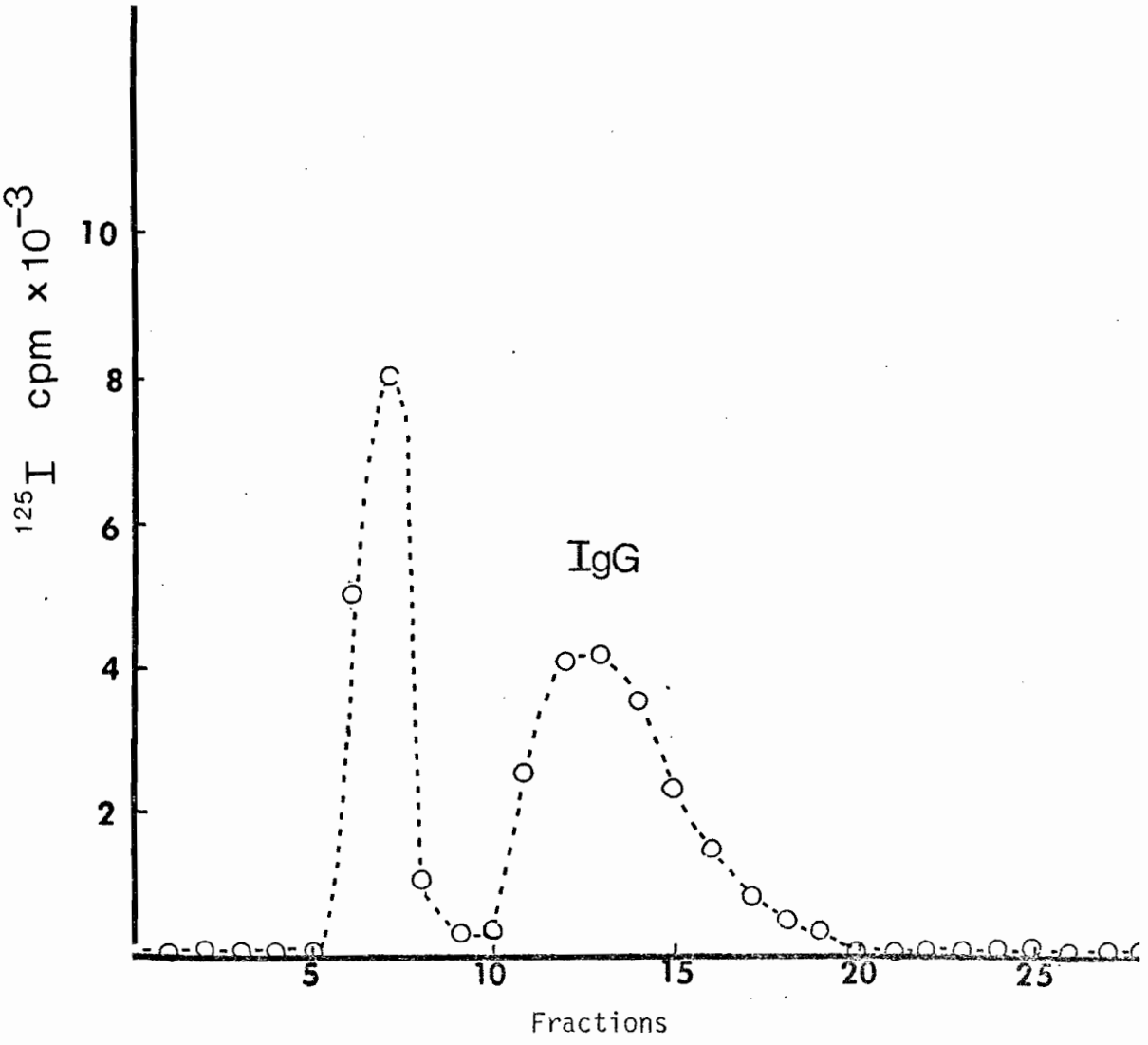


Fig.4-1a

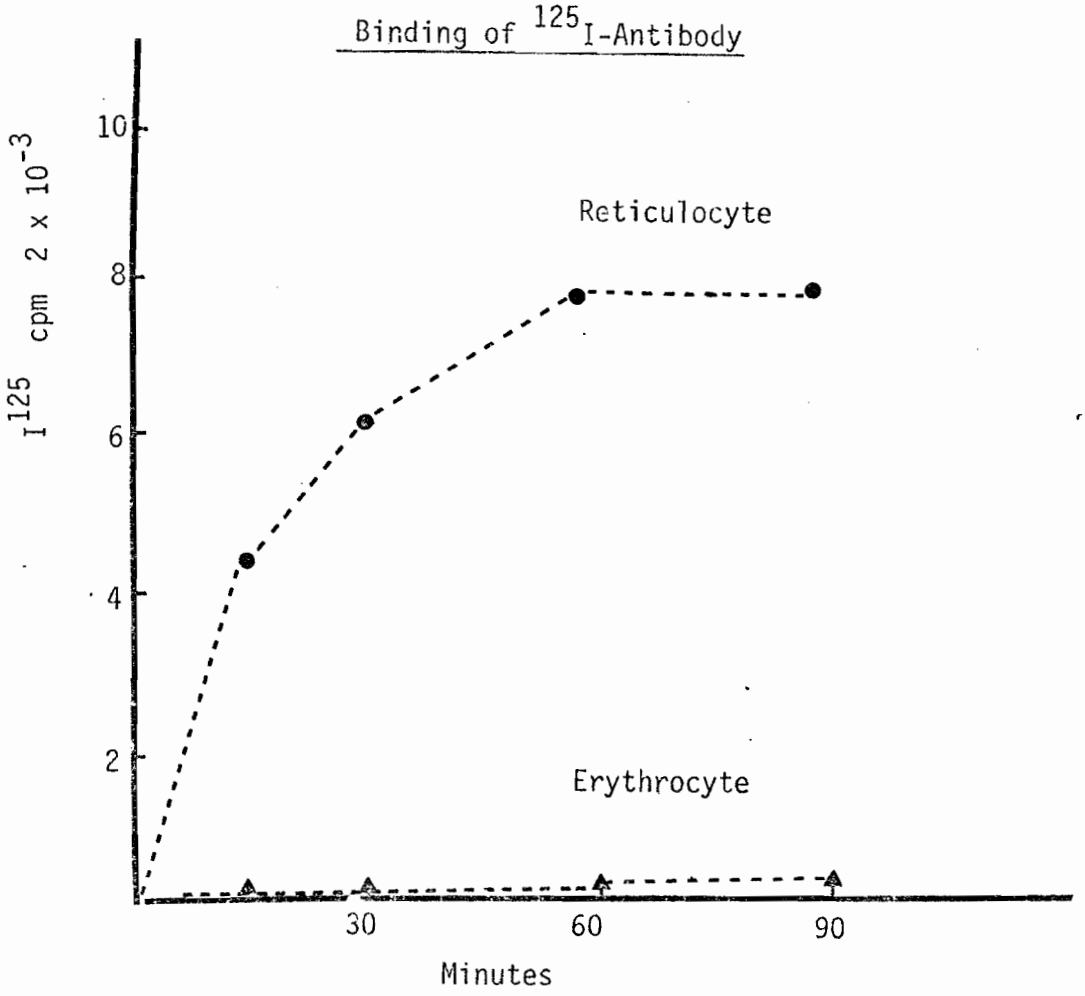

Binding of ^{125}I-Antibody
I^{125} cpm 2 x 10^{-3}
10
8
6
4
2
Reticulocyte
Erythrocyte
30
60
90
Minutes

Fig.4-1b

Fig.4-1. Separation of ^{125}I-antibody Labelled Cell and Time Course of ^{125}I-antibody Binding to the Cell.

2% sheep reticulocyte and erythrocyte suspensions in phosphate buffered saline pH 7.4 were incubated with ^{125}I-anti-transferrin receptor antibody(~.25μg/ml, specific activity ~ 4.5 x 10^5 cpm/μg) at 0°C for various time. At each time point, 0.4 ml of the samples from each suspension was taken and filtered at 4°C through Sepharose 6B columns (1.5 x 7 cm) with phosphate buffered saline pH 7.4, 20 drops per fraction were collected and counted for ^{125}I. (a) is a typical result of the filtration. Two radioactive peaks were well resolved, the first is associated with the cell fractions and the second is free ^{125}I-antibody. The radioactivity associated with the cell fractions is considered as cell bound. (b) is the time course of ^{125}I-antibody binding to reticulocytes or erythrocytes.

incubated at 37°C. At intervals, samples were taken, centrifuged at 12,000 x g for 1 minute to remove intact cells and the supernatants and the cell pellets were counted. Fig. 2 shows the change in radioactivity associated with the cell pellet and the supernatant during the course of incubation at 37°C. Using cells labelled with ^{125}I-antibody at 0°C as the starting material, the radioactivity associated with the cell pellet decreased while that associated with the supernatant increased reciprocally during the experimental period. To assess whether the ^{125}I-material released from the labelled cell at 37°C was degraded protein, samples of the cell free supernatant were treated with trichloroacetic acid (TCA). The results in Fig. 3 show that all the radioactivity released into the medium during the experimental period was precipitated with TCA indicating that the components released had high molecular weights.

Assessment of the Binding Activity of the Released ^{125}I- Antibody

If free ^{125}I-antibody is released, ^{125}I-antibody would be expected to retain at least part of its capacity to bind to fresh reticulocytes. The released high molecular weight material containing ^{125}I was incubated with fresh reticulocytes at 0°C and this binding was compared to that with fresh (never bound) ^{125}I-antibodies. The results in Table 1 show that the binding of the released ^{125}I-material to fresh reticulocytes at 0°C was small compared to the binding seen with fresh antibodies. In fact, it is of same order as the non-specific binding of ^{125}I-antibody to the mature red cell (Table 1). The data indicate that the radioactive material released into the medium from the cell associated ^{125}I-antibody has lost the capacity to rebind to reticulocytes. The loss

Fig.4-2. DISTRIBUTION OF CELL ASSOCIATED AND RELEASED RADIOACTIVITY WITH TIME

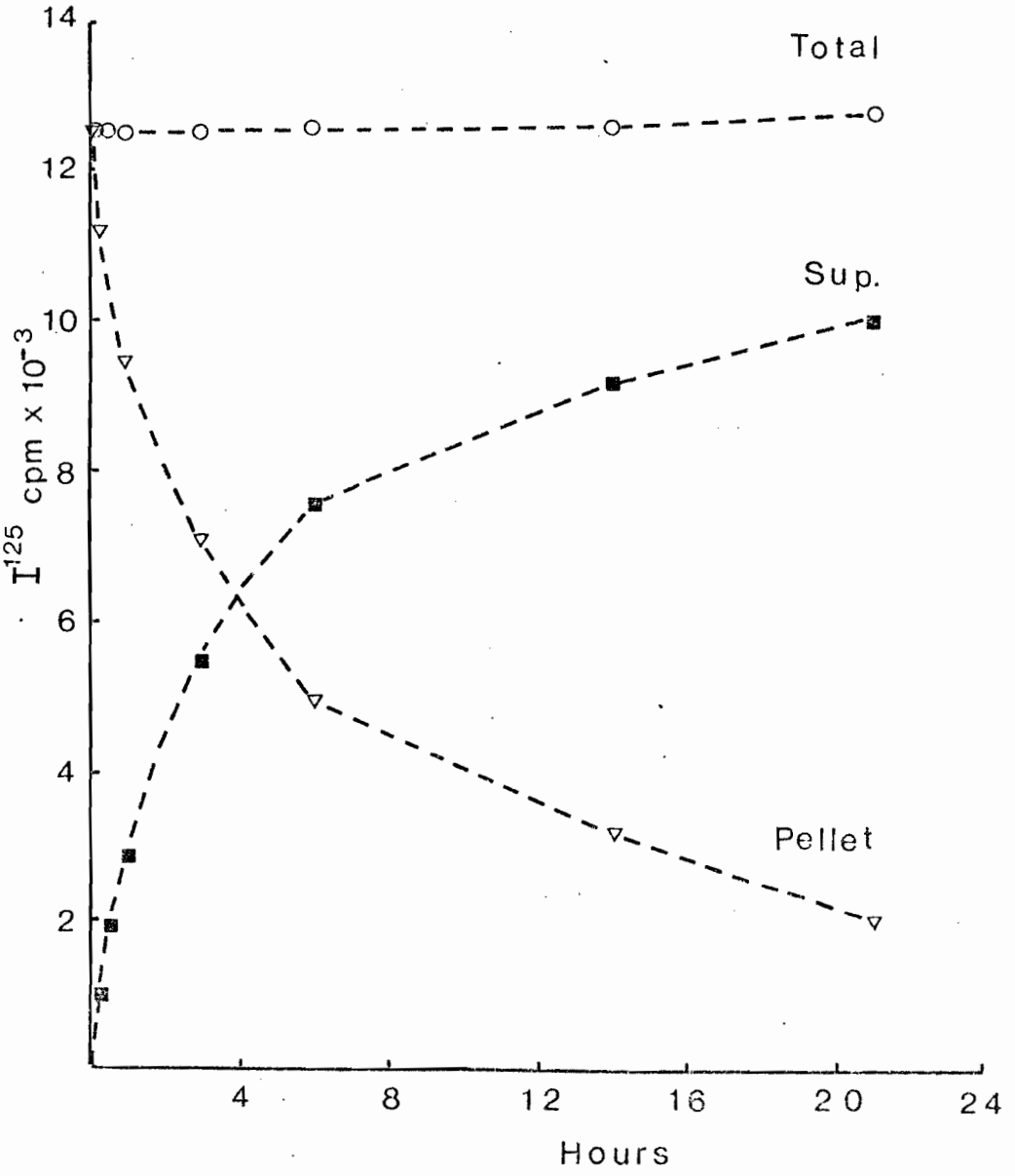


Fig. A-2. Time Course of Cell Bound Radioactivity Released into the Culture Medium

2% sheep reticulocytes in phosphate buffered saline pH 7.4 were incubated with ^{125}I-anti-transferrin receptor antibody (~ 0.4 μg/ml, specific activity ~ 4.5 x 10^5 cpm/μg) at 0°C for 90 minutes. The ^{125}I-antibody labelled cells were obtained as described in Fig. 1. The cells were resuspended into 1% cell suspension with culture medium containing 2% fetal calf serum. This suspension was flushed with 95% O_2 and 5% CO_2 and incubated at 37°C. At various intervals, 0.4 ml samples were taken and centrifuged in an Eppendorff centrifuge for 1 minute. The supernatants and the pellets were counted for ^{125}I respectively.

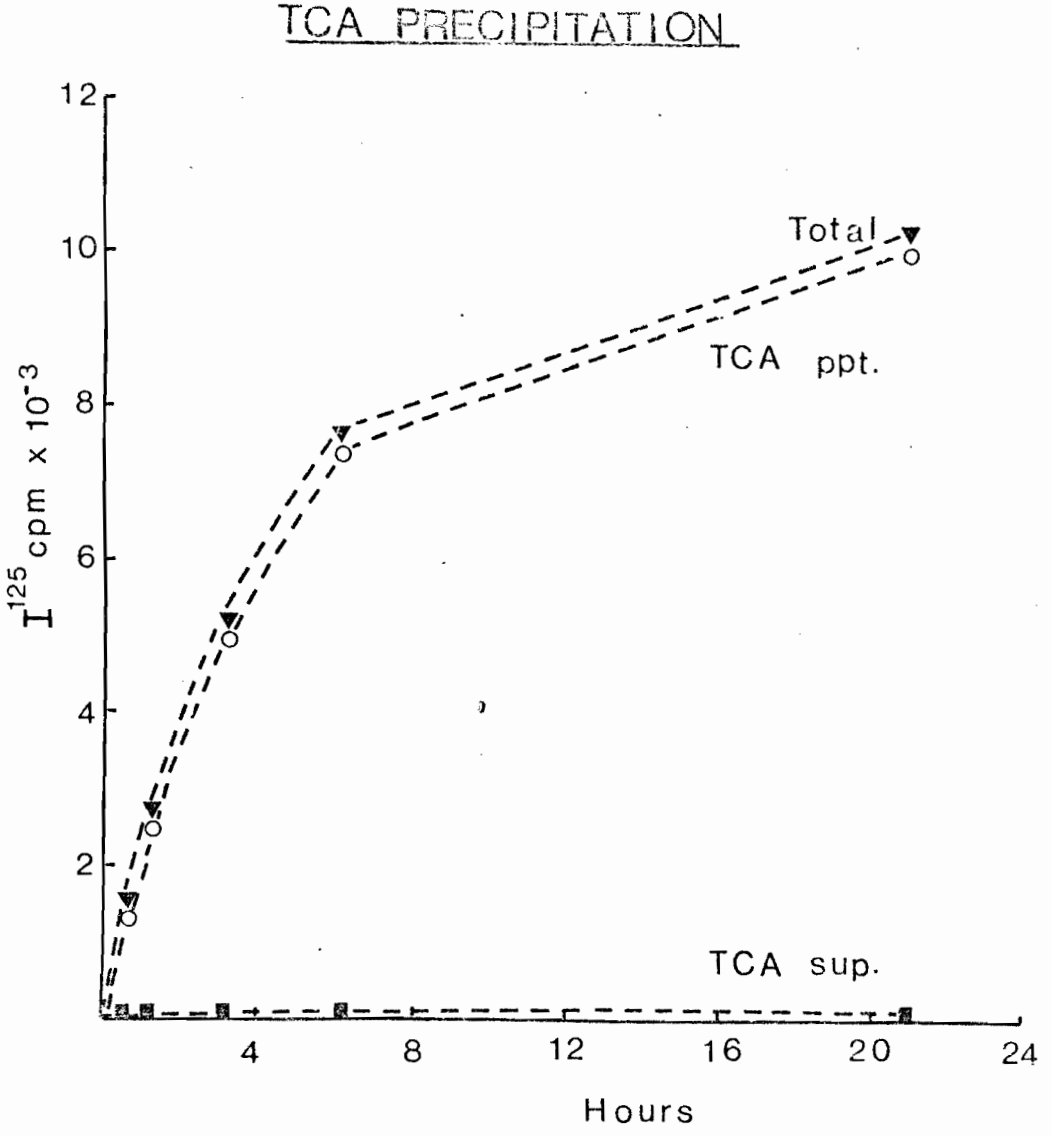


Fig 4-3. TCA Precipitability of the Radioactivity Released into the Medium

To each of the supernatants obtained in Fig. 2, 40 µl of albumin (1 mg/ml) was added, followed by 60 µl of 40% TCA. The samples were mixed and then centrifuged at 12,000 x g for 2 minutes. The supernatants and pellets were counted for ^{125}I.

Table 4-1. Binding Capacity of ^{125}I-Radioactivity Released from ^{125}I-Antibody Labelled Reticulocytes

Source of ^{125}I-radioactivity	cpm bound to fresh reticulocytes or erythrocytes
1. ^{125}I-material derived from a 24 hr culture of ^{125}I-antibody labelled reticulocytes	660 (reticulocytes)
2. fresh ^{125}I-antibody	21,000 (reticulocytes)
3. ^{125}I-antibody derived from incubation of ^{125}I-antibody in cell free medium	18,400 (reticulocytes)
4. ^{125}I-antibody preincubated with sheep erythrocytes	20,000 (reticulocytes)
5. fresh ^{125}I antibody	600 (erythrocytes)

(1) After incubation of ^{125}I-antibody labelled reticulocytes for 24 hours at 37°C the cells were removed by centrifugation and the supernatant collected. To the cell free supernatant was added fresh reticulocytes (2%) and the cells were incubated for 90 minutes at 0°C. The cells were filtered through a Sepharose 6B column (1.5x7 cm) and collected in fractions. Cell associated radioactivity was counted. In the controls,(2) fresh ^{125}I-antibody was incubated with fresh reticulocytes; (3) ^{125}I-antibody was incubated at 37°C without cells in culture medium and after 24 hours the cell free medium was incubated with fresh reticulocytes; (4) ^{125}I-antibody was preincubated at 37°C with sheep erythrocytes for 24 hours, the culture was centrifuged and the supernatant was incubated with fresh reticulocytes; (5) fresh ^{125}I-antibody was incubated with fresh erythrocytes at 0°C for 90 minutes and ^{125}I-antibody bound to the cells was obtained as above. The radioactivity used for binding to fresh reticulocytes or erythrocytes was constant (~74,000 cpm) for all the experiments.

in binding activity is not due to inactivation of the antibody by incubation at 37°C since free ^{125}I-antibody, incubated in the absence of cells, maintained its ability to bind to reticulocytes (Table 1). The experiments thus indicate that TCA-precipitable radioactivity released to the medium is unlikely to be free ^{125}I-antibody which has dissociated from the cells. Moreover, the ^{125}I-antibody preincubated with sheep erythrocytes at 37°C maintained the ability to bind to fresh reticulocytes (Table 1). The particular association of ^{125}I-antibody inactivation with reticulocytes suggested that the released ^{125}I-material might have undergone some alteration by virtue of having been attached to the transferrin receptor of reticulocytes.

The Released ^{125}I-material Has a Molecular Weight Greater than that of Free ^{125}I-antibody.

If the released, TCA-precipitable, radioactivity was partially degraded ^{125}I-antibody but high molecular weight material, then it should be possible to separate the radioactive peptides from free antibody by gel filtration. To examine this possibility the cell-free culture medium containing released ^{125}I-compound(s) was fractionated on Sephadex G-150. It may be seen (Fig. 4) that the majority of the radioactivity was eluted with the fraction corresponding to the void volume. Only a small fraction of the radioactivity was eluted as free ^{125}I-antibodies. Thus, the bulk of the radioactivity released was in a molecular weight form (or forms) higher than that of free antibodies. There was no detectable degradation of ^{125}I-antibody to products of a molecular weight lower than that of the free antibody.

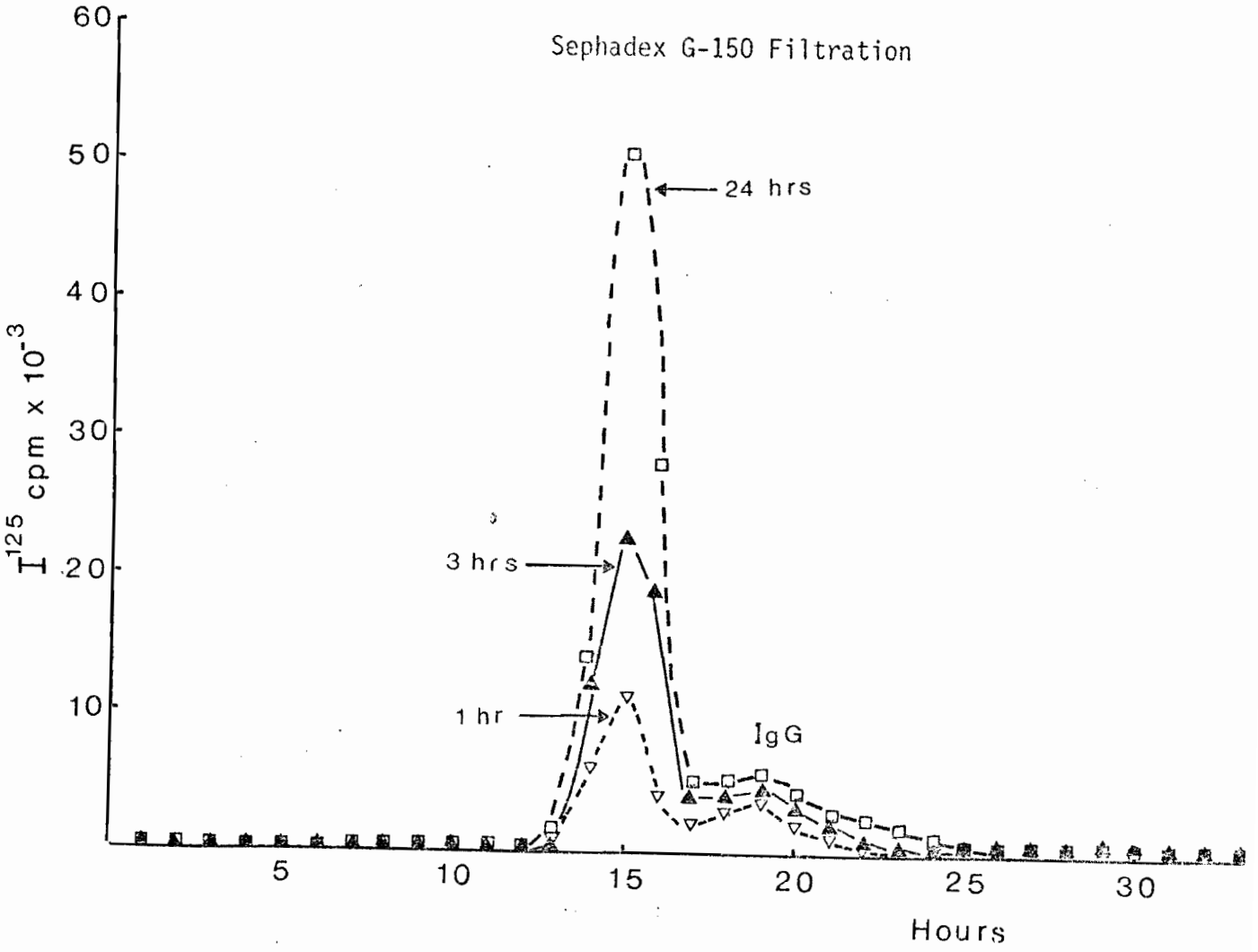


Fig.4-4. Sephadex G-150 Filtration of the Released Radioactivity

Supernatants from ^{125}I-antibody labelled reticulocyte incubated at 37°C for various times were applied to Sephadex G-150 column (1.5x80 cm) and eluted with phosphate buffered saline pH 7.4 2.5 ml were collected each fraction and counted for ^{125}I. The radioactivity is completely recovered.

Release of Transferrin Receptor in Association with ^{125}I-anti-transferrin Receptor Antibody

Since the released radioactivity had lost the ability to bind to fresh reticulocytes, had a higher molecular weight than native antibody, and the antibody is directed against the transferrin receptor, it was considered possible that the released material might be a complex of the transferrin receptor and the antibody directed against it. The association of the receptor with the released antibody would account for the loss of cell binding activity of the antibody. If the ^{125}I-transferrin receptor antibody is released in such a complex, one might expect to isolate the transferrin receptor by purification of the released complex with a protein A affinity column. Protein A is known to have a high specific affinity for the Fc region of the IgG molecule (Forsgren and Sjöquist, 1966). The data in Fig. 5 indicate that ∿75% of the radioactivity released to the medium after 6 hours of incubation is retained by the protein A column. When glycine buffer eluate of the protein A column was subjected to SDS polyacrylamide gel electrophoresis, three polypeptides comigrating with transferrin receptor (∿93K), transferrin (∿78K), and heavy chain of IgG (∿53K) appeared in the gel (Fig. 6). The initial culture medium also contained a number of peptides which were retained by the protein A column, the major component being a peptide with a molecular weight of ∿ 60 x 10^3 characteristic of albumin. Since the culture medium contained 2% fetal calf serum, the peptides appearing in the control medium may be due to an inadequately washed column. Extensive washing of the protein A column is not feasible in these experiments because controls have shown that the ^{125}I-released

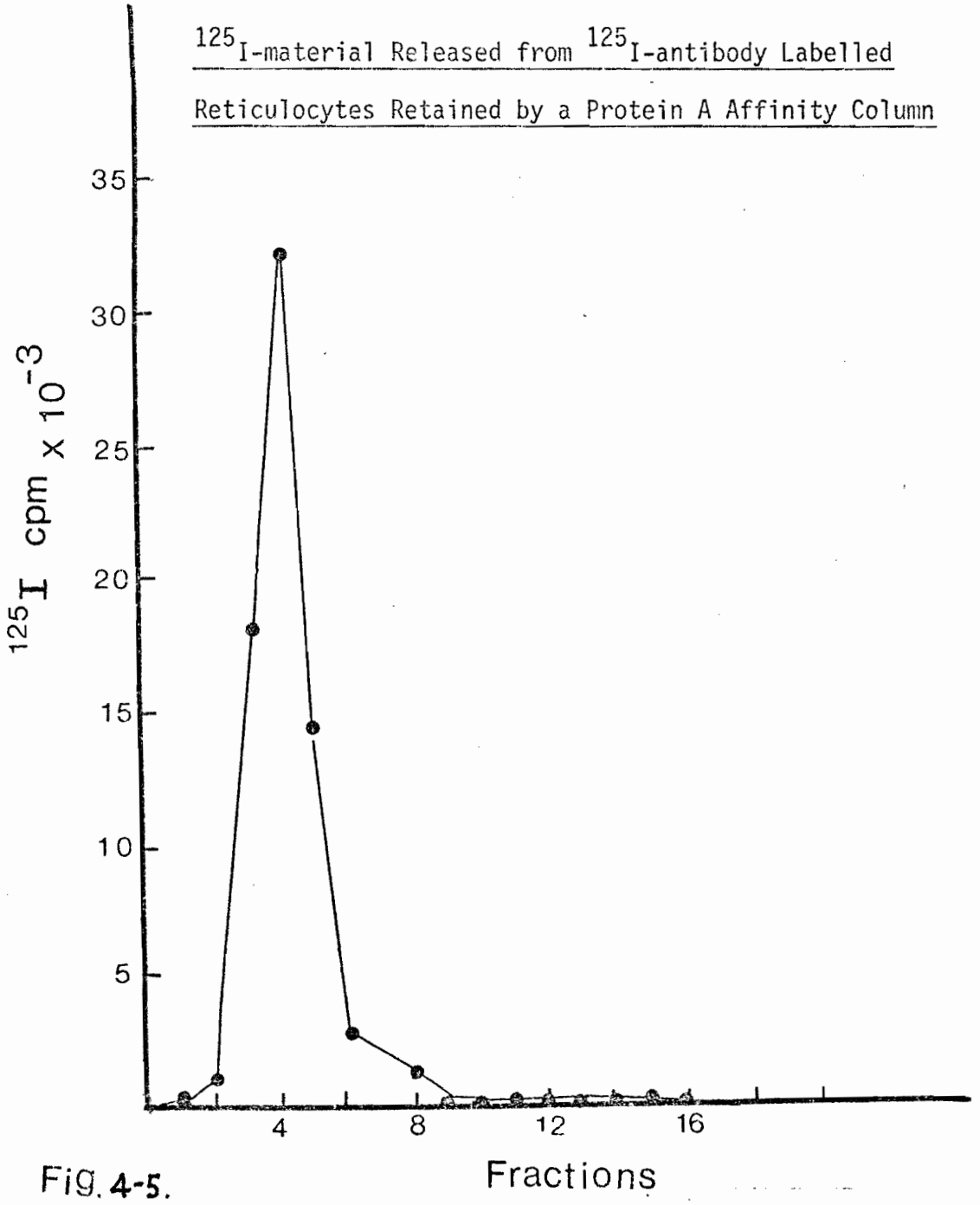

^{125}I-material Released from ^{125}I-antibody Labelled
Reticulocytes Retained by a Protein A Affinity Column
^{125}I cpm x 10^{-3}
35
30
25
20
15
10
5
4
8
12
16
Fractions

Fig. 4-5.

Fig. 4-5. ^{125}I-material Released from ^{125}I-antibody Labelled Reticulocytes Retained by Protein A Affinity Column

2% reticulocytes were incubated with ^{125}I-antibody (~1 μg/ml, specific activity ~1.5 x 10^5 cpm/μg) at 0°C for 90 minutes. This suspension was then filtered through a Sepharose 6B column (2.6 x 12 cm) at 4°C. The cell fraction was collected and resuspended to a 1% cell suspension with culture medium containing 2% fetal calf serum, flushed with 95% O_2 and 5% CO_2, and incubated at 37°C. After 6 hours, 25 ml sample was taken and centrifuged at 12,000 x g. The supernatant (~3 x 10^5 cpm) was applied to a protein A-Sepharose 4B column (2.5 ml) and washed with phosphate buffered saline pH 7.4 until no further radioactivity was eluted (~3 bed volumes). The column was then eluted with 0.1 M glycine buffered saline pH 3.0. 50 drops were collected per fraction, neutralized and counted. ~2.3 x 10^5 cpm (~75% of that applied) was eluted with 0.1 M glycine buffered saline pH 3.0.

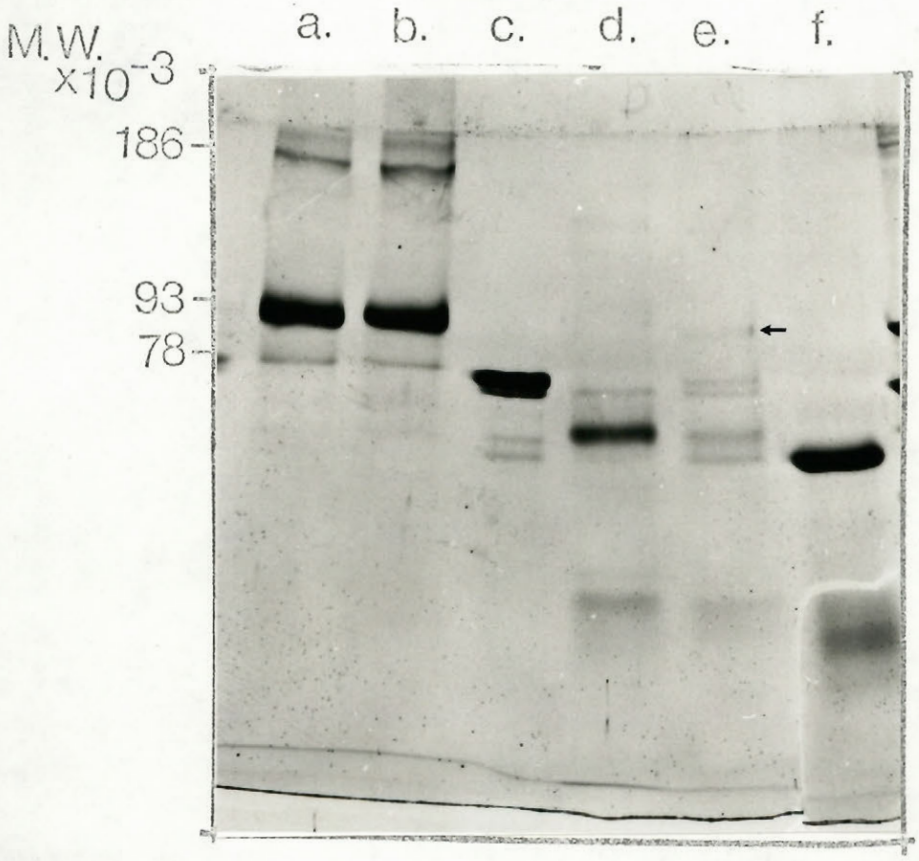


Fig. 4-6

Fig. 4-6. Peptides Released from Reticulocytes Correspond to Transferrin and Transferrin Receptor

a.b. Peptides isolated from sheep reticulocyte membranes by (a) transferrin affinity column, and (b) anti-transferrin receptor antibody immunoaffinity column. The 93K polypeptide corresponds to the monomer of transferrin receptor and 78K to the sheep transferrin; c. sheep transferrin (78K); d. peptides retained by the protein A column from ∿75 ml of the initial culture medium after washing the column with same volume of phosphate buffered saline pH 7.4 as used for the 6 hour culture medium; e. peptides retained by protein A column from ∿ 25 ml of the 6 hour culture medium (Fig. 5); f. free IgG.

For the electrophoresis the eluates from the protein A column were neutralized, dialyzed against H_2O, lyophilized and then dissolved in the electrophoresis buffer in the presence of 2% SDS and 10% mercaptoethanol and electrophoresized in 5% - 15% gradient SDS-polyacrylamide gel. It can be seen (e) there are two polypeptides comigrating with transferrin receptor (93K) (arrow) and transferrin (78K) respectively appears at 6 hour culture medium and these two polypeptides are absent in (d), the initial culture medium served as control. The polypeptide comigrates with the heavy chain of IgG also appears to be more abundant in (e) than in (d). The polypeptides appear both in the initial and 6 hour culture medium are believed to be the polypeptides from the calf fetal serum added to the culture medium and were retained by the column perhaps due to the insufficient wash to remove them when a 3-4 times of the bed volume of PBS was used to wash away the unbound radioactivity. High molecular polypeptides corresponding to the spectrin (M.W. ∿ 200K) is also absent from the 6 hour culture medium.

material was not retained if the column was extensively washed with phosphate-buffered saline. Hence it was not possible to get lower background levels in the control. It should be noted that in the data shown in Fig. 6 three times more control medium was processed than medium derived from the incubation with cells to show that the new peptides appearing were not present in the control medium.

Transferrin Receptor-Antibody Complex is Released as a Vesicle

The release of the transferrin receptor-antibody complex into the supernatant may occur in either of two ways: (1) a soluble protein complex or (2) a membrane vesicle containing the transferrin receptor. To distinguish between these possibilities, a number of experiments were carried out, all of which are consistent with the conclusion that a vesicle was released. (1) The supernatant containing the ^{125}I-antibody transferrin receptor complex released from ^{125}I-antibody labelled reticulocytes after 6 hours in culture was chromatographed on a Sepharose 6B column. From the results shown, ~85% of the radioactivity was eluted in the void fractions. The rest of the radioactivity was coeluted with free IgG (Table 2). (2) The radioactivity eluted in the void volume from the Sepharose 6B filtration was centrifuged at 100 K x g for 1 hour. The major portion (~85%) of the radioactivity was centrifuged down at 100K x g (Table 2), suggesting that ^{125}I-antibodies were contained in a high molecular weight complex. (3) The cell free medium containing ^{125}I-antibody-transferrin receptor complexes was centrifuged to equilibrium (24 hrs at 100K x g) on a 20% - 50% (W/W) sucrose density gradient. Radioactivity was found at a density equivalent to that of 40% sucrose (Fig. 7)

Table 4-2. Distribution of the ^{125}I-labelled Material Released from Cultured ^{125}I-antibody Labelled Reticulocytes

	cpm
Cell free Supernatant	12,390
Sepharose 6B Filtration	
Void Fractions	9,860
Free ^{125}I-antibody Fractions	1,460
Sedimentation at 100K x g	
Supernatant	783
Pellet	8,835

2% reticulocytes in phosphate buffered saline was incubated with ^{125}I-anti-transferrin receptor antibody at 0°C for 90 minutes. The suspension was filtered through a Sepharose 6B column and the cells resuspended in culture medium containing 2% fetal calf serum to a 1% cell suspension and incubated at 37°C. After 6 hours, the cells were removed by centrifugation at 12,000 x g for 1 minute and the supernatant collected and counted. An aliquot of the supernatant was applied to a Sepharose 6B column (1.5 x 80 cm) and eluted with phosphate buffered saline (pH 7.4), 1 ml fractions were collected. The void volume obtained was collected and centrifuged at 100K x g for 1 hour and the supernatant and pellets obtained counted.

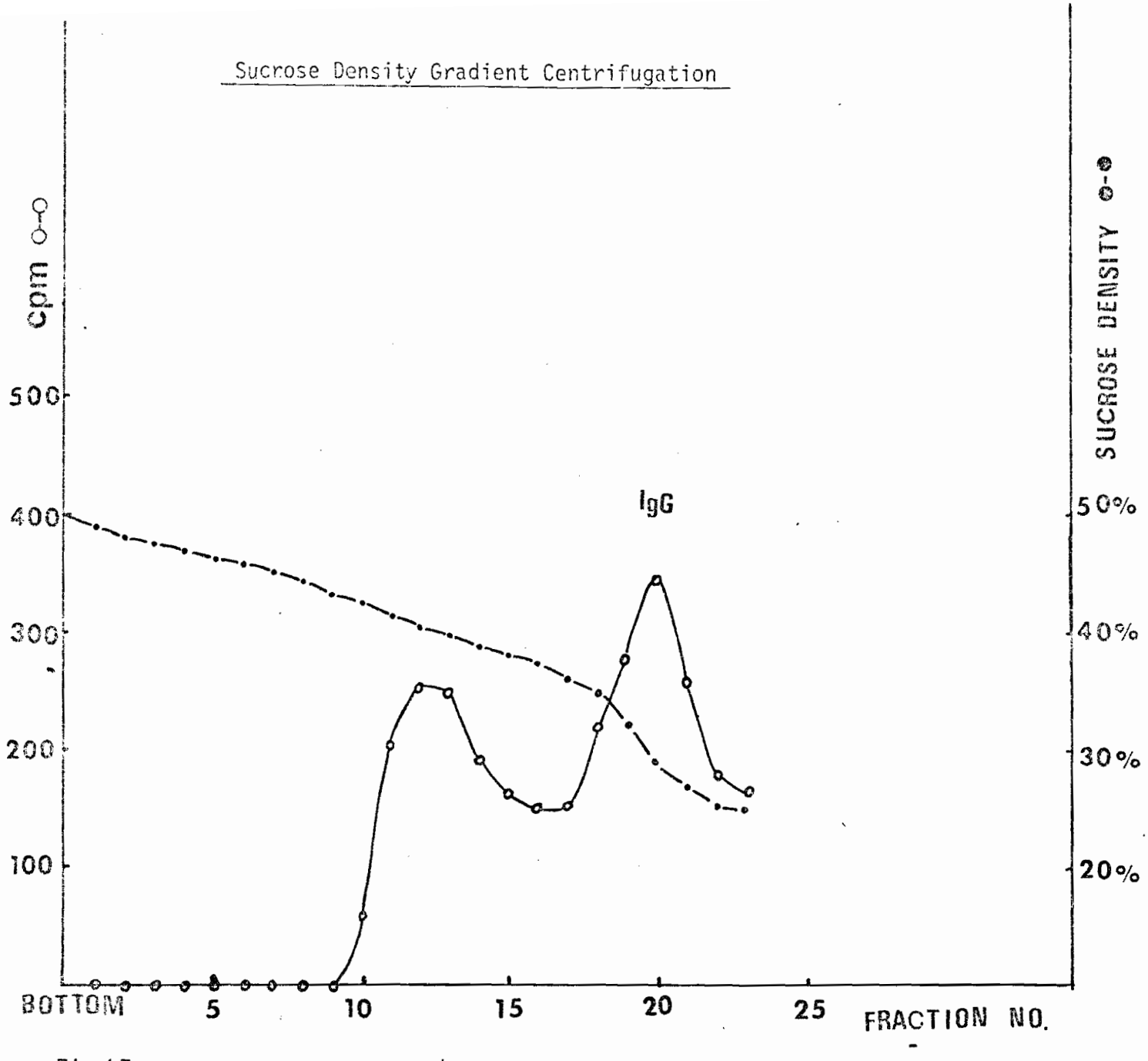

Sucrose Density Gradient Centrifugation
cpm
500
400
300
200
100
IgG
SUCROSE DENSITY
50%
40%
30%
20%
BOTTOM
5
10
15
20
25
FRACTION NO.

Fig 4-7

Fig 4-7. Sucrose Density Gradient Centrifugation of ^{125}I-labelled Materials Released from ^{125}I-antibody Labelled Reticulocytes

^{125}I-antibody labelled reticulocytes were incubated at 37°C for 6 hours. The cell-free supernatant (0.2 ml) was layered on top of a 5 ml cellulose nitrate tube containing 20%- 50% sucrose density gradient and centrifuged at 100K x g at 4°C for 24 hours. The tube was punctured on the bottom, 0.2 ml was collected per fraction and counted for ^{125}I.

and in a peak corresponding to free IgG at a lower density. In many systems it has been shown that plasma membrane vesicles have a buoyant density near 40% sucrose. The high amount of radioactivity at the low density was probably due to dissociation of the antibody from the vesicle during centrifugation. (4) It was possible to detect vesicles in the cell free supernatant using electron microscopy. Uniform membrane vesicles with clustered knobs on their surfaces were seen under EM (Fig. 8) after staining the 100K x g pellet with 1% ammonium molybdate. No such vesicles were observed when the initial medium was processed in the same way. Moreover, similar vesicles (Fig. 9) were isolated by chromatography of the supernatant from a 6 hour culture medium on a protein A column suggesting that the antibody is on the surface of the vesicle. Together, these experiments suggest that vesicles containing the transferrin receptor and the anti-receptor antibody were externalized from ^{125}I-antibody labelled sheep reticulocytes incubated at 37°C.

Antibody Induced Redistribution and Externalization Followed by Direct Immunofluorescence

To assess whether the release of vesicles from the antibody-labelled reticulocytes followed a route analogous to ligand induced internalization or externalization described in many other cell systems (Taylor et al., 1971; Salisburg, Condeels and Satir; Goldstein, Anderson and Brown,1979; Pastan and Willingham, 1981; Nordquist,Anglin and Lerner, 1977; Calafat et al., 1976), reticulocytes were first incubated with FITC-labelled antibody at 0°C. After separation of the cells from free FITC-antibody and resuspension in fresh culture medium, the

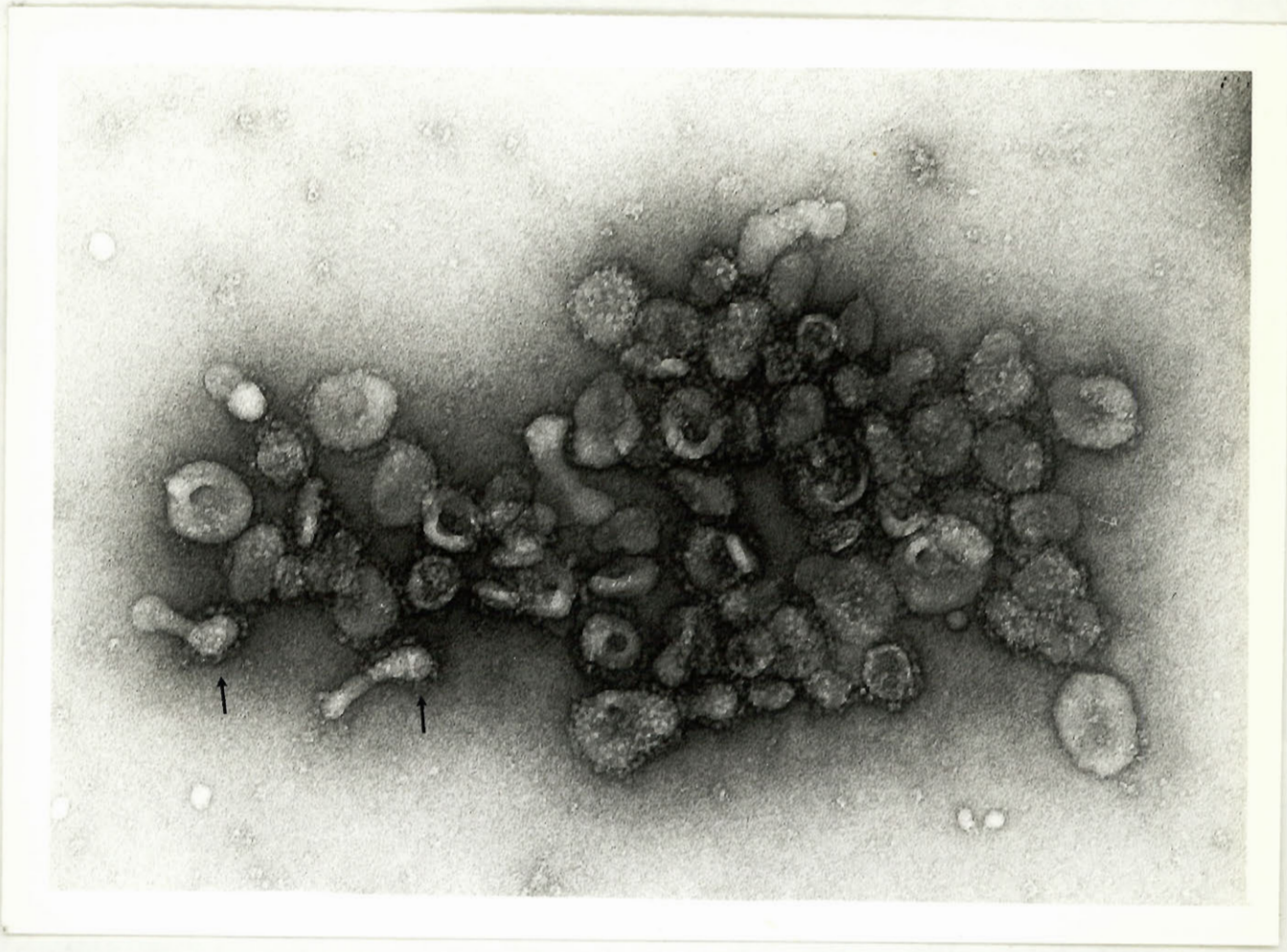


Fig.4-8 Vesicular Appearance of Released ^{125}I-Material from ^{125}I-antibody labelled Reticulocytes.

The supernatant obtained from ^{125}I-anti-transferrin receptor antibody labelled reticulocytes incubated at 37°C for 6 hours was centrifuged at 100 K x g for 1 hour. The pellet fraction was stained with 1% ammonium molybdate and examined under an electron microscope. Vesicles with surface knobs are seen. Some (arrow) have elongated shapes with knobs only at one end. (Magnification 50,800 X 2.5).

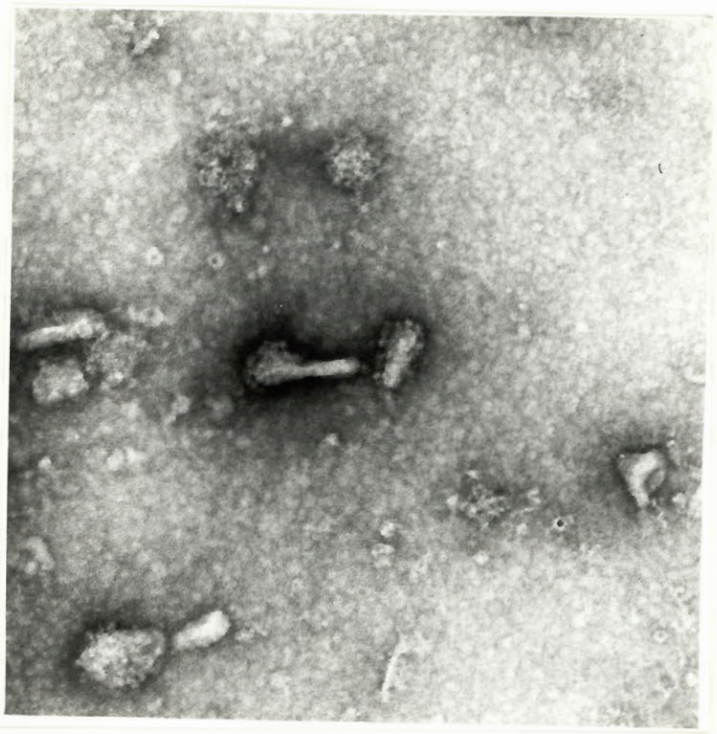
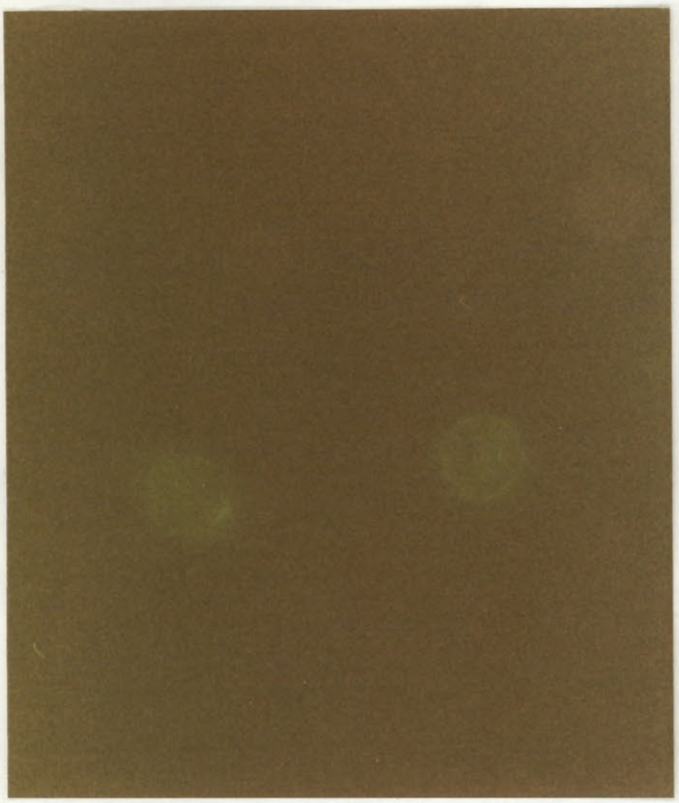


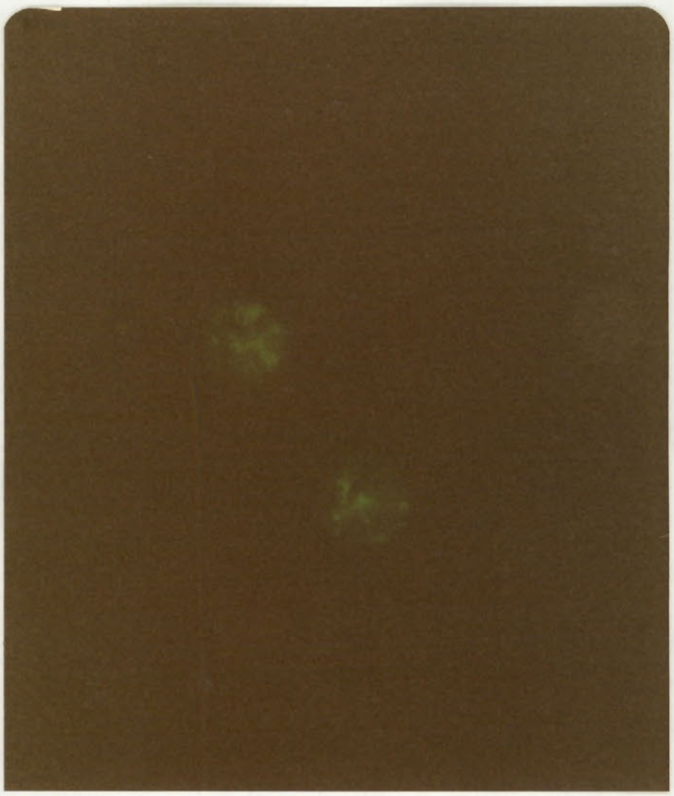
Fig.4-9. Electron Micrograph of Material Retained by a Protein A Affinity Column

The cell free supernatant from ^{125}I-antibody labelled reticulocytes reticulocytes incubated at 37°C for 6 hours was applied to protein A affinity column at room temperature. After washing with phosphate buffered saline pH 7.4 the bound material was eluted with 0.1 M, glycine buffered saline pH 3.0. The eluate was immediately neutralized and centrifuged at 100K x g for 1 hour. The pellet obtained was stained in 5% ammonium molybdate and examined under an electron microscope. (Magnification 50,800 X 2.5).

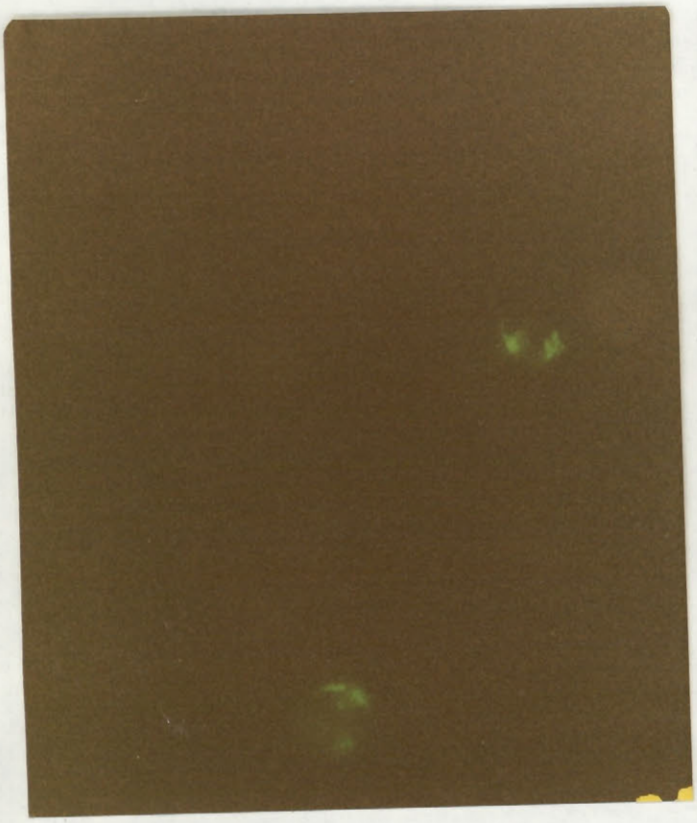
Fig.4-10.



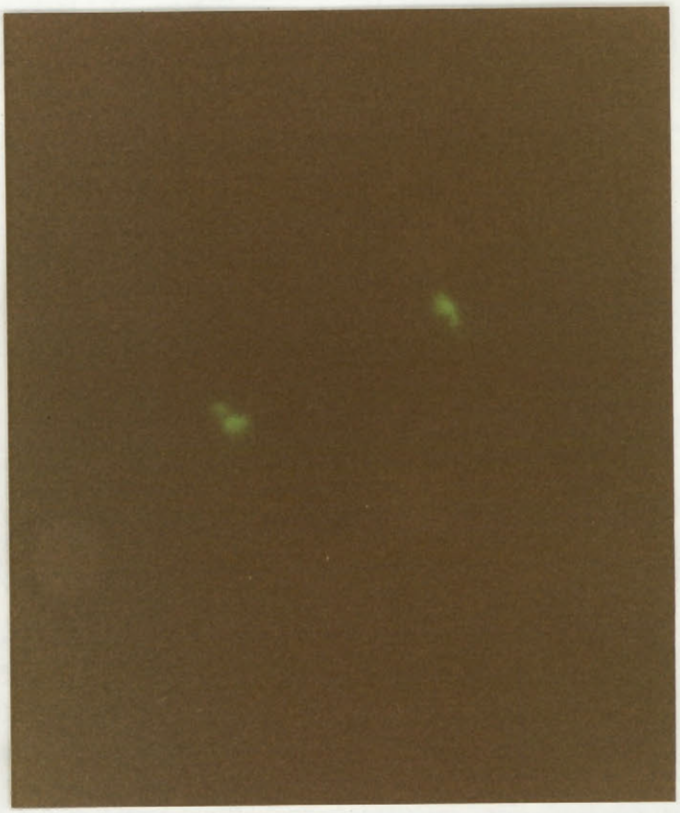
a.



b.



c.



d.

Fig. 4-10 Surface Distribution of FITC-Anti-transferrin Receptor Antibody Sheep Reticulocyte at 37°C

Sheep reticulocytes were labelled with FITC-anti-transferrin receptor antibody at 0°C for 90 minutes. The free FITC-antibody then was removed by centrifugation and the FITC-antibody labelled reticulocytes were resuspended to 1% cell suspension in culture medium containing 2% fetal calf serum and incubated at 37°C. Samples were taken at intervals for examination under fluoresence microscope.

a. Unfixed cells labelled at 0°C or prefixed cells labelled at 37°C

b, c, d. Unfixed FITC-antibody labelled reticulocytes incubated at 37°C.

b. After 30 minutes; c. after 3 hours; d. after 6hours.

(Magnification 1,000 X)

Fig. 4-11. Fluorescent Particles Released from the FITC-antibody Labelled Reticulocyte

Experimental conditions were similar to those described in Fig. 10, except the cell free culture medium after 6 hours at 37°C was examined and photographed under a fluorescence microscope. (Magnification 1,000 X).

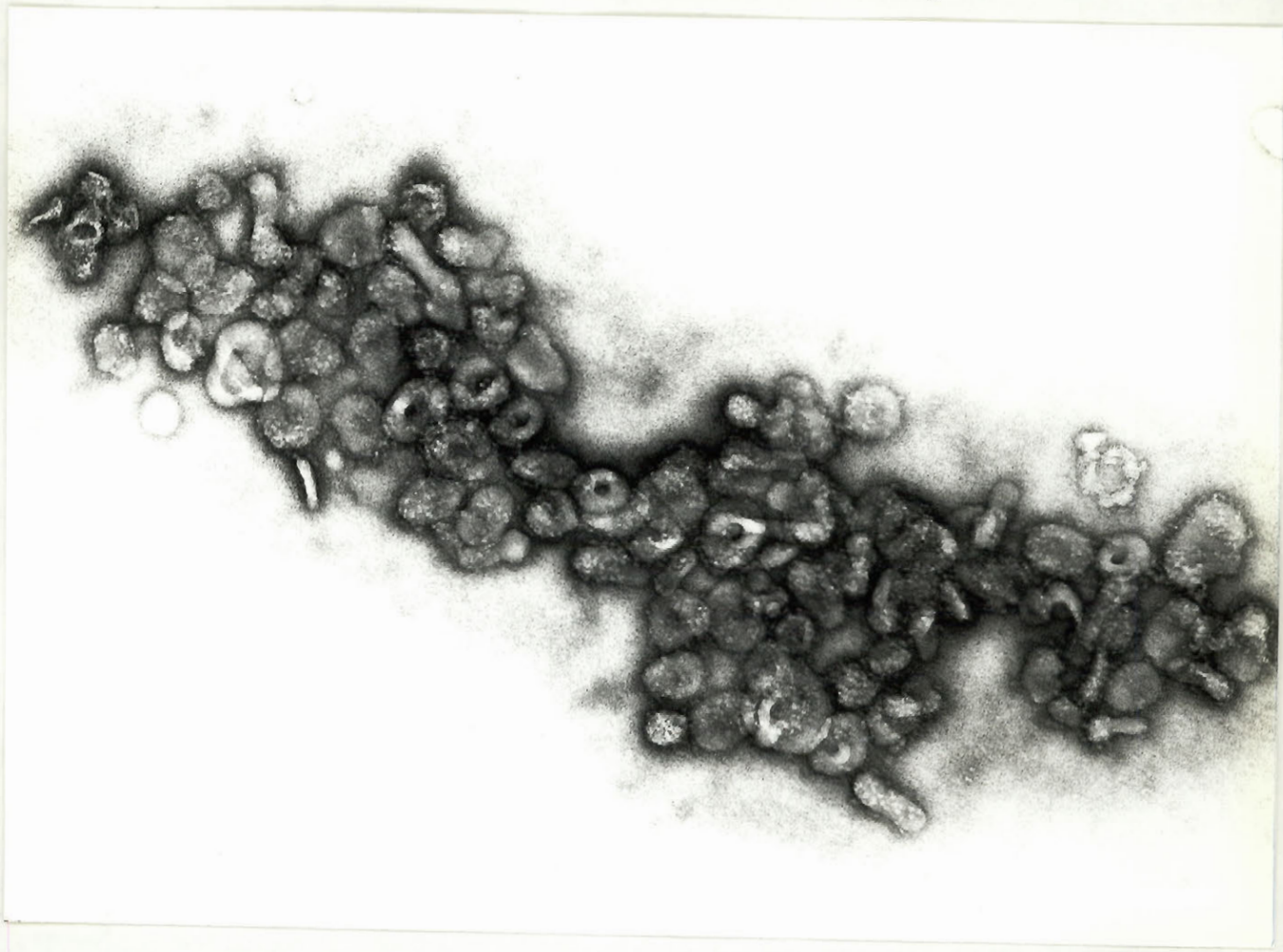


Fig.4-12. Vesicles Released from FITC-antibody Labelled Reticulocytes

After 6 hours of culturing FITC-antibody labelled reticulocytes were centrifuged at 12,000 x g for 1 minute. The supernatant was passed through a Sepharose 6B column (1.5 x 7 cm), eluted with phosphate buffered saline (pH 7.4). The void volume was collected and centrifuged at 100K x g for 1 hour. The pellet fraction was stained as in Fig. 8 for electron microscope. (Magnification 50,800 X 2.5)

Antibody Induced Redistribution and Externalization Followed by an Indirect Immunoferritin Labelling Technique

Using ferritin labelled protein A and purified anti-transferrin receptor antibody to follow the antibody induced redistribution and externalization, the electron micrographs in Fig.13a show that prior to transferring the antibody labelled reticulocyte to 37°C the antibody-transferrin receptor complex was diffusely distributed on the cell surface. However, after incubation at 37°C for 30 minutes the complex formed patches (Fig. 13b), and there was also evidence of vesicle externalization (Fig. 13c). Such a phenomenon of vesiculation was rarely seen in the control experiment where the antibody was absent.

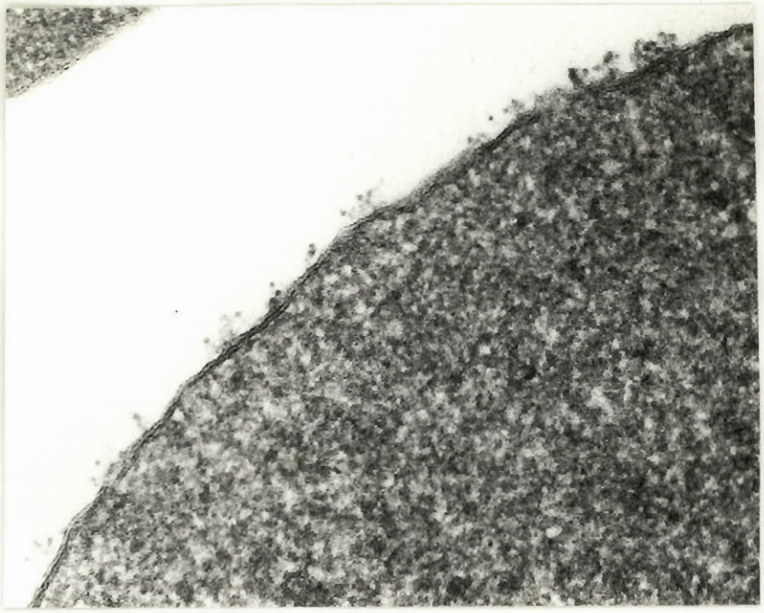
Dissociation of Vesicle Surface Bound Antibody

When the medium containing ^{125}I released vesicles was treated with pH 2.3 glycine-buffered saline, neutralized and then passed through a Sepharose 6B column, most of the radioactivity dissociated from the vesicles and was eluted in the position of free ^{125}I-antibody (Table 3). A similar result was obtained with the supernatant containing the fluorescent particles, i.e. after treatment with pH 2.3 glycine buffered saline, neutralization and chromatography on Sepharose 6B, fluorescent particles were rarely found in the void volume. These experiments indicate that the antibodies (^{125}I-labelled or FITC-labelled) are on the surface of the vesicles.

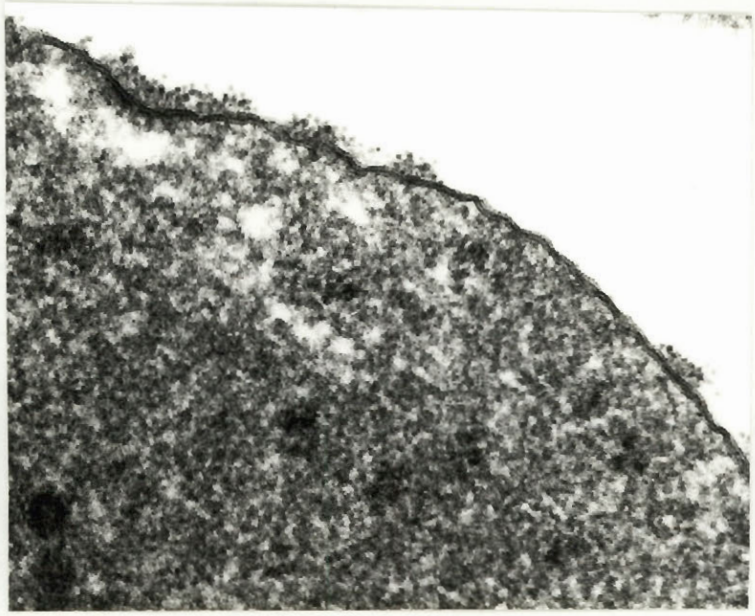
Kinetics of ^{125}I-antibody Induced Externalization

The procedures developed to separate externalized vesicles from cells and free antibodies were applied to a kinetic study of vesicle

a.



b.



c.

Fig.4-13.

Fig.4-13. Antibody Induced Redistribution and Externalization Followed by an Indirect Immunoferritin Labelling Technique

2% reticulocytes were preincubated with purified anti-transferrin receptor antibodies at 0°C for 90 minutes. The cells were filtered through a Sepharose 6B column. The cells were collected, centrifuged and resuspended into 1% cell suspension in ice cold culture medium containing 2% fetal calf serum. This suspension was transferred to 37°C. Prior to transferring to 37°C and after incubation at 37°C for 30 minutes, samples were taken and processed for ferritin-protein A labelling and thin section as described in methods. The results were examined under a Philips model 300 electron microscope. (a) prior to transferring to 37°C; (b) after incubation at 37°C for 30 minutes; (c) vesicle which was undergoing externalization (Magnification 98,625 X2.5).

Table 4-3. Acid Dissociation of ^{125}I-labelled Antibody on the Externalized Vesicles

	Acid Treatment		
	-	+	
^{125}I in void volume	2,100	700	(cpm)
^{125}I in free ^{125}I-antibody fraction	800	2,400	

^{125}I-antibody labelled reticulocytes were incubated at 37°C for 6 hr. The cells were removed by centrifugation and an aliquot of the supernatant of the incubation medium was applied to a Sepharose 6B column (no acid treatment). An identical sample of the supernatant was treated with 0.05 M glycine buffered saline at pH 2.3 for 10 minutes at 4°C, neutralized with phosphate buffer and then applied to the Sepharose 6B column. The column was eluted with phosphate buffered saline (pH 7.4) and the eluate was collected into fractions and counted. Two radioactive peaks are obtained. One is in the void volume and the other coeluted with free ^{125}I-antibody. The radioactivity associated with the void volume was considered as vesicle bound.

externalization. The data in Fig. 14 show the appearance of the vesicle associated ^{125}I in the medium as a function of time. The rate of externalization decreased with time. About 50% of the cell-bound antibodies were externalized in 6 hours under the present conditions. By 21 hours about 80% of the bound ^{125}I-antibodies were externalized. Cell integrity was maintained during the experimental period as indicated by the cellular potassium and the ATP levels (Table 4).

Dissociation of Cell Bound ^{125}I-antibody

To assess whether there was any internalization of ^{125}I-antibody, ^{125}I-samples were taken from ^{125}I-antibody labelled reticulocytes before and after incubation at 37°C. After centrifugation, the cell pellets were resuspended in pH 2.3 glycine buffered-saline and layered on top of a tube containing 5% sucrose in 0.1 M glycine buffered saline pH 2.3 and centrifuged at 4°C. The cell pellets were counted. It was assumed that the surface-bound ^{125}I-antibody would be accessible to the glycine buffer and therefore dissociate from the cell during the centrifugation. The results (Fig. 15) show that there was a small increase in the absolute amount of undissociable radioactivity after the culture was transferred from 0°C to 37°C. The absolute amount of the undissociable radioactivity then decreased gradually with time at 37°C. However, the undissociable radioactivity remained a constant percentage of the total cell associated redioactivity at each time point at 37°C. The undissociable level of radioactivity may reflect a small fraction of bound ^{125}I-antibody which had been internalized.

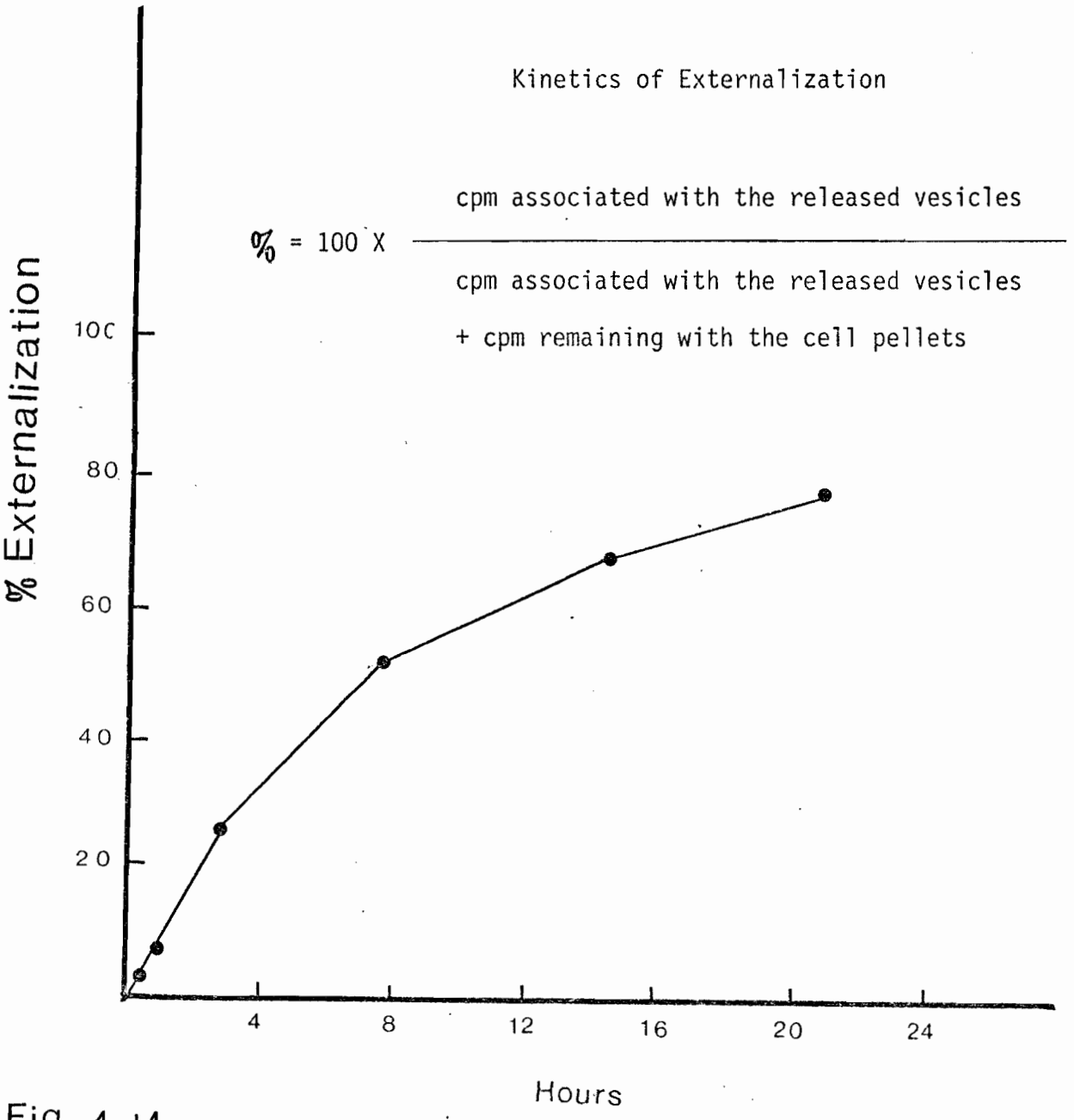

Kinetics of Externalization
% = 100 X
cpm associated with the released vesicles
cpm associated with the released vesicles
+ cpm remaining with the cell pellets
% Externalization
100
80
60
40
20
4
8
12
16
20
24
Hours

Fig. 4-14

Fig.4-14 The Kinetics of ^{125}I-Anti-transferrin Receptor Antibody Induced Externalization in Sheep Reticulocytes

2% sheep reticulocytes in phosphate buffered saline pH 7.4 were incubated with ^{125}I-antitransferrin receptor antibody (~0.4 µg/ml, specific activity ~ 4.5 x 10^5 cpm/µg) at 0°C for 90 minutes. The ^{125}I-antibody labelled reticulocytes were then separated from free ^{125}I-antibodies as described in methods and resuspended to give a 1% cell suspension in culture medium containing 2% serum and incubated at 37°C. At intervals, 0.4 ml samples were taken and centrifuged at 12,000 x g for 1 minute. The supernantant then was filtered through a Sepharose 6B column (1.5x7 cm) 20 drops per fraction were collected and fractions were counted . The radioactivity associated with the void fraction was considered vesicle bound. The vesicle associated radioactivity is expressed as a percentage of the total radioactivity in the vesicles and cells.

Table 4-4 Intracellular ATP and K^+

	ATP mM				
Time (hour)	0	1	3	6	21
+ Antibody	2.0	1.4	1.3	1.1	1.0
- Antibody	2.0	1.5	1.3	1.1	1.1

	(K^+) in mM				
Time (hour)	0	1	3	6	21
+ Antibody	115	121	121	115	109
- Antibody	121	121	127	121	115

2% sheep reticulocytes in phosphate buffered saline (pH7.4) were incubated with purified antibody (~1 µg/ml) at 0°C for 90 minutes. The free antibody was removed by filtration as described in methods. The cells were resuspended to a 1 % suspension in culture medium containing 2% fetal calf serum and incubated at 37°C. At intervals, samples were taken and analyzed for ATP and K^+ as described in methods.

Dissociation of Cell Bound Radioactivity by

Acid Glycine Buffered Saline

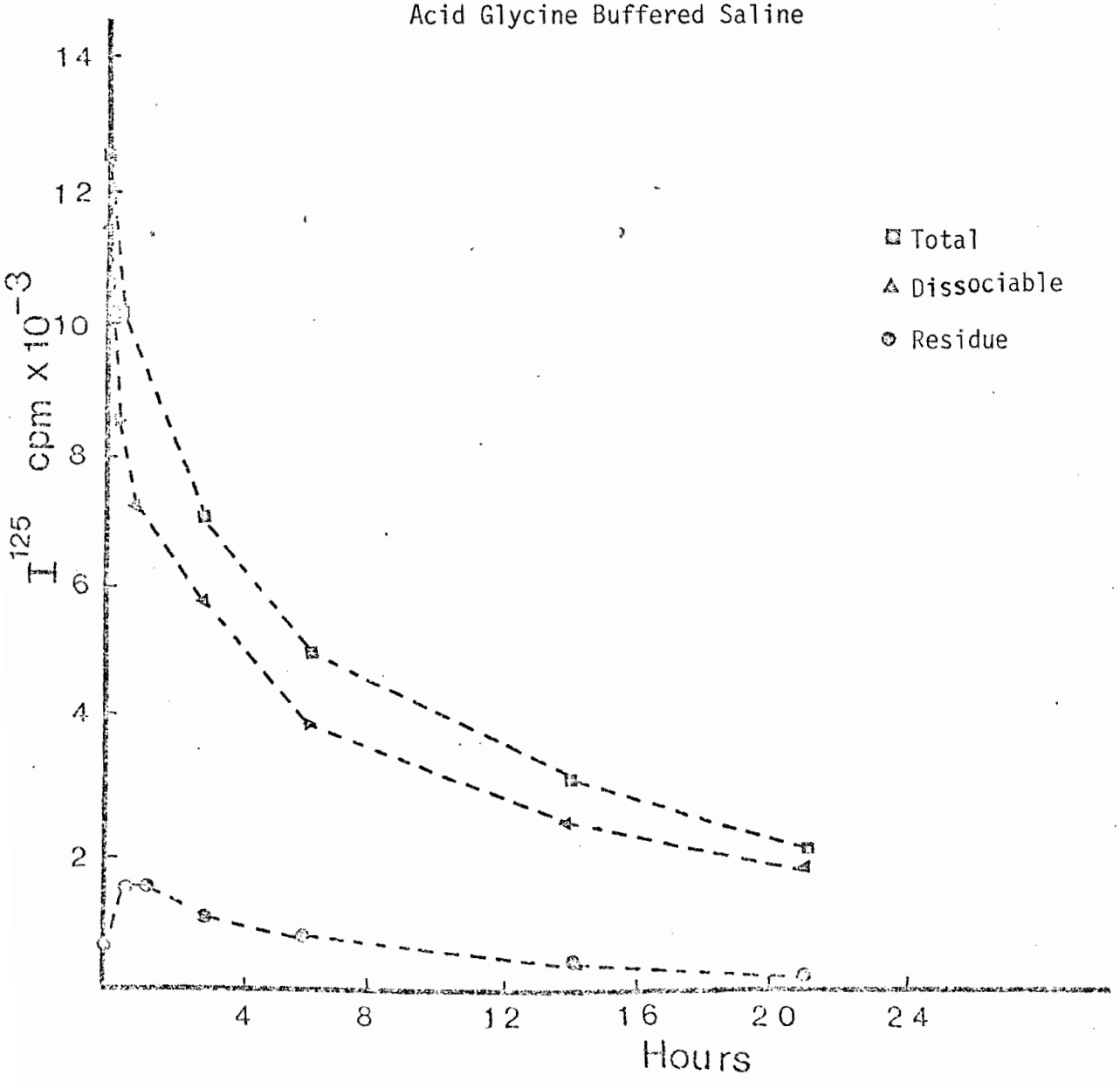


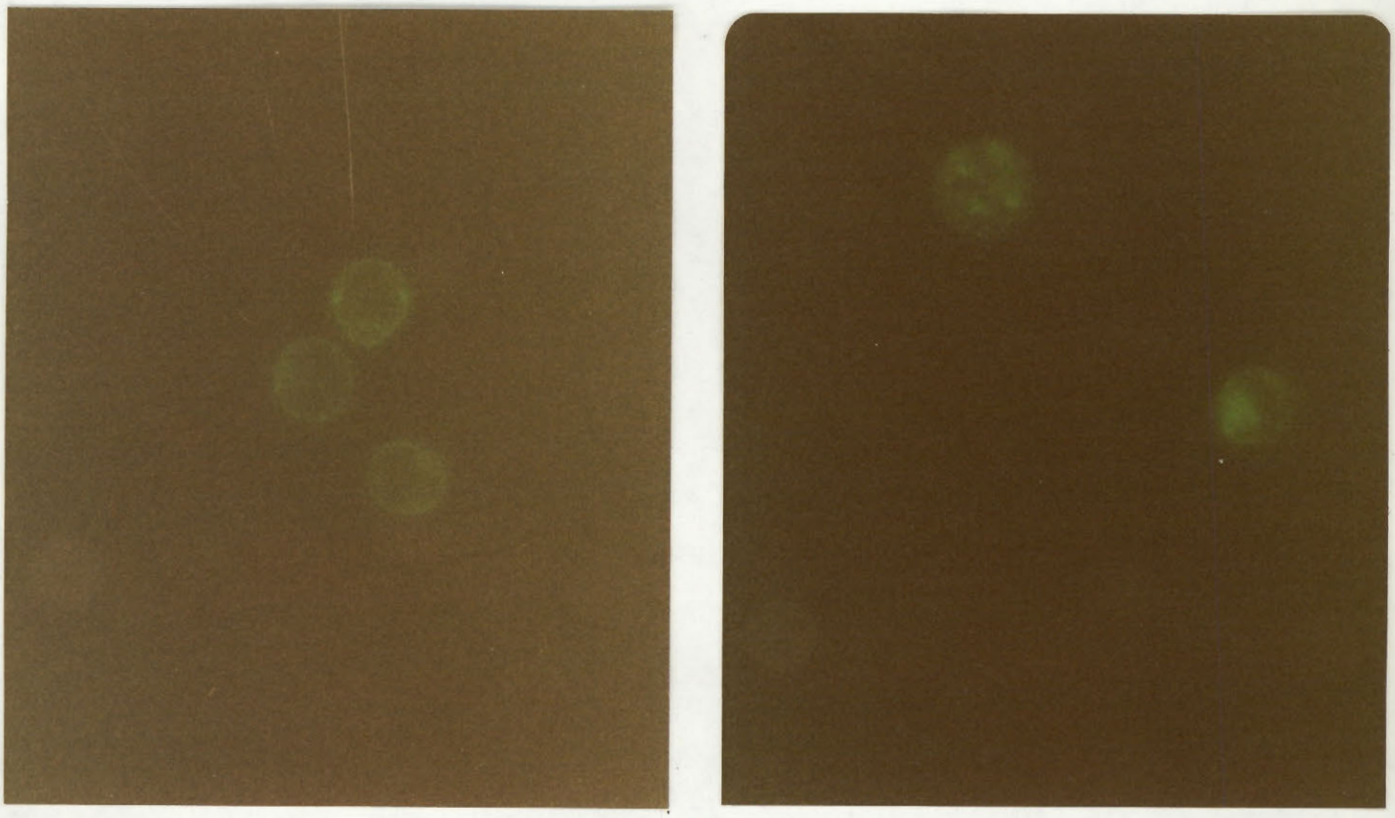
Fig. 4-15

Fig 4-15 Dissociation of Cell Bound Radioactivity by Acid Glycine Buffered Saline

Samples of the culture were taken at intervals and centrifuged at 12,000 x g for 1 minute. The cell pellets obatined were resuspended in 0.4 ml PBS (pH 7.4) at 0°C and an additional 0.4 ml of ice cold 0.2 M glycine buffered saline (pH 2.3) was added. The sample was layered on top of a 15 ml tube containing 12 ml of 5% sucrose in 0.1 M glycine buffered saline (pH 2.3) and centrifuged at 4°C at 6,000 rpm using a Sorvall HB4 rotor. The pellets obtained were counted. The dissociable radioactivity was obtained by substracting the remaining cell (residue) bound radioactivity from the total radioactivity in the sample prior to centrifugation. ■ total radioactivity; ▲ dissociable by acid glycine buffered saline; ● radioactivity associated with the residue.

Metabolic Requirement for the Antibody Induced Redistribution and Externalization

The distribution of immunofluorescence on the reticulocyte surface remained diffuse during incubation with FITC-antibody at 0°C or during incubation in phosphate-buffered saline (pH 7.4) at 37°C. In contrast, absence of serum in the culture medium did not affect the redistribution (Fig. 16). The experiments suggest that metabolic activity was required for the induced redistribution of the receptor. The incubation conditions which reduced redistribution of the immunofluorescence also reduced the externalization of ^{125}I-labelled antibodies (Fig. 17) suggesting that the redistribution and externalization are linked events and it appears that externalization of the antibody-receptor complex follows the redistribution of the complex. Moreover, transferrin was shown not to affect the externalization (Fig. 17).



a. b.

Fig. 4-16. Metabolic Requirement for FITC-antibody Induced Redistribution on the Surface of Sheep Reticulocytes

Experimental conditions were similar to those described in Fig. 10, except that phosphate buffered saline (pH 7.4) or serum free culture medium was used to replace the control, serum containing, culture medium. The incubation was carried out at 37°C for 30 minutes.

a. cells in phosphate-buffered saline.

b. cells in serum free culture medium.

Labelled cells in control culture medium maintained at 0°C for several hours also showed a uniform distribution of immunofluorescence on their surface as cells in a.

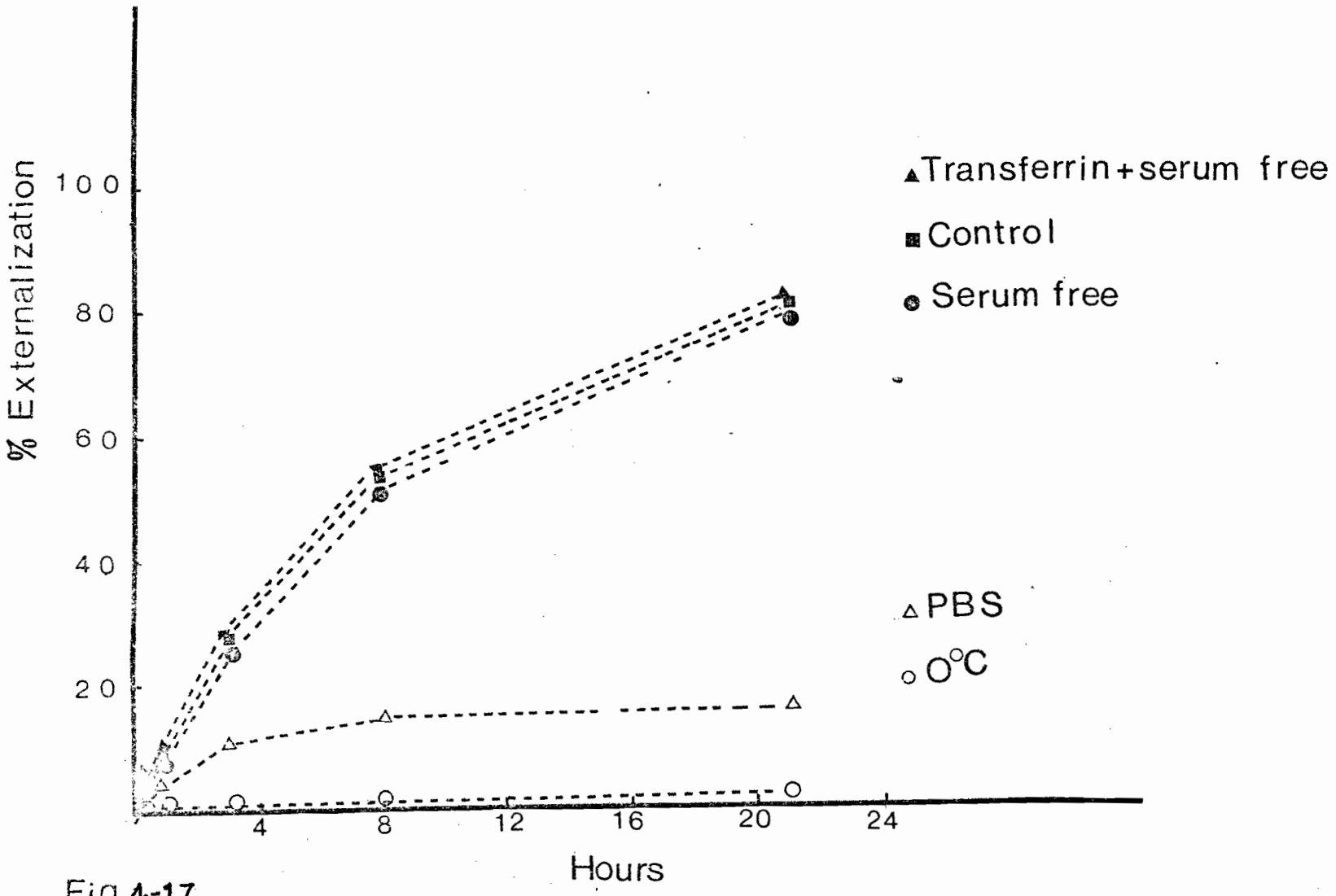

Metabolic Requirement for Externalization
% Externalization
100
80
60
40
20
4
8
12
16
20
24
Hours
▲Transferrin+serum free
■Control
●Serum free
△PBS
○O°C

Fig.4-17

Fig. 4-17. Metabolic Requirements for the Externalization of the Cell bound Radioactivity

Experiments were conducted similarly as described in Fig. 14 except that (■) culture medium containing 2% fetal calf serum (control) was replaced with (△) phosphate buffered saline (pH 7.4); or (●) serum free culture medium; or (▲) serum free culture medium containing transferrin (100 µg/ml). (○) serum plus culture medium was used but incubation was carried out at 0°C.

DISCUSSION

The results presented demonstrate that the anti-transferrin receptor antibody is able to induce redistribution, followed by externalization, of transferrin receptors in sheep reticulocytes. The phenonmenon follows a pattern similar to that observed in anti-Ig or anti-IgM induced redistribution and internalization in the B lympyocyte or the culture B lymphoblastoid cell, namely: 1) binding of the antibody; 2) selective clustering, patching and/or capping and; 3) externalization of the antibody-receptor complex (instead of internalization in that induced by anti-Ig or anti-IgM). Patching or capping did not precede vesicle formation.

As shown by fluorescence or electron microscopy, the transferrin receptor was initially diffusely distributed on the reticulocyte surface. At 0°C or with prefixed cells, the fluorescence or immunoferritin remains diffuse in a manner analogous to that reported with antibodies or other ligands in lymphocytes or other cells (Schreiner and Unanue, 1976; Salisbury, Condeelis and Satir, 1980; Goldstein, Anderson and Brown, 1979; Pastan and Willingham, 1981; Singer et al., 1978). When warmed to 37°C, the antibody-receptor complex started to redistribute on the cell surface. Unlike the antibody induced capping in B lymphocytes or cultured B lymphoblastoid cells, which usually required less than 30 minutes at 37°C (Taylor et al., 1971; Salisbury, Condeelis and Satir, 1980), the redistribution of the antibody-transferrin receptor complex into caps on sheep reticulocytes took several hours at 37°C.

It has been proposed that lateral mobility of surface receptors in reticulocytes is limited to certain mobile domains in the membrane,

while other areas of the membrane are immobile, (Tokuyasu, Schekman and Singer, 1979; Zweig and Singer, 1979). The present data, which show that the antibody-transferrin receptor complex is capable of forming patches and/or caps suggest that there is substantial lateral mobility of the transferrin receptor in sheep reticulocyte plasma membranes.

Along with redistribution on the cell surface, the antibody-transferrin receptor complex was externalized from the cell surface through the release of vesicles. The conclusion that vesicles were released was based on the observations that (1) the particles released may be centrifuged down in 1 hour at 100K x g; (2) the centrifuged particles may be visualized in the EM after negative staining and they had a vesicular appearance (Fig. 8, 9, 12); (3) these particles appeared in the void volume of a Sepharose 6B column; (4) the ^{125}I-containing particles banded at a sucrose density of ~40%; (5) electron microscopic study showed that vesicle was undergoing externalization.

That the released vesicles contain antibody, transferrin and transferrin receptor is shown by the observations that (1) the vesicles were retained by protein A columns indicating the presence of IgG; (2) the eluates of the protein A column contained polypeptides which comigrated with transferrin receptor and transferrin as detected by PAGE. Coupled with their appearance under EM and the knowledge that antibodies are on the vesicles, it is tempting to speculate that the knob-like structures on the vesicles surface may represent the anti-transferrin receptor antibody.

Kinetic studies using ^{125}I-antibodies and immunofluorescence studies using FITC-antibodies showed that release of antibody-containing

vesicles started when the reticulocytes with bound antibody were warmed to 37°C and continued throughout the period during and after patch (or cap) formation. It has been shown that patch (or cap) formation does not necessarily precede internalization of anti-surface antibody in lymphoid cells (Schreiner and Unanne, 1976; Linthicum and Sell, 1974; Salisbury, Condeelis and Satir, 1980). The rate of externalization decreased with time and did not appear to follow simple first order kinetics. It is not known what factors affect externalization although the process appeared to depend on metabolic activity.

If the antibody-receptor complex clusters in the membrane are to undergo externalization, the clustered domains have to extrude outwardly first and then fuse at the base to form sealed vesicles. Fig. 8, 9, 12 show that some externalized vesicles can be seen where one end is clustered with knob-like structure and the other end is free from knobs. The knob-free end may represent the base where membrane fusion occurs during the induced externalization. It has been proposed that the erythrocyte membrane associates with the underlying cytoskeletal system through the integral membrane proteins (for review see Branton, Cell, 1981). Prior to externalization and membrane fusion the integral membrane proteins must first dissociate from the underlying network and the released vesicles should be free from spectrin. The results obtained are consistent with this prediction since by gel electrophoresis of the released vesicles no spectrin is detected. Previous reports have also shown that membrane vesicles released from erythrocytes with increased intracellular Ca^{2+} (Allan et al., 1976) or ATP depletion (Lutz et al., 1976); vesicles endocytosed in erythrocyte ghosts (Hardy and Schrier,

1978; Hardy, Bensch and Schrier, 1979); and vesicles internalized in reticulocytes after exposure to Con A (Tokuyasu, Schekman and Singer, 1979) are all deficient in spectrin. All these observations suggest that during vesicle formation and prior to externalization or internalization the plasma membrane becomes detached from the cytoskeleton at the site of vesicle formation.

Whether there is internalization of antibody was not established. The dissociation experiments (Fig. 15) suggested that a small percentage of radioactivity may be internalized during the incubation at 37°C. However, the gradual decrease of the absolute amount of undissociable radioactivity suggested that the internalized radioactivity may eventually become surface bound. The undissociable radioactivity remained a constant fraction of the total radioactivity bound to the cell. After 21 hours incubation at 37°C only 2% of the initial radioactivity remained non-dissociable.

It has been shown that anti-receptor antibody binding may trigger the same molecular interactions as the binding of several physiological ligands (for example, Beisiegel et al., 1981; Schlessinger, Van Obberghen and Kahn, 1980). Although it is unlikely that anti-transferrin receptor antibody binding to sheep reticulocytes will mimic the physiological effects of transferrin binding (i.e. iron delivery), both antibody binding and transferrin binding may trigger the same type of processing (such as redistribution of the receptor on the cell surface membrane and vesicle formation). Whether binding of transferrin, like binding of anti-transferrin receptor antibodies, will lead to the externalization of a vesicle containing transferrin and the receptor

is not known. Current data (Hemmaplardh and Morgan, 1976; Martinez-Medellin et al., 1972; Sullivan and Weintraub, 1976; Karin and Mintz, 1981) based on experiments of short duration (~1-2 hours) suggest that transferrin and the receptor are internalized. However, it is possible that after longer intervals, incubation with transferrin may eventually lead to a loss of receptor similar to that seen with the antibodies. Since it is known that the transferrin receptor disappears from the cell surface during the maturation process of the reticulocyte, the changes triggered in the membrane by the antibodies against the receptor may provide important clues on the processing of transferrin by the reticulocytes and on the in vitro maturation process of the reticulocyte.

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GENERAL DISCUSSION

An earlier report by Schulman and Nelson (292) demonstrated that rabbit reticulocytes have surface antigens which are absent from the erythrocyte. The experiments showed specific agglutination of rabbit reticulocytes by guinea pig antiserum raised against rabbit reticulocytes and preabsorbed with rabbit erythrocytes. In the present work, the existence of the reticulocyte surface specific antigens in sheep reticulocytes was shown by immunofluorescence, radioimmunoprecipitation, immunoaffinity chromatography, and a radioimmunobinding assay with sheep erythrocyte preabsorbed rabbit anti-sheep reticulocyte antiserum or purified rabbit antibodies to the sheep reticulocyte surface specific antigens.

The sheep reticulocyte surface specific antigens appear to be largely integral membrane proteins since detergent is required to solubilize the antigens.

Using immunoprecipitation of detergent solubilized ^{125}I-labelled plasma membranes, it was shown that the amount of ^{125}I-labelled immunoprecipitate decreases gradually with time in culture. After four days in culture, very little specific immunoprecipitate was detected. The correlation between the disappearance of these surface antigens and the membrane associated functions such as Na^+-dependent amino acid transport and transferrin binding during reticulocyte maturation indicates that these antigens may represent the Na^+-dependent amino acid transporter or transferrin receptor or both.

To test this possibility, the antibodies to sheep reticulocyte specific antigens were first purified. The purification involved the use of

intact reticulocytes to absorb the antibodies from the sheep erythrocyte preabsorbed antiserum.

The antibodies absorbed to reticulocytes were eluted from the cell surface and further purified by protein A affinity chromatography. The use of intact reticulocytes to absorb out the antibodies assures that the antibodies obtained are directed against reticulocyte surface antigens. Although two of the steps involved in the purification required exposure of cells and antibodies to acidic conditions, the immediate neutralization of the acid presumably prevented the denaturation of the antibodies and cell lysis. The biological activity of the purified antibodies is evident from the immunofluorescence, immunoaffinity chromatography, and immunobinding experiments.

Three polypeptides corresponding approximately to 186,000, 93,000, and 78,000 M.W. respectively were purified from reticulocyte but not erythrocyte plasma membrane when using the purified antibodies coupled to sepharose 4B. The molecular weights estimated in this work differs from that estimated by radioimmunoprecipitation using Staphylococcus aureus followed by gel electrophoresis (molecular weights approximate to 200,000, 95,000, and 85,000). The discrepancy between the two molecular weight estimates may be due to the different procedures used to estimate the molecular weights.

The estimated molecular weights of the polypeptides isolated by the immunoaffinity column are very close to the molecular weights reported for transferrin receptor and transferrin by other investigators (260,262,265,274,277,280,281,282).

The conclusion that the reticulocyte specific surface antigen is the transferrin receptor is based on the observations that (a) the

polypeptides isolated by the immunoaffinity column co-electrophoresed in the SDS-polyacrylamide gels with polypeptides isolated with a transferrin affinity column, (b) prepassage of the material through one column removed all material capable of binding to the second column, (c) ^{125}I-tyrosyl peptide mapping indicates that the 186,000 polypeptide is a dimer of the 93,000 polypeptide and the 78,000 polypeptide is sheep transferrin, and (d) the control experiments showed that the antibodies are not directed against transferrin.

Knowing the increasingly important role played by the transferrin receptor in the proliferation of cells (268,272,274,275,276,277,278), antibodies against the transferrin receptor may provide a powerful probe to study the possible molecular mechanism for transferrin receptor function and modulation during cell growth. Moreover, despite the presence of transferrin receptors on normal cells such as erythroid cells and placental trophoblasts (264,265,266,267), the relative abundance of the transferrin receptor on tumor cells growing in vivo or in vitro compared to that in normal tissue (268,275,279) makes the transferrin receptor a possible marker to detect a growing tumor cell (275,284). Indeed, anti-transferrin receptor antibody has been used as a carrier of toxin to direct the toxin to the growing tumor cells thereby specifically killing the cells in vitro (284). Anti-transferrin receptor antibody has also been shown to inhibit the growth of human melanoma cells in nude mice (284). All the data indicate that anti-transferrin receptor antibodies may have potential in cancer chemotherapy.

Although monoclonal antibodies against the transferrin receptor of malignant cells (272,274,275) or polyclonal antibodies prepared by using

conventional methods (283,293) have been prepared by several investigators, the procedure used here for preparation of anti-reticulocyte surface specific antibodies (anti-transferrin receptor antibodies) is simple and reproducible, providing a good way to obtain the antibody to study the behaviour of the transferrin receptor.

The conclusion that the 186,000 polypeptide shown on the SDS-gel is dimer of the 93,000 polypeptide is consistent with that of other investigors (260,272,275,280,281,282) who have suggested that the active form of the receptor may be a disulfide linked dimer of the 93,000 polypeptide.

Although in some studies reported (293), anti-transferrin receptor antibody has been shown to inhibit iron uptake or transferrin binding, the current studies show that these antibodies do not bind to the receptor at the same site as transferrin. Thus when the solubilized membrane components (transferrin receptors) are retained by a transferrin affinity column, the column will retain the antibody. No antibody is retained in the absence of the membrane components (transferrin receptors). This principle is also true for the retention of transferrin by the immunoaffinity column. These results are consistent with the conclusion arrived at by others that excess transferrin did not inhibit the binding of monoclonal antibody to extracts of leukemic cells (274) and may explain the earlier experiment of Schulman and Nelson (292) that transferrin did not inhibit the agglutination of rabbit reticulocytes by guinea pig anti-rabbit reticulocyte specific antiserum.

Since transferrin was coisolated with transferrin receptor by the transferrin affinity column, it suggests that the transferrin receptor has more than one binding site for transferrin. This is

consistent with recent evidence that transferrin receptor has two binding sites for transferrin (282).

That the transferrin receptor spans the plasma membrane was suggested by the accessibility of the receptor both from cytoplasmic and external surfaces to lactoperoxidase catalyzed ^{125}I-iodination. Many cell surface receptors have been shown to be transmembrane, e.g. acetylcholine receptor (294). The transmembrane structure is important because it provides a basis for transmembrane control of ligand-receptor interaction.

Anti-surface receptor antibody has been shown to be useful in the study of the behaviour of the surface receptor when challenged with ligand. For example, a monoclonal antibody to the low density lipoprotein receptor has been demonstrated to mimic the low density lipoprotein in that the antibody also induces the low density lipoprotein receptor to undergo redistribution and internalization (156). Anti-insulin receptor antibody also cocapped with insulin on cultured human lymphocytes (155). Moreover, anti-insulin receptor antibody has also been shown to mimic the physiolgical function of insulin (150,151,152,153,154). Therefore, anti-transferrin receptor antibody may provide a good tool to study the behaviour of transferrin receptor not only in the transferrin-transferrin receptor interaction but also the possible mechanism of transferrin receptor disappearance during reticulocyte maturation.

The anti-transferrin receptor antibody-transferrin receptor complexes were diffusely distributed on the cell surfaces initially as were shown by immunofluorescence technique at 0°C or with prefixed cell. However, when the unfixed FITC-antibody labelled reticulocytes were warmed up to 37°C, the antibody-receptor complexes redistributed into

clusters, patches, and/or eventually into caps (usually bipolar or tripolar). Uniform fluorescent vesicles were continous released from the cell surface into the medium during and after the patch and/or cap formation in cells labelled with FITC-antibody. The externalization was followed by a kinetic study using ^{125}I-antibody. The externalization of the antibody-receptor complex appears to be associated with the redistribution since incubation at 0°C or replacing culture medium with phosphate buffer saline pH 7.4 reduces externalization of antibody-receptor complexes as well as redistribution. Patch or cap formation do not appear to be required for externalization. This antibody induced phenomenon is similar to the anti-surface Ig or surface IgM antibody induced capping and internalization in B lymphocytes (74) and cultured B lymphoblastoid cells (141) respectively. In the case of antibody (75) or many other ligand (113,114) induced redistribution and internalization, the antibody or ligands are internalized and then presumbly degraded in the lysosomes. However, instead of internalization and degradation in lysosomes, the anti-transferrin receptor antibody-receptor complex of sheep reticulocytes is externalized. Although the reasons are unknown, differences between the behaviour of reticulocytes and other cells may be related to the loss of intracellular structures in the reticulocyte. Whether the anti-transferrin receptor antibody also cause externalization of the transferrin receptor in vivo is not known.

Externalization of the cell bound antibody seemed complete since more than 80% of the cell bound ^{125}-antibody was externalized after 21 hours of culture in vitro and most of the remaining cell bound radioactivity was surface bound(Fig. 14, 15, chapter 4).

However, as shown from Fig. 15 (chapter 4), there is a small increase in the nonelutable radioactivity by acidic glycine buffered saline when the ^{125}I-labelled cells were transferred to 37°C. The amount of nonelutable radioactivity decreased gradually but was maintained at a constant fraction of the cell associated radioactivity. One possible suggestion is that this nonelutable radioactivity may represent the antibody-receptor complex which was internalized and therefore became inaccessible to the acidic glycine buffered saline (pH 2.3). If this interpretation is correct, then this nonelutable amount of material may represent a steady state between internalized and externalized antibody-receptor complexes and that the antibody-receptor complex may be internalized and then recycle back to the plasma membrane and eventually externalized.

When the vesicles isolated by protein A affinity column were electrophoresed in SDS-polyacrylamide gel, it was shown that in addition to the background peptides which were present in the initial culture medium the major peptides appearing were peptides which coelectrophoresed with the transferrin receptor, transferrin and antibody. The relative absence of other reticulocyte membrane peptides provides biochemical evidence that the transferrin receptor and the antibody are selectively clustered into vesicles and then externalized. The presence of transferrin on the released vesicles may be due to transferrin which was bound to the reticulocytes during incubation in the serum containing medium.

Two lines of evidence suggest that transferrin may induce a phenomenon similar to that of the antibody. First, the coisolation of peptides which coelectrophoresized with transferrin in the antibody

induced externalized vesicles. Second, the kinetic studies (Fig. 17, chapter 4) which showed that transferrin did not affect the antibody induced externalization. Whether transferrin binding also results in the externalization of transferrin-receptor complexes is not yet known. If transferrin binding results in the externalization of transferrin-receptor complexes in a similar way to that induced by antibody, the question then will be how this can be reconciled with other reports which showed the internalization of transferrin-receptor complexes during iron transport (131,271,288,289,290,291). Both events may, of course, occur if the transferrin-receptor complex is first internalized and then recycles back to the plasma membrane where it is eventually externalized. This hypothetical model of antibody and transferrin-receptor complex pathway is consistent with the transferrin-receptor pathway proposed by Morgan in iron transport (241). In Morgan's hypothesis, the iron-transferrin-receptor complex is internalized and, after release of the iron carried by the transferrin, the transferrin-receptor complex then recycles back to the plasma membrane where the transferrin is released. However, the receptor remains in the membrane. In the present work, the data suggest that receptor is externalized from the membrane.

The hypothetical model for transferrin induced redistribution and externalization may also reflect the normal mechanism for the disappearance of the transferrin receptor during reticulocyte maturation. The ability of reticulocytes to synthesize proteins is almost at an end. The continuous binding of transferrin to the receptor will eventually result in loss of all the transferrin receptor on the cell surface during reticulocyte maturation.

In conclusion, antibody induced redistribution and externalization of membrane proteins in sheep reticulocytes not only provides an example of ligand induced redistribution and externalization in normal cells but may also be used as an excellent model to study ligand-receptor interaction across the membrane.

Contributions to Original Knowledge

1. Isolation and identification of anti-sheep reticulocyte surface specific antibodies as anti-transferrin receptor antibodies.
2. Isolation and identification of sheep reticulocyte surface specific antigens (proteins) as transferrin receptors.
3. Demonstration that the transferrin receptor disappears during sheep reticulocyte maturation in vitro.
4. Demonstration that the transferrin receptor is a transmembrane protein in sheep reticulocytes.
5. Demonstration that the antigenic sites differ from the transferrin binding sites on the transferrin receptor of sheep reticulocytes.
6. Demonstration that the transferrin receptor has a dimeric structure by showing that ^{125}I-tyrosyl peptide maps of the 186K and 93K polypeptides from a SDS-polyacrylamide gel are nearly identical.
7. Demonstration with immunofluorescence and immunoferritin labelling technique that anti-transferrin receptor antibody-transferrin receptor complexes undergo redistribution on the surface of sheep reticulocytes.
8. Demonstration that anti-transferrin receptor antibody-transferrin receptor complexes undergo externalization using ^{125}I-labelled and FITC labelled antibodies or with immunoferritin labelling technique.
9. Isolation of the externalized vesicles by sepharose 6B filtration.
10. Demonstration that the antibody containing vesicles are free from most plasma membrane proteins including spectrin but contain three polypeptides identified as transferrin receptor, transferrin and antibody.

11. Electron microscopic studies which show the uniform size of the vesicles and their unusual structure.
12. The dependence of antibody induced redistribution and externalization on metabolic activity.

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