VISUALIZATION AND CHARACTERIZATION OF CELL SURFACE GLYCOPROTEINS ON MOUSE NEUROBLASTOMA CELLS

by

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ABSTRACT

The cell surface glycoproteins of mouse neuroblastoma cells were visualized using fluorescent lectins in combination with fluorescent light microscopy and SDS-gel electrophoresis, lectin-microsphere conjugates in combination with scanning electron microscopy, and iodinated lectins. The lectins used in these studies were: Concanavalin A (ConA) which is specific for α-D-mannose and α-D-glucose groups, wheat germ agglutinin (WGA) which binds to N-acetylglucosamine oligomers and *Ricinus communis* agglutinin (RCAI) which is specific for D-galactose residues.

All three lectins were found to have over 10^7 high affinity ($K_d = 2 \times 10^{-7}$ M) binding sites on the neuroblastoma cell surface. These sites were found to be densely and randomly distributed over the surfaces of cells which were fixed with glutaraldehyde before labelling or labeled at 4°C. However, when the cells were labeled at 23°C or 37°C, the lectin receptors were found to undergo redistribution. All three lectins were internalized by the cells in an energy-dependent process when the cells were treated with the lectins for 60 min or more at 37°C. However, the patterns of redistribution for the different lectin receptors were not the same.

When cells were labeled with ConA, the receptors were shown to patch and then clear from the surface of the cells. Once the label had completely cleared from the cell surface, the cells could not be labeled with additional ConA, even when the first labelling was done using ConA at concentrations well below saturation. However, when cells were treated in the same way with WGA or RCAI, a heavy, uni-

form display of marker was seen on the cell surface at all times. It was only when the cells were briefly labeled with these lectins and then incubated in buffer that it became apparent that these receptors could also patch and clear from the cell surface. In addition, in order to clear all the receptors for these two lectins from the cell surface, it was necessary to label the cells with saturating concentrations of lectin. Thus, labeled and unlabeled ConA receptors on the neuroblastoma cells undergo co-ordinate redistribution whereas labeled WGA and RCAI receptors redistribute independently of their unlabeled counterparts. Studies with drugs which disrupt the various cytoskeletal elements suggested that the microfilament system played a role in the co-ordinate redistribution of ConA receptors.

Double labelling studies with both different iodinated and fluorescent lectins indicated that the binding sites for WGA were not associated with those for ConA. WGA binding sites did appear to be directly associated with many of the binding sites for RCAI. Studies with ConA and RCAI were not carried out because it was shown that ConA binds to RCAI.

Using the fluorescent lectins in combination with SDS-gel electrophoresis, the lectin-binding polypeptides from the neuroblastoma cell membranes were identified and characterized. Plasma membranes were purified from the neuroblastoma cells using hypotonic disruption followed by differential and isopycnic gradient centifugation. The membrane preparation showed a 10-fold increase in the specific activity of two plasma membrane markers and little contamination by other cell components. The membranes were dissociated in SDS and run on SDS-polyacrylamide gels to separate out the different polypeptides. The gels were fixed and stained with fluorescent lectins. WGA and RCAI were both found to bind

almost exclusively to a single polypeptide with a molecular weight of 30,000 daltons. This polypeptide also stained strongly for carbohydrate after periodate oxidation. ConA, on the other hand, bound to over 20 polypeptides, most of which had molecular weights between 60,000 and 120,000 daltons. However, when the cells were made to internalize all their ConA receptors and their membranes isolated and run on gels, four of the ConA-staining polypeptides were found to be absent as compared to membranes from untreated cells. Thus, it appears that only four of the ConA-binding polypeptides seen on the gels were available for ConA binding at the cell surface. These polypeptides also labeled with ^{125}I when intact cells were treated with ^{125}I and lactoperoxidase.

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ABBREVIATIONS

5-BrdU	bromodeoxyuridine
BSA	bovine serum albumin
Bt_2cAMP	dibutyryl cyclic AMP
ConA	concanavalin A
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
DTBP	dimethyl-3-3'-dithiobispropionimidate
FCS	fetal calf serum
Fl	fluorescein
D-glcNAc	N-acetyl-D-glucosamine
H-CAB	HEPES-ConA buffer
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IgG	immunoglobulin G
ma	milliampere
MEM	minimal essential medium
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NP-40	Nonidet P-40
PAS	periodic acid-Schiff base
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
RBC	red blood cell
RCAI	*Ricinus communis* agglutinin (120,000 daltons)
RCAII	*Ricinus communis* agglutinin (60,000 daltons)
Rh	rhodamine

rPBS	ricin phosphate-buffered saline
SEM	scanning electron microscope
SDS	sodium dodecyl sulfate
T-CAB	Tris-ConA buffer
TEM	transmission electron microscope
TEMED	N, N, N', N'-tetramethylenediamine
TCA	trichloroacetic acid
WGA	wheat germ agglutinin

Chapter 1

INTRODUCTION

The unique property of life is the individuality of single living organisms (1). This individuality implies the existence of a physical boundary which separates a living organism from its environment. Without this boundary, or membrane, an organism would be indistinguishable from its surroundings. Thus, the plasma membrane of cells must have very ancient origins, indeed. As well as delineating the living organism, the membrane must also be able to permit a certain amount of interaction between the organism and its environment.

The Plasma Membrane

The existence of the plasma membrane and many of its properties were deduced long before it was actually visualized and isolated. By the late 1800's it had been shown that a barrier surrounded cells which made them only semi-permeable; water molecules could freely penetrate into the cell but many other molecules could not (2, 3). The relative ease of penetration of different molecules was found to parallel their lipid solubility, suggesting the semi-permeable barrier was composed of a thin layer of lipid (2, 3). However, not all the properties of membranes corresponded

to those of a soap bubble, the basic model for lipids in an aqueous environment, which suggested that the membrane contained something else besides lipid. This something else was thought to be protein. These observations led to the development of a model of membrane structure by Davson and Danielli in 1934 (2). The central feature of this model was a continuous bimolecular lipid layer arranged so that the hydrophilic portions of the lipids extended towards the aqueous environment while the hydrophobic portions met in the middle. The lipid bilayer was thought to be coated on both sides by a layer of adsorbed protein. Further studies showed that small, polar, organic molecules could penetrate into cells at a much faster rate than could be accounted for by their lipid solubility (2). This led to the suggestion that the plasma membrane was transversed by small, aqueous channels or pores. This idea as well as new knowledge of protein structure led to a revised model of membrane structure proposed by Danielli in the mid 1950's (2, 3). The central feature of this model was still a more or less continuous lipid bilayer. In addition, protein-coated pores were pictured as penetrating through the bilayer. The protein was thought to be in two layers at each lipid:water interface; a layer of bound hydrophobic protein more or less interwoven with the lipid and a second layer of loosely attached, absorbed protein, probably in extended β sheet form. This unit membrane model held up until the early 1970's as the most acceptable theory of membrane structure. The idea of a lipid bilayer was consistent with the experimental evidence. The problem lay with the nature of the membrane proteins and this was its downfall. A continuous unfolded sheet of protein would have large numbers of hydrophobic amino acids exposed to the environment, a thermodynamically unsatisfactory condition (4). In

addition, the hydrophilic portions of the phospholipids of the lipid bilayer were found to be susceptible to enzymatic attack and did not appear to be complexed to proteins to the extent suggested by the unit membrane model (5). Indeed, most of the membrane porteins were found to be extremely heterogeneous, globular and with regions of high hydrophobicity (4). These and other observations led to the development by Singer of the fluid mosaic model in the early 1970's (4, 5, 6, 7). In its simplest form, the plasma membrane was visualized as a two-dimensional solution consisting of a mosaic of integral proteins embedded in a fluid lipid bilayer with peripheral proteins loosely attached to both surfaces (see Figure 1.). The integral membrane proteins, which make up 70-80% of the membrane proteins, are globular in structure and amphipathic in nature (6, 7). This means that the most stable structure is one in which the hydrophobic regions of both the phospholipids and integral proteins are sequestered together within the centre of the membrane while their hydrophilic groups are maximally in contact with the aqueous environment at both faces of the membrane (7, 8). The integral proteins are extremely heterogeneous both within a single membrane and between different types of membranes (5, 6, 7, 8). Some integral proteins can span the entire membrane whereas others project from only one side or the other. In addition, some of these proteins can exist together in complexes. Complexes of this type could provide the pores known to penetrate the membrane. Most membrane-associated enzymes, antigenic sites, transport proteins and drug, hormone and lectin receptors can be classified as integral proteins (7). Integral membrane proteins do not appear to change their orientation with respect to the cell surface once they are inserted into

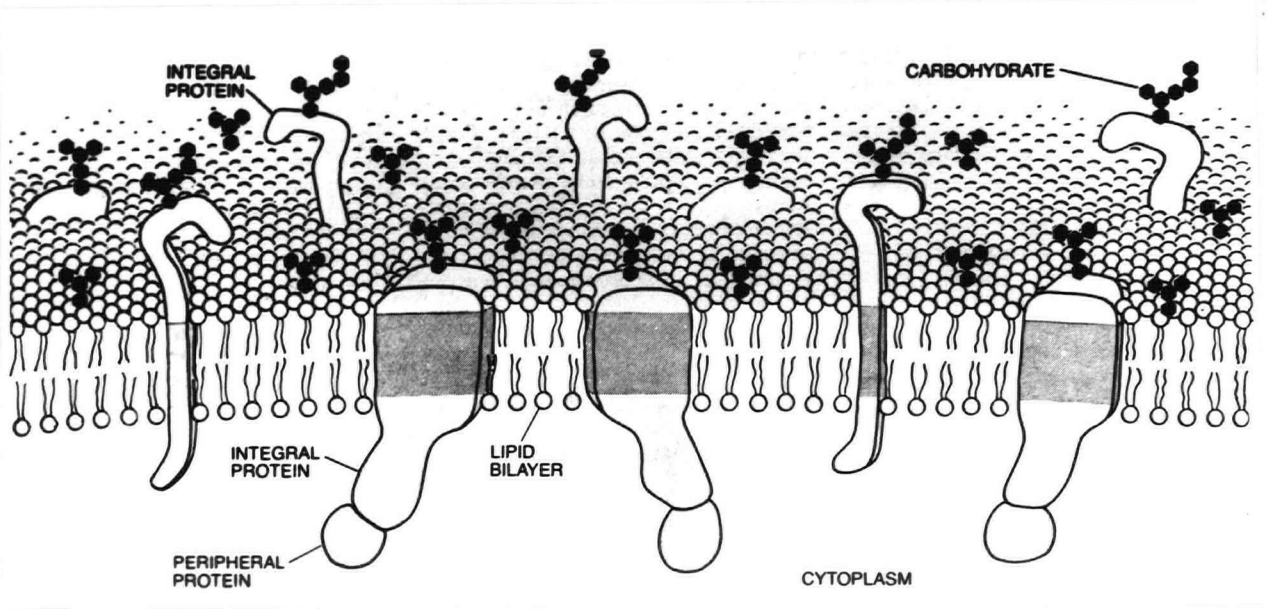


Figure 1. Fluid mosaic model of the plasma membrane.

the membrane (5, 6, 7, 8). In other words, they do not rotate from one side of the membrane to the other. The major effect of this phenomenon is the asymmetric distribution of membrane components. Thus, the carbohydrate portions of membrane glycoproteins and glycolipids are localized exclusively on the outer surface of the plasma membrane. Many serve as receptors for antibodies, hormones, lectins, viruses and other agents (6). One of the best understood integral proteins is glycophorin, the major glycoprotein of the human erythrocyte membrane (8). A clearer picture of the nature of integral proteins may be achieved by a brief discussion of the results of studies on this protein. Glycophorin is, by weight, 60% carbohydrate and 40% protein, has a molecular weight of 31,000 and consists of a single polypeptide chain of 131 amino acids. It is organized into three distinct domains. The NH_2 terminal region (64 residues) contains predominantly hydrophilic amino acids and all sixteen of the oligosaccharide chains. This part of the glycophorin molecule extends outside the cell and serves as a receptor for several lectins and influenza virus. It also contains the MN and ABO blood-group antigens. The second section of the protein, made up of 32 nonpolar amino acids, is highly hydrophobic and is tightly associated with the lipids within the interior of the membrane. The COOH-terminal section of glycophorin contains 35 predominantly hydrophilic amino acids and is exposed at the cytoplasmic face of the red blood cell (RBC) membrane.

The peripheral proteins (7), on the other hand, are only weakly bound to the surfaces of cell membranes and do not appear to interact with the membrane lipids. Unlike the integral proteins, the peripheral proteins can be eluted from membranes by mild treatments which only disrupt ionic interactions.

There is also evidence for a partial asymmetry in the distribution of the membrane lipids, at least in the erythrocyte (8). In this case, phosphatidylcholine, sphingomyelin, cholesterol, and glycolipids are more concentrated in the outside face while phosphatidylserine and phosphatidylethanolamine are found mainly in the inner face of the lipid bilayer.

One of the major consequences of the fluid mosaic structure of membranes is the ability of the membrane components to move laterally in the plane of the membrane (5, 6, 7, 8). The fluidity of the lipid bilayer seems to depend on a number of factors, including the fatty acid composition of the membrane, and the temperature, but is relatively uniform throughout the membrane (phospholipid diffusion constant, $D = 10^{-8}$ $cm^2 \cdot sec^{-1}$) (8). Measurements of the lateral mobility of different membrane proteins have found that not all proteins move at the same rate. Indeed, some proteins appear to diffuse freely whereas others move more slowly, or in some cases, not at all (5, 6, 8). This implies that there is more involved in the movement of the membrane proteins than the fluidity of the lipid bilayer. Nicolson (5) has proposed several mechanisms whereby the control of membrane protein mobility could be brought about. Two of the more important of these mechanisms are: (a) the association of membrane components in the plane of the membrane giving rise to aggregate formation and (b) the association of membrane components with "restraining" molecules at the inner or outer membrane surfaces. Cell junctions are probably the best known examples of restricted movement brought about by association of membrane components (5). However, the second mechanism is the most important with respect to general control of membrane protein

mobility since most large protein aggregates consist of only one or two protein species and only occupy a small percentage of the total cell surface (5, 7). In most of the cases studied so far the restraining elements appear to be the cytoskeletal proteins associated with the inner surface of the cell membrane. A few of these, such as spectrin which is associated with the inner surface of the red blood cell membrane, fall into the class of peripheral proteins (8). The membrane-associated cytoskeletal proteins include a number of proteins which are also a part of the contractile system of skeletal muscle cells (8, 10, 11). The microfilaments (60 Å diameter) can be found closely associated with the cytoplasmic surface of the cell membrane (9, 10). They contain predominantly actin, the major protein of the thin filaments of skeletal muscle. Myosin, the major protein of muscle thick filaments, has been found associated with actin in the microfilaments as has tropomyosin (9, 10). This suggests that the cytoskeletal elements may be capable of undergoing contraction. The microfilaments are thought to be attached in places to the cell membrane (10, 11). This attachment may be direct or indirect and probably involves one or more integral proteins. One of the proteins which has been implicated in this association is α-actinin, which forms the Z line to which the thin filaments attach in skeletal muscle (10, 11). Microtubules (250 Å diameter), made up of tubulin, are also considered to be membrane-associated cytoskeletal proteins (9). However, they appear to be localized more deeply within the cell than the microfilaments and do not seem to connect directly to the cell membrane (9, 10). A diagram of one proposed arrangement of these proteins is shown in Figure 2.

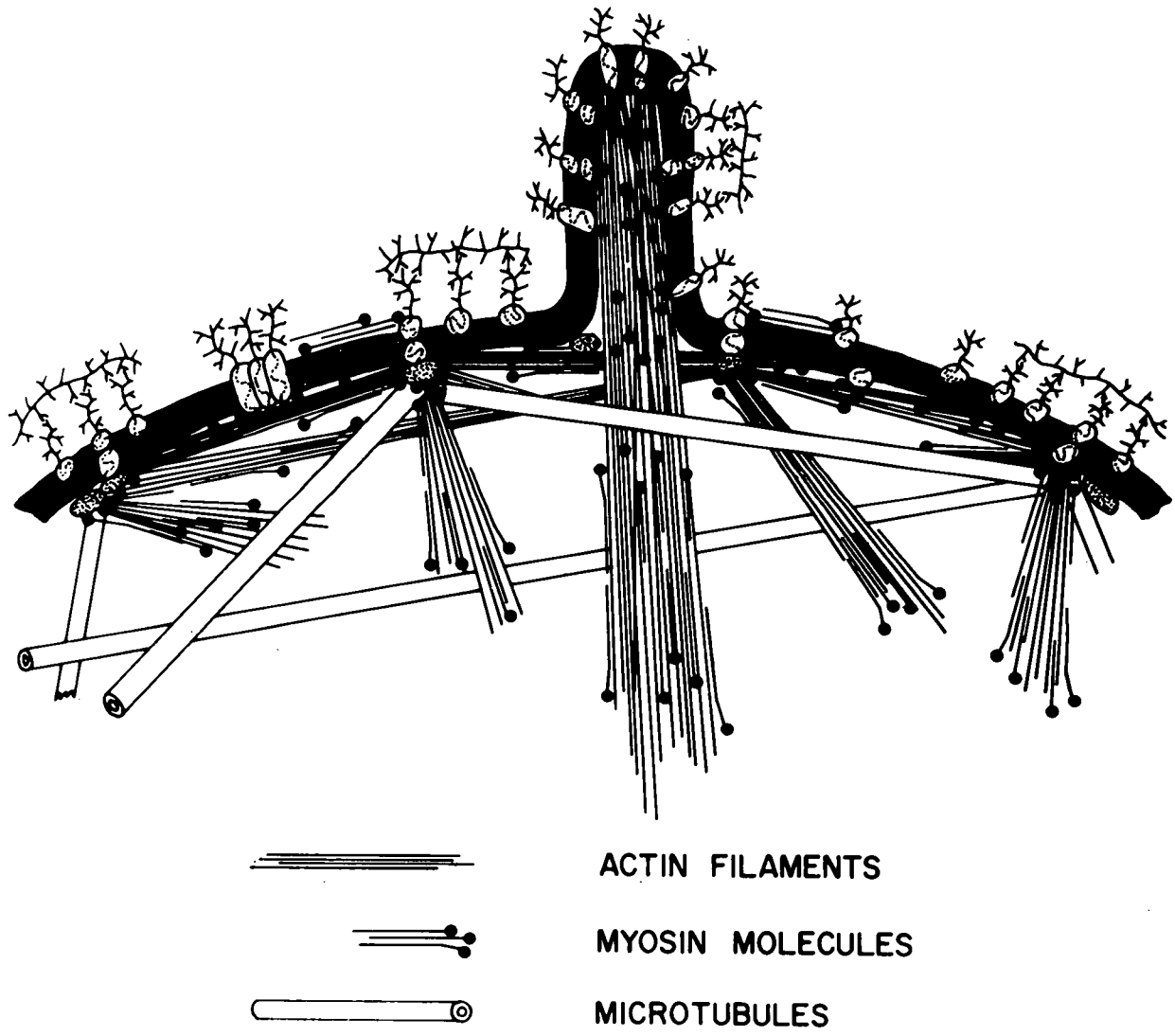


Figure 2. One model of the interactions between membrane-associated microtubule and microfilament systems involved in transmembrane control over cell surface receptor mobility and distribution. The microfilaments are thought to be contractile whereas the microtubules are envisaged as skeletal elements (from (5)).

Membrane constituents such as bound water and divalent cations may also play important roles in the control of membrane protein organization (8).

The outside surface of cells is often not smooth but rather can have a variety of membrane elaborations (8). These are particularly common on tissue culture and lymphoid cells and include microvilli (slender, finger-like projections), blebs (small spherical projections), filopodia (extremely long, thin projections) and lamellopodia, folds or ruffles (broad flattened projections).

Cell Surface Labelling Techniques

A number of techniques have been developed to study the topography of selected membrane proteins. In general, these techniques all use methods to specifically tag ligands which have been found to bind to cell surface receptors. The techniques can be divided into two broad classes: radiochemical and microscopic. In order to discuss these techniques the example of lectins (proteins that bind to specific oligosaccharide sequences) will be used. However, the methodology is just as applicable to antibodies or hormones. As a matter of fact, many of the procedures were originally developed for antibodies.

The introduction of a radioactive label into a ligand molecule allows quantitative analyses of ligand-cell interactions to be made. Studies with radioactively labeled ligands can provide information on the number of ligand receptor sites and the strength of the ligand binding to these sites. They can also be used to obtain information on the relative disposition of receptors for different ligands. The most common

radioactive label incorporated into lectins is iodine; generally ^{125}I ($t_{1/2}$ = 60 days), although ^{131}I ($t_{1/2}$ = 8 days) has also been used (12). Lectins, particularly ConA, have also been labeled by acetylation using radioactive acetic anhydride (^{14}C or ^{3}H) (12). However, specific activities are generally lower than with ^{125}I and acetylation often has a greater effect on proteins than iodination of tyrosine residues. Lectins have been iodinated by both chemical and enzymatic techniques (12). The former uses chloramine T, a strong oxidizing agent which may damage some proteins. The latter method uses lactoperoxidase, an oxidizing enzyme, in conjunction with a peroxide generator; either glucose oxidase, another enzyme, or hydrogen peroxide. After iodination, the free iodine must be separated from the labeled protein. The preferred method for doing this is affinity chromatography since this assures that the iodinated lectin is biologically active (12). However, gel filtration or dialysis can be used when affinity columns are not practical (12). In order to use iodinated lectins in binding studies, the lectin should be shown to be homogeneous and free of contaminating proteins. It is also very important to ascertain that the iodinated proteins retain their biological activity (12).

Labelling studies are performed by incubating cells, or isolated cell membranes, with varying concentrations of iodinated lectin in the absence and presence of a specific sugar inhibitor of lectin binding or, more generally with unlabeled ligand (12). Specific binding is then calculated to be the difference in the amount of radioactivity bound in the two experiments. The number of receptors for a given ligand can be determined directly from the binding curve if saturation can be obtained (12). However, the number of receptor sites as well as binding affinities are

generally calculated using the Scatchard equation (105). Estimates of numbers of binding sites assume that only one ligand combines with one receptor (12). Since this may not always be the case, especially with lectins which are often multivalent, values for numbers of binding sites may be off by factors of 2 or more (12). In addition, different cells within a population may bind different amounts of label so that the value obtained for numbers of binding sites is simply an average (12).

The microscopic techniques for studying cell surface receptors can be divided into three groups depending on the instrument they are used with: fluorescent light microscope, scanning electron microscope (SEM) and transmission electron microscope (TEM). In order to study cell surface receptors with any of these instruments the ligand in question must be tagged with a marker which can be "seen" using that instrument. For light microscopy, the ligands are conjugated to fluorescent dyes (13, 14). The two commonly used dyes are fluorescein isothiocyanate (Fl) which emits a green fluorescence when excited and tetramethyl rhodamine isothiocyanate (Rh) which gives off a red colour (13). Conjugation of these dyes to lectins or other ligands is relatively simple (13, 14). The dye is mixed with the lectin at alkaline pH and after several hours the dye-lectin conjugate can be separated from free dye by dialysis, gel filtration or affinity chromatography (13, 14). It is assumed that enough dye is used in the reaction so that there is no free lectin. The dye to protein (F:P) ratio can be determined with a spectrophotometer and used to characterize the conjugate (13, 14). Acceptable ratios range from 1 to 3. Alternatively, the absorbance ratios of protein to dye can be used and should range from 1.5-3.8 for Fl and 2.5-6 for Rh (18). Cells can then

be labeled with the fluorescent lectin conjugate and examined in a light microscope equipped with a fluorescent light source and the appropriate filters. Controls must be run to assure that the fluorescence is due to cell labelling (13). This technique is rapid, convenient, can be used to sample a large number of cells and gives a general pattern of label distribution (13, 14, 18). However, the resolution is low (individual fluorescent lectin molecules cannot be seen) and it is very difficult to correlate the cell surface morphology with the fluorescent labelling of the cell surface receptors (14).

The detection of cell surface receptors with the SEM requires the conjugation of ligands to macromolecular markers. The size and shape of the markers are their most important characteristics for this type of study (15). The markers must be uniform in size and shape, they must be large enough to be visible in the SEM (10 nm or greater) and small enough so as not to obscure cell surface morphology (15). A number of both biological macromolecules and synthetic particles have been used as markers. These include: hemocyanin, ferritin, tobacco mosaic virus, bacteriophages, silica spheres, gold granules and polystyrene, polymethacrylate and copolymer methacrylate microspheres (15). Lectins can be conjugated to these markers both covalently and noncovalently depending on the marker (15). In some cases (e.g. gold granules), lectins and other proteins will adsorb non-specifically onto the surface of the marker although this is often not a very stable interaction. Most of the time, lectins or other ligands are coupled covalently to the markers, usually with glutaraldehyde as the cross-linker (15). This can be done directly (one-step) where ligand and marker are simply mixed with glutaraldehyde or indirectly (two-step) where

the marker is first activated and after removal of excess glutaraldehyde, the ligand is added (15, 18). Free ligand can be separated from the ligand-marker conjugate by gel filtration or sucrose density gradient centrifugation (15). Generally, more than one ligand is bound per marker. The number can be controlled, though, so that a given preparation has a fairly uniform ligand:marker ratio. Cells can then be labeled with the ligand-marker conjugate. Controls must be run not only to assure that the ligand-marker conjugate is binding specifically but also to check for non-specific binding of the free marker. Sample preparation is more involved than for light microscopy but still fairly rapid. After labeling, cells are fixed with glutaraldehyde, dehydrated and critical point dried (15, 102). This procedure preserves the "native" cell surface morphology which would be distorted by surface tension if the solvent were simply allowed to evaporate from the cell surface. The samples are then coated with gold or gold-palladium to prevent charging in the SEM and mounted on stubs (115). The SEM allows a three dimensional examination of at least half the surface of a cell at a level of resolution which permits identification of the individual ligand-marker conjugates. Thus, the display of individual receptors over a large area of a cell can be seen at one time. In addition, it is possible to make correlations between cell surface morphology and cell surface receptors. Nevertheless, the technique does have drawbacks which lie mainly in the large size of the markers with respect to the size of the individual cell surface receptors. This limits the resolution of the receptors and makes precise analysis of receptor distribution patterns difficult. Also, since one marker generally carries at least several ligands one conjugate may label a number of receptors. In

addition, steric hindrances may prevent the labelling of some receptors entirely (15).

Labelling techniques for the TEM utilize electron dense markers such as ferritin in order to make the ligands visible to the electron beam (16, 17). Conjugation of ligands to ferritin is similar to the conjugation of ligands to SEM markers. However, because of the higher resolution obtainable with the TEM it is more important to achieve a low ligand:ferritin ratio, preferably 1:1 (16, 18). Free ligand is removed by ultracentrifugation or gel filtration (16, 17). Cells are labeled as in the other techniques including the appropriate controls to assure the specificity of the labelling. However, to prepare cells for examination in the TEM is a long and involved procedure (16, 18) requiring multiple fixation steps, dehydration, infiltration, embedding in plastic and thin sectioning. Although this technique provides higher resolution of individual cell surface receptors than either the light or scanning electron microscopes, and permits a correlation between receptor distribution and internal structures, only a limited correlation of labelling with overall cell surface morphology is possible (16, 17, 18). This is due to the requirement for thin sections ($\leq$ 0.1 μM) in order to view a sample in the TEM. This requirement means that only a very small portion of the entire cell surface can be examined at any one time. In addition, exactly what portion of the cell surface a given section represents is often difficult to tell. Nevertheless, a general distribution pattern can be determined as with the fluorescent light microscope. The relatively large size of the TEM markers may affect the resolution as discussed for the SEM.

Cells can be labeled either in suspension or attached to a substrate. They can be fixed before labelling to immobilize surface components or labeled without fixation which will allow redistribution of cell surface receptors (15, 18). For fixation, aldehyde fixatives are generally used. These cross-link proteins but leave lipids largely unmodified (18). Carbohydrate receptors are generally unaffected by these fixatives but cell surface antigens may be altered (18).

Thus, a number of techniques are available for determining the organization of cell surface receptors in the plasma membrane. The choice of technique depends upon the information that one wants to obtain. Ideally, a combination of techniques should be used so that different kinds of information can be obtained and then integrated into a more comprehensive understanding of cell surface receptor organization.

Distribution and Mobility of Lymphocyte Cell Surface Receptors as a Model System

The native distribution, and dynamic behaviour of cell surface components has been best characterized in lymphocytes. The studies on lymphocytes have been the basis for subsequent research in many other systems and thus a review of the basic principles behind these studies and the major conclusions which have been reached will be useful in understanding the rationale behind my experiments. The usual membrane components examined in these studies were the cell surface receptors for a variety of agents which can interact specifically with the lymphocyte cell surface. With the lymphocytes, as with many other cells, these receptors have been

predominantly either cell surface antigens or lectin-specific glycoproteins. The two most commonly studied receptors on lymphocytes are cell surface immunoglobulin (IgG) and the binding sites for Concanavalin A (ConA), a lectin.

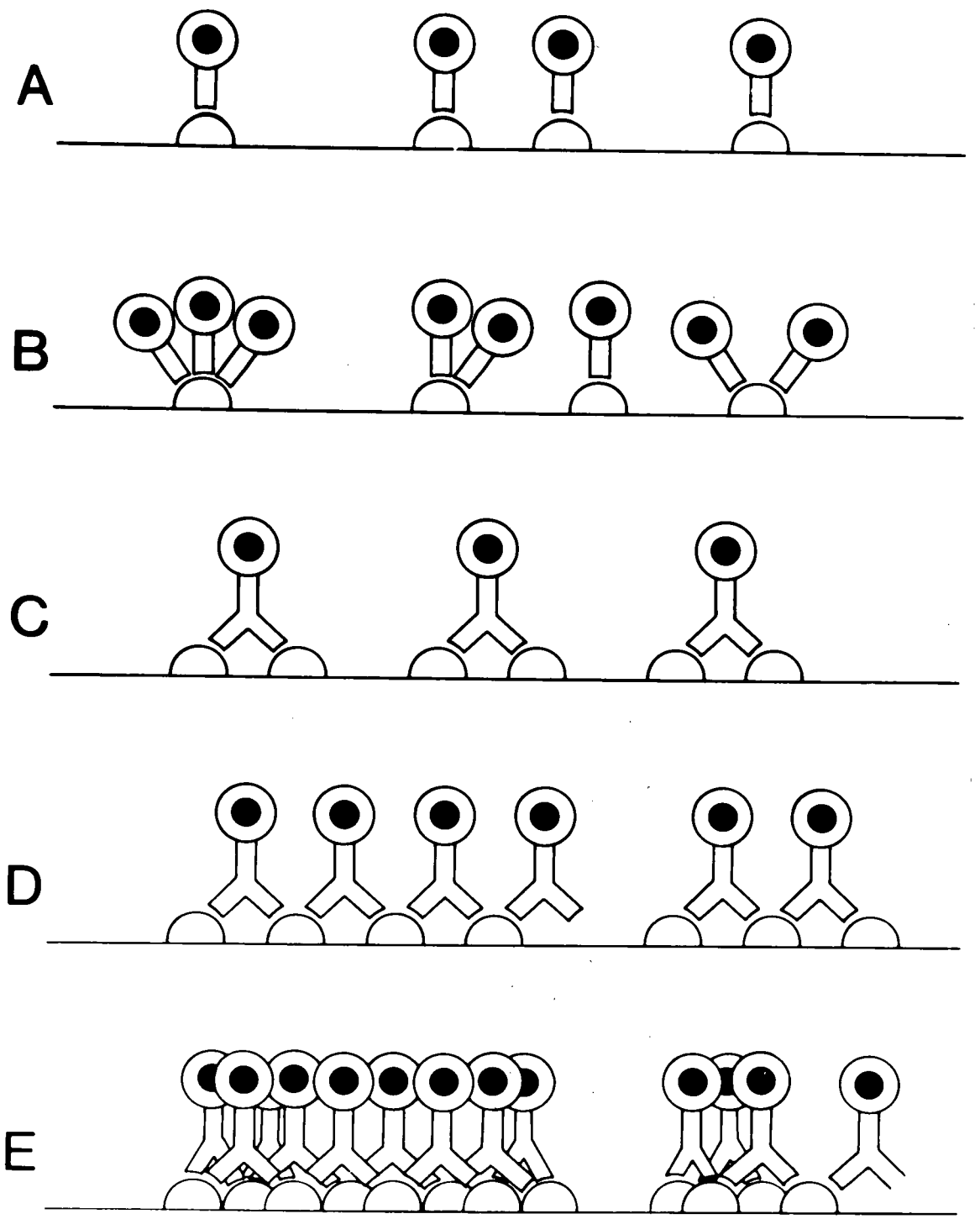
The native distribution of cell surface receptors for IgG, ConA and many other agents on lymphocytes has been found to be uniform and dispersed (5, 6, 8, 18, 19, 26). Here, the native distribution can be defined as that arrangement of cell surface receptors observed on cells which have been fixed with aldehyde before labelling. Although fixation may slightly alter the normal distribution of cell surface components as it cross-links them, this is probably below the resolution of even the TEM (18). With lymphocytes, all other conditions (e.g. labelling at 4°C) have led to only a slowing down and not a complete immobilization of receptor movement. This normal distribution of cell surface receptors is seen as a uniform ring of fluorescence in the fluorescent light microscope using fluorescently-tagged ligand (24, 27), a uniform coating of ferritin-ligand conjugate in the TEM (28, 32) and a homogeneous distribution of marker-ligand conjugate over the entire cell surface in the SEM (98).

When lymphocytes are not fixed before labelling, the cell surface receptors can redistribute. It is this phenomenon which has substantiated the concept of the membrane as a fluid mosaic as well as generating innumerable experiments and theories in itself. The first step which occurs in the redistribution of cell membrane receptors in lymphocytes, as well as other cells, is called patching. It is generally thought to occur only when the receptor is cross-linked into a two-dimensional network by a

multivalent ligand (19, 26). This is seen as the segregation of labeled surface components into discrete aggregates which are separated by areas of cell membrane free of label and can best be observed using either the TEM or SEM since patches are often not resolved at the level of the light microscope. Since patching does not require metabolic energy (5, 6, 8, 18, 19) it has been considered to be mainly a diffusional process. Hence, the rate of patch formation for a specific cell membrane receptor will be affected by the mobility of that receptor especially if the receptor concentration is low. This in turn can depend on the viscosity of the lipid-protein mosaic, the size and shape of the moving receptors and the interaction of the cyto-skeleton (if any) with the receptors. Lateral diffusion constants have been measured for several cell surface receptors on a few cell types and range from a rapid 10^{-9} cm^2 sec^{-1} found for muscle cell surface antigens (20) to a slow $3 \cdot 10^{-11}$ cm^2 sec^{-1} to $8 \cdot 10^{-12}$ cm^2 sec^{-1} for ConA receptors on mouse fibroblasts (21) and rat myoblasts (22). Since all these values are considerably slower than the diffusion constant for bulk lipid and cover a large range, they suggest that both the size of receptor macromolecules and the interaction of these receptors with cytoskeletal elements do play roles in mobility. Nevertheless, even using a diffusion constant of 10^{-11} cm^2 sec^{-1}, it can be estimated (18, 19) that the time required for labeled receptors to collide and form patches would be much shorter than the time period generally used in labelling experiments. However, the size and number of patches and rate of formation of larger aggregates may be affected by the diffusion rates of both labeled and unlabeled receptors. For instance, at low temperatures surface IgG forms only patches whereas at 37°C much larger aggregates occur (26). The most

important factors influencing patch formation appear to be the valency of ligand with respect to the cell surface receptor and the valency of the receptor with respect to the ligand (5, 8, 19, 26). The importance of ligand valency has been shown in several studies (19). In one such study, de Petris and Raff (23) looked at the distribution of several cell surface antigens on mouse thymocytes using unlabeled monovalent antibody to a cell surface antigen followed by either a monovalent anti-IgG-ferritin conjugate or a divalent anti-IgG-ferritin conjugate. With the monovalent antibody-ferritin conjugate, the distribution of ferritin molecules remained dispersed or clustered only slightly, even at 22°C whereas the distribution of ferritin was highly patched when the divalent antibody-ferritin conjugate was used, even at 6°C. The role of receptor valency in patch formation is probably at least as important as that of ligand valency. This is apparent when the distribution patterns of different lymphocyte receptors are looked at under comparable experimental conditions. Multivalent surface IgG molecules form large dense patches (19) whereas low valency antigens form small, loose patches (19) and monovalent antigens such as Thy-1 form no patches at all (23). The redistribution patterns resulting from various combinations of ligand and receptor valencies are shown in Figure 3. Since the interaction between ligand and receptor is reversible the redistribution patterns will also be influenced by the equilibrium constant of the reaction and the concentration of the reactants (18, 26). Hence a high dissociation constant would be expected to promote formation of smaller patches. Both extreme antigen (24) and antibody excess (25) would have the same effect.

Figure 3. Schematic drawing of the different labelling and redistribution patterns of cell surface receptors possible depending on the valency of the reagents. (A) Monovalent ligand, monovalent receptor; no redistribution. (B) Monovalent ligand, polyvalent receptor; minimal redistribution, some clustering. (C) Divalent ligand, monovalent receptor; some redistribution but labelling patterns appear the same as in (A). (D) Divalent ligand, divalent receptor; redistribution into linear chains. (E) Divalent ligand, polyvalent receptor; redistribution into two-dimensional patches (from (18)).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Cap Formation. Capping which, at least on lymphocytes, usually follows patch formation occurs by mechanisms quite different from those involved in patching (5, 6, 18, 19). Whereas patch formation is essentially a passive, diffusional process, capping is a very active one in which diffusion probably does not play a major role. Formation of caps and probably also large patches (18) occurs only in metabolically active cells at temperatures above 12-15°C and is visualized as the displacement of all the labeled surface components towards one pole of the lymphocyte (5, 6, 8, 18, 19). It is inhibited by agents which block ATP formation such as sodium azide (6, 8, 19). With lymphocytes, at least 15 cell surface receptors have been found to cap (19), albeit with extremely variable efficiencies (5, 19, 26). Cap formation has been most thoroughly studied with surface IgG and ConA receptors and the ensuing discussion will concentrate on these. The ConA receptors, in particular, are molecularly heterogeneous and can include cell surface IgG molecules. Capping occurs only if the cell surface components are first patched, although this is not sufficient to induce caps (19). Monovalent ligands, which do not give rise to patches, also do not cap (24, 27) and conditions which reduce or block patch formation such as ligand or receptor excess similarly affect cap formation (19, 24, 26). In addition, many cell surface antigens only cap when a double label of antibody is used (26). A few exceptions to this have been reported, notably (28). Caps can form very rapidly, especially if the cells are first induced to form patches at 4°C and then warmed to 37°C (5, 19). The rate of capping of cell surface receptors seems to vary among different lymphoid cell types and between different receptors on the same cell (6, 8, 19, 26). Capping can occur on both immobile cells

with little or no changes in cell shape and on mobile, but not necessarily moving cells accompanied by alterations in cell shape (19, 26). Formerly, this was thought to occur by two different mechanisms but more recent studies (11, 19) suggest that they may simply be two facets of the same mechanism. When capping on lymphocytes is accompanied by a change in cell shape this is visualized as the formation of a cytoplasmic protrusion, the uropod, on which the cap is found (5, 6, 8, 18, 19, 24, 26, 27). Microvilli are often associated with the uropod, as well (19). The change in cell shape was found to be only transient during surface IgG capping on lymphocytes and reversed as the ligand-receptor complex was cleared from the cell surface (29). Cell mobility can also be induced by capping (8,9).

One of the more interesting types of experiments which have come out of the capping phenomenon are those on the physical interdependence of different membrane components. In a typical experiment of this kind one of the two membrane receptors in question is allowed to cap completely at 24°C or 37°C by labelling with the appropriate ligand conjugated to Fl. The cells are then cooledto 0°C to prevent further capping and labeled with a ligand conjugated to Rh that is specific for the second receptor (18). If the two receptors are independent, then the capping of the first one should not affect the distribution of the second one, whereas if they are related the two receptors should co-cap (18, 19). The mutual independence of a large number of lymphocyte surface components has now been shown (e.g. 27, 30). One of the more important conclusions which can be derived from this type of study is that capping cannot occur simply by overall backwards flow of the whole membrane since if it did then

all the surface components, labeled and unlabeled, would co-cap (19). Some lymphocyte cell surface components have been found to co-cap. There are several explanations for this and examples of all have been observed. The most obvious reason for co-capping is that the two different ligands share the same receptor (e.g. 19, 31). Another possibility is that with certain ligands, such as ConA, which bind to a heterogeneous group of components some of these components may be receptors for other ligands (e.g. 6, 19, 32). The third explanation is that the two different receptors become associated due to some change induced in the membrane organization by the labelling of one of the receptors (19). This change in membrane organization may be related to the cytoskeleton and will be discussed further.

The metabolic dependence of the capping process suggests that it should be considered in terms of the whole membrane complex including the underlying cytoskeleton. A good deal of evidence has now accumulated which implicates the cytoskeleton in the capping process (5, 6, 8, 19, 26). Until recently, most of the evidence for cytoskeletal involvement came from studies using drugs which specifically disrupt the different cytoskeletal elements. The cytochalasins, in particular cytochalasin B, at moderate doses (10^{-5} M) inhibit cell movement, cytoplasmic streaming and membrane movements by impairing the function of microfilaments (5, 6, 8, 19). The degree of inhibition of capping by cytochalasin B is highly variable, depending both on the type of lymphocyte and the type of receptor (5, 19). For example, the capping of ConA receptors in splenocytes is completely inhibited (34, 35) whereas that of surface IgG and H-2 antigens is only partially blocked (28, 34, 36). Structures sensitive to

cytochalasin B are also required to maintain caps once they have been formed. Both ConA and surface IgG caps were found to disperse rapidly in the presence of cytochalasin B (34). This suggests that not only cap formation but also cap maintenance are highly dependent on a functional, internal microfilament system. It also implies that caps are not necessarily single, highly cross-linked aggregates of labeled receptors but are rather separate patches held together by intracellular controls (19).

Ca^{+2} plays a major role in the control of muscle contraction and likewise could be considered for a role in nonmuscle microfilament-regulated movement. However, the results so far reported are not clear. High levels of intracellular Ca^{+2} have affected capping in some systems but not others (5, 6, 8, 19).

The role of microtubules in capping is not clear. Again most studies on their role in the capping process have been done with drugs (e.g. colchicine, vinblastine) which affect their function (37). In all cases so far examined, microtubule-disrupting drugs alone have been found to have no inhibitory effects on capping (5, 6, 8, 19). However, when both microtubule- and micro-filament-disrupting drugs have been used together the inhibition of capping is much greater than that seen with cytochalasin B alone but the effect is still highly dependent on the type of cell and receptor (5, 6, 8, 19). The implications of this are still not clear.

The role of microtubules in the capping process becomes even more confusing when certain studies with ConA are considered. Several groups (34, 35, 37, 39) have shown that treatment of lymphocytes at 20-37°C with high (> 10 μg/ml, usually $\geq$ 50 μg/ml) doses of tetravalent ConA

prevented the subsequent capping of surface IgG and other cell surface antigens. In addition, high concentrations of ConA also inhibited its own capping. The inhibitory effect of ConA on the capping of its own and other cell surface receptors could be relieved by microtubule-disrupting agents (37, 38). These results suggested that ConA had some effect on the microtubule system which "locked" certain receptors into place and prevented their capping (39). This was found to occur even when only a limited portion of the cell surface was in contact with ConA as when cells were bound to ConA-derivitized solid supports (40).

It is apparent then that the cytoskeletal system can control capping but exactly how this occurs is not known. A number of theories have been put forward based on the evidence just discussed but none of them seem to satisfactorily explain all the data (5, 6, 8, 19, 38, 39). However, since such a large number of cell surface receptors are known to cap it appears highly improbable that they all bind specifically and directly to either microfilaments or microtubules. Thus, it is more appealing to conceive of an indirect interaction via one or more membrane molecules which directly associate with the cytoskeleton and which can be induced to interact with certain cell surface receptors once they are labeled. Singer *et al.* (11) have recently provided more direct evidence for the involvement of the microfilament system in capping. Using antibodies to actin and myosin in conjunction with fluorescent light microscopy they showed that when ConA was induced to form a cap on lymphocytes there was an accompanying concentration of both actin and myosin beneath the ConA cap. Tubulin did not redistribute along with the actin and myosin probably because the microtubules were disrupted by cold shock. Patching of

ConA receptors was also accompanied by the redistribution of actin and myosin; in this case into small "subpatches" immediately underneath the ConA patch. A general model for capping has been proposed based on these and earlier data. In this model, (Fig. 4) one or more integral membrane proteins are thought to be bound directly to actin. The clustering of any one type of cell surface receptor would lead to a binding of clusters to the integral protein and an energy-independent transmembrane linkage of the receptor cluster to actin and myosin on the inside surface of the cell. These actin- and myosin-linked clusters would then be concentrated into a cap by an energy-dependent contraction of the actomyosin system of the microfilaments (11). This model still leaves many questions unanswered, particularly the role of microtubules. Although it has been suggested (11) that the dose dependent inhibition of capping by ConA (38, 39) is an isolated phenomenon, this has not been shown conclusively. Hence, capping in lymphocytes is a complex phenomenon which depends not only on relatively simple parameters such as ligand and receptor valency but also the much more complicated cytoskeletal system. Most evidence suggests that it is mainly dependent on an intact microfilament system but when this is disrupted, the microtubules may become important (8, 11, 19).

What happens to the capped complexes? Generally, they appear to be either internalized or shed. Shedding involves the release of the complex into the medium in an intact or partially degraded form (18, 19). Internalization, or endocytosis, is more complex and involves the uptake of the capped ligand-receptor complex into the cell. Although endocytosis of ligand-receptor complexes often occurs following cap formation, capping is not required for it to occur (19). Clustering of receptors is to a cer-

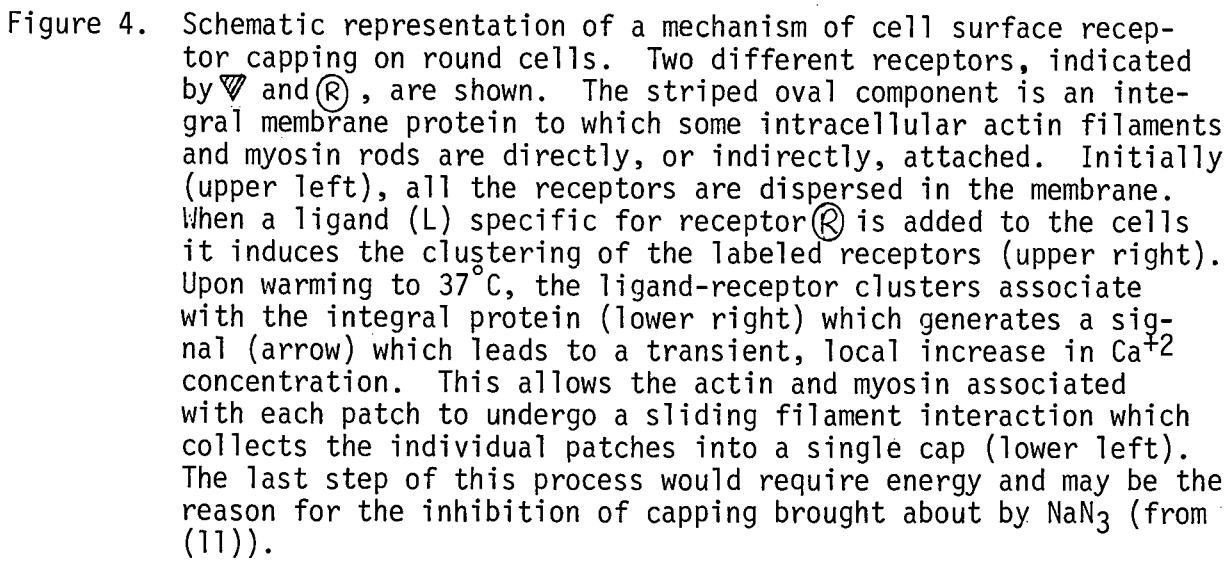
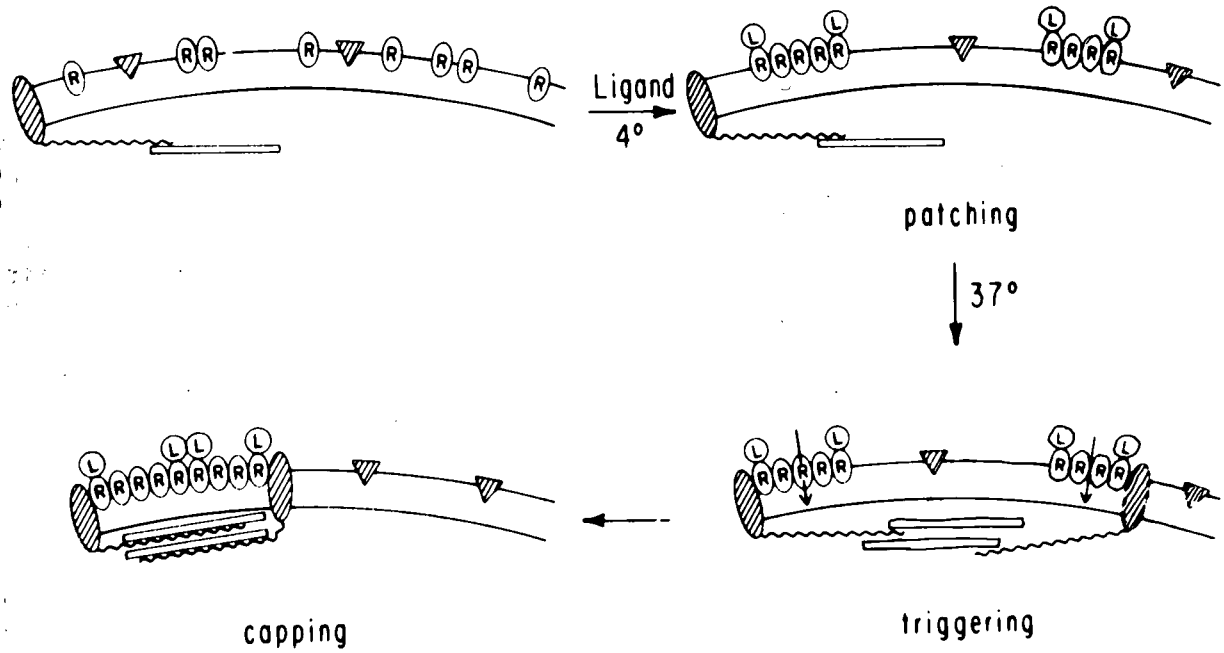


Figure 4. Schematic representation of a mechanism of cell surface receptor capping on round cells. Two different receptors, indicated by ▽ and Ⓡ, are shown. The striped oval component is an integral membrane protein to which some intracellular actin filaments and myosin rods are directly, or indirectly, attached. Initially (upper left), all the receptors are dispersed in the membrane. When a ligand (L) specific for receptor Ⓡ is added to the cells it induces the clustering of the labeled receptors (upper right). Upon warming to 37°C, the ligand-receptor clusters associate with the integral protein (lower right) which generates a signal (arrow) which leads to a transient, local increase in Ca^{+2} concentration. This allows the actin and myosin associated with each patch to undergo a sliding filament interaction which collects the individual patches into a single cap (lower left). The last step of this process would require energy and may be the reason for the inhibition of capping brought about by NaN_3 (from (11)).

AN HYPOTHETICAL MECHANISM FOR CAPPING



tain extent, however (19). The internalization of caps or patches is one form of adsorptive pinocytosis and thus it can be used to describe this general phenomenon (41). The uptake of caps or patches appears to be a local event with invagination of the labeled membrane being followed by the pinching off of small vesicles (19, 41). After interiorization the vesicles often fuse with lysosomes located in the area of the golgi apparatus (41). Neither microtubule nor microfilament-disrupting drugs block endocytosis of receptor-ligand complexes in lymphocytes. However, there is some evidence that both may affect the rate of internalization and the movement of the intracellular vesicles (19, 41). Endocytosis does require metabolic energy but the exact source of the energy and how it is used are still in doubt (41). Pinocytosis can occur at low temperatures although it is markedly slowed. In fibroblasts, the rate of internalization is directly proportional to the incubation temperature from 2°C to 38°C (42).

What is the function of cap formation? Although it is most highly studied on lymphocytes, it has also been observed in many other systems including protozoa and the cellular slime molds (8, 19, 26). The suggestion has been made that it simply reflects the ongoing activity of a mechanism which performs a basic function common to many cell types such as cell movement (8, 19). Nevertheless, the capping phenomenon provides a clear example of both the fluid and complex nature of the plasma membrane.

The Role of Cell Surface Glycoproteins in Cellular Functions

The plasma membrane glycoproteins have been found to play a number of important roles in the "life" of cells. Indeed, it has been

suggested that, at least on erythrocytes, there are no cell surface proteins which are not glycoproteins (47). Carbohydrates can make up from 2-10% of the total dry weight of the plasma membrane (43). However, the oligosaccharide side chains of glycoproteins are formed from relatively few monosaccharides. Sialic acid, N-acetyl- D-glucosamine, N-acetyl-D-galactosamine, D-galactose, D-mannose and L-fucose are the only sugars found (44). Although glucose is by far the most abundant sugar in mammalian cells, it is not found in membrane glycoproteins (45). This has been taken as evidence for the importance of these glycoproteins in cell behaviour since a lot of glucose would probably make specificity difficult. The oligosaccharide chains are linked to protein through asparagine, serine or threonine. They are created from the monosaccharides by the formation of glycosidic bonds between the reducing end of one sugar and a hydroxyl group of another in either an α or β configuration. This can give rise to a large number of different possible linkages. The oligosaccharides can be extremely complex with numerous side chains and thus the structure of only a few have been determined. The glycoproteins are extremely heterogeneous in size with molecular weights ranging from 29,000 to over 300,000 daltons (46). Early work on cell surface glycoproteins, where the effect of the treatment of cells with neuraminidase on certain cellular functions was examined, indicated that the loss of cell surface glycoproteins brought about significant changes in a wide variety of cells (43). These changes included alterations in adhesive properties, alterations in enzyme activities and the loss of receptors for a number of viruses and bacteria (43). A number of cell surface antigens have now been found to be glycoproteins and these include the blood-group specific antigens, histocompatibility

antigens, cell surface immunoglobulin and certain tumor specific antigens. The membrane receptors for several polypeptide homones, including insulin, have also been shown to be glycoproteins (45). Alterations in cell surface glycoproteins have been found in a number of different types of cells following transformation (50). For instance, fibronectin, which has received a lot of attention recently as a cell surface protein that is lost following transformation of fibroblasts, is a glycoprotein. (However, there is evidence that the carbohydrate portion is not required for biological activity but does protect against proteolytic degradation (49).) Since adhesive properties are known to be altered following transformation, studies on transformed cells have provided evidence for a more general role for cell surface carbohydrate in cellular adhesion and morphology (46, 50). Frazier and Glaser (51) have recently reviewed a number of systems in which cell surface glycoproteins have been implicated in intercellular adhesion. These include cell-cell interactions during the development of the chick retina, sponge reaggregation and the aggregation of cells during the life cycle of the cellular slime mold. The latter phenomenon appears to be mediated by carbohydrate-binding proteins which are released by the individual cells and specifically cross-link cells of the same species to give the end product, a coherent multicellular slug. Cell surface glycoproteins have also been implicated in mating and fertilization. The sea urchin is one of the best understood examples of this. Glycoprotein receptors have been found on the surface of unfertilized sea urchin eggs and these are recognized by carbohydrate-binding proteins on activated sperm in a species-specific manner. Cell surface glycoproteins

have also been implicated in cell homing patterns and membrane transport (44, 46, 50).

In spite of the evidence of this long list, the importance of cell surface glycoproteins in cellular function is really only beginning to be realized. The development of tools and techniques over the past few years which enable one to specifically look at glycoproteins has greatly stimulated research in this field. One of these tools is the lectins which will be discussed in the next section.

Lectins

Lectins were first identified in the late 1800's as a red blood cell (RBC) agglutinating activity found in certain plant extracts (52). By the 1930's this agglutinating activity had been localized to specific proteins in the extracts which were capable of binding to sugars on the RBC surface (12). Lectins from hundreds of different sources have now been described (52, 53) but only twenty or so have been isolated in a pure form (54). It is now recognized that lectins can be found not only in plant extracts but also fungi, the hemolymph of a variety of invertebrates including the horshoe crab, sea hare, snail, lobster, earthworm and oyster, fish ova, cellular slime molds, electric eels, toad eggs and rabbit liver (53).

The property which has made the lectins into an important tool in membrane research is their ability to bind tightly but reversibly to specific carbohydrate groups without modifying them chemically (12, 53). Hence, they can be used as specfic probes for cell surface glycoproteins.

The simplest and most easily studied interaction of lectins with cell surfaces is agglutination, particularly hemagglutination (52, 54). Measurement of agglutination can be done by simply mixing a suspension of cells with increasing dilutions of a lectin, observing the reaction under a microscope and noting the minimal lectin concentration required to give agglutination (12). When red blood cells are used a titer of agglutinating activity can be obtained even more easily with microtiter plates where non-agglutinated cells settle but agglutinated cells do not (12). Hemagglutination, because of its simplicity, is often used to test for the presence of a lectin during purification and it is also important for studying the affinity of a given lectin for different mono- and oligosaccharides (12). Agglutination, in general, is the simplest method for determining the presence of receptors for a specific lectin on a cell type of interest (12).

Originally, lectins were purified using the traditional methods of protein chemistry (52). Now, however, they are usually purified using the technique of affinity chromatography (17, 52). This is possible because of their carbohydrate-binding specificity. For example, ConA can be highly purified from a crude jack bean extract simply by chromatography on Sephadex, a glucose polymer and a sugar to which ConA binds (53). After removal of unbound proteins, the ConA can be specifically eluted with a glucose solution. With other lectins, whose specificities differ from that of ConA, special affinity columns must be prepared by the covalent coupling of an oligosaccharide or glycoprotein bearing the appropriate sugar moiety to an insoluble matrix (53).

The sugar specificities, structures and physical properties of a number of lectins are known but only those relevant to my research will be discussed. These are listed in Table I.

ConA is probably the best studied lectin. It is specific for oligosaccharides containing α-D-glucose or α-D-mannose at their non-reducing ends (52, 53, 54, 55). ConA can also bind to internal α-D-mannose groups (52, 54, 55). The lectin is composed of identical subunits, each of 26,000 daltons, which can associate into dimers or tetramers depending on the pH, temperature and monomer concentration (55). This makes for some confusion in quantitative binding studies. Below pH 5.6 ConA is always a dimer, whereas above pH 5.6 it is a dimer at 0°C, a tetramer at 37°C and a mixture at 23°C (53, 55, 56). Above neutral pH, it forms higher molecular weight aggregates in a time-dependent fashion (52). Each ConA subunit has one carbohydrate binding site and one site each for the binding of Ca^{+2} and Mn^{+2} (53, 55). Both ions are needed for biological activity, probably because they stabilize the sugar binding site (55). The complete amino acid sequence and three-dimensional structure of ConA (53, 54, 55) is known but is not within the scope of this discussion.

Wheat germ agglutinin (WGA) binds to N-acetyl-D-glucosamine (D-glcNAc) and sialic acid residues at the non-reducing ends of oligosaccharide chains (52, 53, 54, 55). It will also bind to internal D-glcNAc groups (53). WGA has a much higher affinity for dimers of N-acetyl-D-glucosamine and this is often considered as the actual group to which it binds (17). WGA is a dimer but reports on its molecular weight are conflicting. Most recent reports place the monomer molecular weight at 17,500 (53, 55), although at least one puts it at 11,500 (17). There are two specific binding sites for $(D\text{-glcNAc})_2$ per WGA monomer (53).

Table I

Properties of Purified Lectins

Lectin	Saccharide Specificity	Molecular Weight	Subunit Structure		Glycoprotein
			Molecular Weight	Number	
Concanavalin A	α-D-mannose α-D-glucose	104,000	26,000	4	No
Lentil lectin	α-D-mannose α-D-glucose	42,000- 63,000	18,000 8,000	2 2	Yes
Ricinus communis agglutin I	D-galactose	120,000	29,500 37,000	2 2	Yes
Ricinus communis agglutinin II	D-galactose N-acetyl-D-galactosamine	60,000	29,500 34,000	1 1	Yes
Ulex europeus lectin I	α-L-fucose	n.d.	n.d.	n.d.	Yes
Wheat germ agglutinin	N-acetyl-D-glucosamine	36,000	18,000	2	No

Taken from Goldstein and Hayes (55).

n.d. - not determined.

Ricinus communis agglutinin (RCAI) a lectin isolated from castor beans, has its binding specificity directed towards terminal β-linked D-galactose groups (52, 53, 54, 55). With a molecular weight of 120,000, it consists of two pairs of nonidentical subunits; one pair of molecular weight 37,000 and one pair of 29,500 molecular weight (52, 53, 55). Recently, evidence has been presented (57) which suggests that only one of the two subunit pairs has sugar binding sites. Ricin (or RCAII), another lectin also found in castor beans, is a toxin as well as an agglutinin (52). It binds to terminal N-acetylgalactosamine groups as well as β-D-galactose residues (52, 55). RCAII, with a molecular weight of 60,000, is also composed of two nonidentical subunits with molecular weights of 29,500 and 34,000 (52, 55). As with RCAI, only one of the two subunits appears capable of binding carbohydrate (57).

Ulex europeus lectin I is specific for terminal α-L-fucose groups (52, 55). The molecular weight, subunit composition and valency are not well characterized although it has been purified to homogeneity (55). Unlike the other lectins described it is blood-group specific, binding only to type O RBC (52, 54, 55)

Lentil lectin is very similar to ConA in its sugar specificity but its binding affinity for α-D-glucose and α-D-mannose is substantially less (54). The structure is also different, the lectin being a tetramer of two pairs of nonidentical subunits (two 18,000 molecular weight and two 8,000 mol. wt. polypeptides) (55)

Several of the lectins discussed are themselves glycoproteins. These include lentil lectin, the *Ricinus communis* lectins and *Ulex europeus* lectin (17, 53).

Although the lectin specificities are generally given in terms of simple sugars, these are not what the lectin binds to on the cell surface. As already mentioned in the section on glycoproteins, the oligosaccharide groups of membrane glycoproteins can be extremely complex. The interaction of lectins with cell surface oligosaccharides is much stronger than that with simple sugars (51). This has been shown using glycopeptide fragments isolated from cells treated with proteolytic enzymes (51). Kornfeld *et al.* (58) obtained a glycopeptide from the surface of human RBC using this technique. On a molar basis, this glycopeptide was 60,000 times more inhibitory for the hemagglutinating activity of *Phaseolus vulgaris* lectin than the specific simple sugar N-acetyl-D-galactosamine and 300 times more inhibitory than mannose was for lentil lectin (54).

As mentioned earlier, agglutination of cells is a convenient tool for measuring lectin activities. It has also sparked a good deal of interest and controversy as a phenomenon in its own right. Many different types of cells have been found to agglutinate in the presence of lectins and it has often been observed that significant changes occur in the agglutinability of these cells following neoplastic transformation, infection with certain nononcogenic viruses and some developmental processes (52, 53, 54). The most heavily studied aspect of these findings is the enhanced agglutinability of many fibroblastic and lymphoid cells seen following transformation (52, 53, 54). The significance of this observation is still not understood since many variables have been found to affect agglutination and the importance of each of these is not known (52, 53, 54). These factors include properties of the lectin such as its valency, its binding affinity and its size as well as properties of the lectin receptor

and the cell membrane as a whole (11, 52, 53, 54). The increased agglutinability of transformed cells does not appear to be due to an increase in the number of lectin binding sites or an alteration in the "native" distribution of lectin receptors on the cell surface (53). Recent work suggests it may be related to changes in the cytoskeletal organization seen in transformed cells but the data are not yet conclusive (5, 6, 8, 11, 53).

Another interesting property associated with lectins is their ability to stimulate mitogenesis in normal lymphocytes (12, 53). When lymphocytes are treated with a mitogenic lectin (e.g. ConA, lentil lectin, *Ulex europeus* lectin) they change from quiescent, non-dividing cells to actively growing and proliferating ones (12). This includes an increase in size and an increase in the rate of many metabolic processes (53). How do lectins do this? The first step is obviously the binding of the lectin to the cell surface. This may be the only step required since it has been found that lectins covalently attached to large supports, which should prevent their entry into cells, can still stimulate mitogenesis (12, 53). Simple binding alone is probably not enough however, since although both B and T lymphocytes bind equal concentrations of ConA, only T cells respond (53). Here again, the cytoskeleton is beginning to be implicated (53).

The question that often arises at this point is why? Certainly lectins are a useful tool for studying plasma membrane glycoproteins but what does ConA do for the jack bean? Some evidence has now accumulated for several functions for lectins in plants. One function is in the protection of plants against the spread of viral infection and another is in

the binding of symbiotic rhizobia to plant roots to form root nodules (53). In addition, as discussed earlier, lectins have been isolated from animal cells and these have been implicated as adhesive agents in a number of systems (52).

Neuroblastoma Cells

A thorough understanding of the nervous sytem has been the goal of many scientists for a considerable period of time. One aspect of this has been to learn as much as possible about the structure and function of individual nerve cells. This proved extremely difficult until techniques for culturing tissues *in vitro* were developed. Then, explants of various regions of the central nervous system could be obtained and used to study the properties of a select portion of the cells of the nervous system. Although the explants proved very valuable to certain types of studies, they presented a number of problems when it came to looking at the properties of individual cells. The cultures usually consisted of large clumps containing several different types of neural cells which were extremely difficult to separate and the explant cells did not divide in culture so any study had to be short term. In 1969 the adaptation of a murine neural tumor to tissue culture as clonal cell lines was reported by two groups (59, 60). These cell lines have come to provide an excellent source of nerve-like cells of a single type which can survive indefinitely in culture. The tumor, or neuroblastoma (C1300), from which the tissue culture cells were cloned had arisen spontaneously in the spinal cord region of a mouse in 1940. A large number of clones have now been isolated which express

nerve-like properties to varying degrees (61). In the research to be discussed a single clonal line (Neuro-2a), established in 1969 by Klebe and Ruddle (60), was used. The original tumor cells were round, highly undifferentiated and did not have much in common with nerve cells (59). However, when the cells were put into culture, under conditions where they could attach to a surface, they often "differentiated" (59, 60). This means that they developed long processes, or neurites, reminiscent of neural axons and dendrites (morphological differentiation), their cell bodies and nuclei increased in size, their membranes became electrically excitable (electrical differentation) and they showed marked increases in the activities of a number of neural enzymes (biochemical differentiation) associated with the metabolism of neurotransmitters (e.g. tyrosine hydroxylase, choline acetyltransferase, acetylcholinesterase) (59, 60, 61). Although the neuroblastoma cells would differentiate spontaneously, this generally occurred with only a small percentage of a given clonal population. Hence, a good deal of effort was put into experiments to find conditions which could bring about a high level of differentiation in a culture of cells. A number of treatments which bring about differentiation have now been described and these include: serum deprivation (cells are routinely grown in 10% serum) (62, 63), cAMP analogues (usually dibutyrylcyclic AMP (Bt_2cAMP)) (61, 64), prostaglandins (61), inhibitors of cyclic nucleotide phosphodiesterase (61), x-irradiation (61), 5-bromodeoxyurdine (5-BrdU) (65), dimethyl sulfoxide (DMSO) (66), haemin (67), dexamethasone (68), low pH (69) and N-methyl-N'-nitro-N-nitrosoguandine (MNNG) (70), a potent mutagen and carcinogen. With all of these agents, except haemin, a 24 to 96 hour treatment is required before differentiation reaches a maximal level. The

morphological differentiation brought about by these agents has generally been found to be reversible (62, 63, 64, 65, 66, 67, 68, 69), except with MNNG (70). However, there is some argument with respect to Bt_2cAMP. One group maintains that after a 3-4 day treatment with this chemical the differentiated morphology cannot be reversed (61), whereas several other groups have not observed this (64, 70). How these diverse agents can all bring about differentiation is not clear although it is clear that differentiation brought about by one agent may not be the same as that brought about by another. For example, in one line of neuroblastoma cells, DMSO brings about a high degree of morphological and electrical differentiation with very little effect on neural enzyme activites and no effect on intracellular cAMP levels (71). Prostaglandins, phosphodiesterase inhibitors and Bt_2cAMP cause only moderate morphological and electrical differentiation in this line but bring about a substantial increase in the activities of neural enzymes and the intracellular cAMP levels (71). Thus, the agent that one chooses should reflect the questions that are being asked.

Serum withdrawal was used to bring about differentiation in most of the research to be discussed so the effects of this treatment will be discussed in more detail. Differentiation by serum withdrawal was first described by Seeds *et al.* (62) and Schubert *et al.* (63). Both groups found it to be a rapid process with neurite formation beginning within two hours after serum withdrawal. Although 0% serum gave the most rapid rate of differentiation, up to 2% serum could be used without altering the percentage of cells (80%) which differentiate. Schubert (63) described in detail the morphological events following serum withdrawal. Within 5 minutes after addition of cells to serum-free medium they attached to the dish and by 15 minutes became extensively flattened and put out numerous

spine-like projections. Neurite formation began after 2 hours and continued for several days. By 24 hours, the cell bodies had become larger and less flattened.

Two groups (72, 73) have looked at the intracellular appearance of undifferentiated and differentiated neuro-2a cells using the TEM. The undifferentiated cells have little obvious cytoskeleton with few neurofilaments, microfilaments or microtubules (72). They also lack dense bodies and dense core vesicles which are thought to serve as catecholamine stores in nerve cells (72). The differentiated cells have many neurofilaments, microtubules and microfilaments in the neurites as well as some in the cell body, which is indicative of a more highly developed cytoskeleton, and numerous dense bodies and dense core vesicles suggesting an increase in the intracellular level of catecholamines (72, 73).

A number of agents have also been found which can block or reverse morphological differentiation and these have given some insight into what cellular processes are required. Protein synthesis inhibitors can prevent differentiation (61, 63) whereas RNA synthesis inhibitors cannot (61, 62, 63). Microtubule- and microfilament-disrupting agents completely block and/or reverse differentiation (61, 62, 63, 74, 75). The requirement for microtubules appears to be filled by the assembly of preformed tubulin subunits (75) and the requirement for microfilaments may also be dependent on the assembly of preformed subunits rather than *de novo* protein synthesis. Cells can also be prevented from morphologically differentiating by growing them under conditions where they can't attach to a substrate (63). Although a number of agents which induce differentiation block cell division this does not seem to be a prerequisite for differentiation

(63). Recently, it has been shown that the cessation of proliferation of serum-deprived neuroblastoma cells is not what it seems (77). The cells actually do continue to proliferate but they also die at a higher rate so that viable cell counts remain relatively constant suggesting an inhibition of growth.

Several groups (78, 79, 80, 81) have reported changes in the glycoprotein composition of neuroblastoma cell membranes following differentiation. This was generally manifested as in increase in glucosamine or fucose incorporation into certain glycopeptides (78, 79) or glycoproteins (80, 81) following differentiation. In some cases,(79, 81) this was correlated with a change in protein composition as well but in others (80) no change in protein composition was evident. The significance of these changes are not known.

Thus, the neuroblastoma cell lines can provide a convenient model to study the organization and behaviour of integral membrane proteins in a differentiated "nerve" cell and the alterations in these proteins as the cells differentiate.

Chapter 2

MATERIALS AND METHODS

Reagents

The sources of the chemicals were: N, N, N', N'-tetramethylenediamine (TEMED) was purchased from Aldrich Chemical Co.; ACS scintillation fluid from Amersham; acrylamide, Bis (N, N-methylenebis-acrylamide), Coomassie brilliant blue R-250 from Bio-Rad Laboratories; sodium dodecyl sulfate (specially pure) from British Drug Houses, Ltd.; tetramethyl rhodamine isothiocyanate from Becton, Dickenson and Co.; castor beans from Buckerfields; β-mercaptoethanol from Eastman Kodak; fluorescein isothiocyanate from ICN Pharmaceuticals; RCA 120 from Miles; basic fuchsin, certified from Matheson, Coleman and Bell; Sepharose 4B and 6B and Sephadex; G-100 and G-200 from Pharmacia; glutaraldehyde (25% aqueous) from Polyscience; bovine serum albumin, Concanavalin A (salt-free), dansyl hydrazine, D-galactose, glycine, N-acetyl-D-glucosamine, α-methyl mannoside, ovomucoid, Trizma base and wheat germ lipase from Sigma Chemical Co. N-acetyl chitobiose was prepared from chitin as described by Rupley (156). All other chemicals were of reagent grade.

Ricin phosphate buffered saline (rPBS) (82) contained 11.69g NaCl, 0.19g NaH_2PO_4 and 0.96g $Na_2HPO_4 \cdot 7\ H_2O$ per liter of distilled water (dH_2O) and adjusted to pH 7.2. Phosphate buffered saline (PBS) was composed of 8.0g NaCl, 0.2g KCl, 0.29g KH_2PO_4 and 2.17 g $Na_2HPO_4 \cdot 7H_2O$ per liter of

dH_2O and adjusted to pH 7.4. Dulbecco's phosphate buffered saline (DPBS) (95) contained 8.0g NaCl, 0.29g KH_2PO_4, 0.29g KCl, 2.17g $Na_2HPO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ and 0.2g $MgCl_2 \cdot 6H_2O$ per liter of dH_2O at pH 7.4. Tris-ConA buffer (T-CAB) was made up of 8.77g NaCl, 0.11g $CaCl_2$, 2.42g Tris, adjusted to pH 7.4, 0.02g $MnCl_2$ per liter of dH_2O.

Lectin Preparation

RCAI and RCAII were purified from castor beans by affinity and gel filtration chromatography according to the method of Nicolson and Blaustein (82). 200g of castor beans were suspended in 1500 ml ricin phosphate buffered saline (rPBS) and homogenized on high in a Waring blender to obtain a uniform suspension. The suspension was stirred for 2.5 hr. at room temperature (23 ± 1°C) and then centrifuged at 12,000 x g for 30 min. The clear supernatant was removed with care being taken to avoid disturbing the upper layer of fat and filtered twice through glass wool. The solution was brought to 60% saturation with ammonium sulfate while stirring at 4°C and then allowed to sit overnight at 4°C to permit complete precipitation of the lectin. The solution was then centrifuged for 30 min at 12,000 x g and the supernatant discarded. The pellet was dissolved in 200 ml rPBS and dialyzed overnight against rPBS at 4°C to remove the ammonium sulfate. Half the dialysate was applied to a Sepharose 4B column (2.6 x 100 cm) at 4°C or 24°C which had been washed with rPBS. The column was washed with more rPBS until the absorbance at 280 nm (A_{280}) of the eluant fell below 0.05. The lectins were then eluted with 0.2M D-galactose in rPBS and the peak tubes pooled and dialyzed extensively against rPBS at 4°C to remove

the sugar. The two lectins were separated by size on a Sephadex G-100 column (1.6 x 60 cm) which was washed and eluted with rPBS.

WGA was purified from wheat germ lipase by affinity chromatography according to the method of Marchesi (83). 1g of lipase in 50 ml of distilled water (dH_2O) was homogenized in a dounce homogenizer, heated to 60°C for 15 min and then chilled. The solution was centrifuged at 10,000 x g for 10 min, filtered through glass wool and diluted 1:1 with 0.5 M NaCl, 0.1 M sodium phosphate, pH 7.0. The preparation was applied to an ovomucoid-Sepharose column (see ref. 86) which had been washed with 0.25M NaCl, 0.5 M sodium phosphate, pH 7.0 and the column was further washed with this buffer until the A_{280} of the eluant fell below 0.05. The WGA was eluted with 0.1M acetic acid and the peak tubes pooled and dialyzed extensively against PBS.

The concentration of all lectins was estimated by the absorbance at 280 nm, assuming an $E^{1cm}_{1mg/ml}$ of 1.4 (18). The purity was confirmed by electrophoresis on 5.6% SDS-polyacrylamide gels. The ability of the lectins to agglutinate human blood cells was used to monitor their presence during purification and their hemagglutination titers were used as a measure of their biological activity. These titers (with human RBC, type A) were typically for the purified lectins (1 mg/ml): RCAI, 1/1024; RCAII, 1/32; and WGA, 1/64.

Fluorescent Lectin Preparation

Lectins were conjugated to fluorescein isothiocyanate (Fl) or tetramethyl rhodamine isothiocyanate (Rh) by reacting the lectins with Fl (0.05 mg/mg lectin) or Rh (0.03 mg in DMSO/mg lectin) in 0.02M sodium

carbonate (pH 9.0) containing 0.01 M specific inhibitory sugar (D-galatose for RCAI and RCAII, α-methyl mannoside for ConA and N-acetyl-D-glucosamine for WGA) for 2 hrs at 24°C. After overnight dialysis against PBS for WGA, RCAI and RCAII or Tris-ConA buffer for ConA to remove the sugars, the fluorescent lectins were separated from free dye and inactive lectin on the appropriate affinity columns (0.5 x 2 cm): ovomucoid-Sepharose for WGA, Sepharose 4B for RCAI and RCAII and Sephadex G-200 for ConA. The lectin-dye mixtures were applied to the columns which had been washed in PBS (RCAI, RCAII, WGA) or Tris-ConA buffer (ConA) and the columns were further washed until the eluant was colourless. The fluorescent lectins were then specifically eluted: WGA with 0.1 M acetic acid; RCAI and RCAII with 0.2M galactose; ConA with 0.2 M α-methyl mannoside and the peak tubes were pooled and dialyzed against PBS or Tris-ConA buffer. Lectin and dye concentrations were determined from the absorbances at 280 nm and 495 nm for Fl or 515 nm for Rh (94).

$$\text{for Fl: conjugates} \quad [\text{Lectin}](\text{mg/ml}) = \frac{OD_{280} - 0.35\ OD_{495}}{E^{1\ cm}_{1\ mg/ml}\ \text{at 280 nm}}$$

$$\text{for Rh: conjugates} \quad [\text{Lectin}](\text{mg/ml}) = \frac{OD_{280} - 0.56\ OD_{515}}{E^{1\ cm}_{1\ mg/ml}\ \text{at 280 nm}}$$

Fluorescent lectin preparations typically had OD 280/495 ratios of 1.0-2.0 for Fl-lectins and OD 280/515 ratios of 1.5-2.0 for Rh-lectins. Hemagglutination titers were also determined after conjugation of the lectins with the dyes and were unchanged from those found for the lectins before conjugation.

Fluorescent Microscopy

The fluorescence of the fluorescent dye conjugated lectins was tested by labelling the beads used for the affinity chromatography of the lectins with the fluorescent lectins and then observing them under the fluorescent light microscope. Thus, Sephadex G-200 was labeled with ConA, ovomucoid-Sepharose with WGA and Sepharose 4B with RCAI and RCAII. In these experiments, the beads, in PBS, were treated for 10 min at 23°C with the fluorescent lectin (100 μg/ml). They were then washed three times by centrifugation and examined under a fluorescent light microscope. The specificity was checked by doing the labelling in the presence of the appropriate saccharide inhibitor (0.1 M D-galactose for RCAI and RCAII, 0.1 M α-methyl mannoside for ConA and 0.01 M N-acetyl chitobiose for WGA).

Double labelling studies were also carried out in order to test for lectin-lectin interactions. The beads in PBS were first labeled for 10 min at 24°C with a nonfluorescent lectin (100 μg/ml) and after washing as described above, they were treated with a second, fluorescent lectin (100 μg/ml), also for 10 min at 24°C, washed as before and examined under the microscope (e.g. RCAI plus Sepharose 4B followed by Fl-ConA). In all cases, controls were run simultaneously as described for single labelling studies. For RCAII, the labelling of Sepharose 4B was carried out at 4°C. Some labelling studies were also done using fluorescent lectins in both steps (e.g. Fl-RCAI followed by Rh-ConA).

Light Microscopy

For light and fluorescent microscopy a Leitz Dialux microscope equipped with phase contrast optics and the appropriate filters and incident

light excitation for fluorescent light microscopy was used. Samples were sandwiched between a glass slide and a coverslip and were photographed on Kodak Tri-X film for 0.5 sec for phase contrast pictures and 45 sec for fluorescent pictures.

Protein Synthesis Inhibition Assay

The toxicity of the *Ricinus communis* agglutinin preparations was tested by assaying their ability to inhibit protein synthesis in tissue culture cells (87). Cells grown in 35 mm tissue culture dishes in complete medium were treated for 1 hour at 37°C with RCAI (10-50 µg/ml), RCAII (10 µg/ml) or PBS in complete medium. They were then washed three times in leucine-free medium and incubated for 1 hr at 37°C in ^{3}H-leucine (3 µCi/ml) in leucine-free medium. The cells were washed three times in Dulbecco's PBS and solubilized in 2.0 ml 1N NaOH. The solubilized cells were scraped off into test tubes and 0.5 ml of each sample was used to determine protein concentration. The remaining 1.5 ml was precipitated with excess, cold 10% TCA, chilled for 1 hour at 4°C and the TCA precipitate collected on glass fiber filters. The filters were dried, placed in scintillation vials with 10 ml ACS scintillation fluid and counted on a Nuclear Chicago liquid scintillation counter.

Red Blood Cell Membranes

Red blood cell membranes were prepared after the method of Dodge *et al.* (96).

Continuous SDS-Polyacrylamide Gel Electrophoresis-Fairbanks System (84)

Reagents: Acrylamide, Bis, 10 x buffer (0.4 M Tris-acetate, 0.2 M sodium acetate, 0.02 M EDTA, pH 7.4), overlay solution (0.1% SDS, 0.15% ammonium persulfate, 0.05% TEMED), running buffer (0.04 M Tris-acetate, 0.02 M sodium acetate, 2.0 mM EDTA, pH 7.4, 0.2% SDS), 20% SDS, 1.5 % ammonium persulfate and 0.5% TEMED.

Procedure: A 5.6% polyacrylamide gel was formed by dissolving 5.6g acrylamide and 210 mg Bis in 50 ml dH_2O. 10 ml 10 X buffer, 5 ml SDS, 10 ml ammonium persulfate and dH_2O to 95 ml were added, the solution was deaerated for 10 min on a vacuum line and then 5.0 ml of TEMED were added. The solution was immediately pipeted into 20 glass tubes (0.6 x 15 cm) which had one end sealed with Parafilm. The gel was gently overlaid with overlay solution and allowed to polymerize for 1 hr at 23°C. After the gels had polymerized, the overlay solution was removed, the tubes were placed in a Savant tube gel apparatus and the upper and lower reservoirs were filled with running buffer. Samples (25-50 µl) were applied with a microsyringe and electrophoresis was carried out at 23°C at a constant current of 8 ma/gel until the dye front was 0.5 cm from the bottom of the gel (2.25 hr). The gels were then removed from the glass tubes, the dye front was marked with india ink and the gels were stained as described below.

Sample preparation: Samples were dissolved in an equal volume of denaturing buffer (20 mM Tris-acetate, pH 8.0, 4% SDS, 40% sucrose and 0.0025% bromophenol blue). In some cases, β-mercaptoethanol was added to a final concentration of 2.0%.

Discontinuous SDS-Polyacrylamide Gel Electrophoresis-Laemmli System (85)

Reagents: Acrylamide solution (30% (w/v) acrylamide and 0.8% (w/v) Bis in dH_2O), separating gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS), stacking gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS), running buffer, 5x concentrated (0.125 M Tris, 0.96 M glycine, pH 8.3, 0.5% SDS), 10% (w/v) ammonium persulfate and TEMED.

Procedure: Different separating gel acrylamide concentrations were used for different experiments and so the protocol for the preparation of the different gels is given below:

Volume (ml) per 30 ml Solution

% gel	Separating Gel Buffer	dH_2O	Acrylamide Solution
6	7.5	16.5	6.0
7.5	7.5	15.0	7.5
9	7.5	13.5	9.0
10	7.5	12.5	10.0

In all cases, the buffer, acrylamide and water were mixed and deaerated before the addition of 30 µl TEMED and 150 µl ammonium persulfate. The gel solution was rapidly injected between two glass plates (14 x 17.8 cm) separated by a 1.5 mm thick spacer and mounted on a Bio-Rad model 220 slab gel apparatus. The gel solution was injected to 1 cm below the bottom of a well-forming comb, overlaid with t-butanol and allowed to polymerize for 1 hr at 23°C. The t-butanol was then removed, the top of the gel rinsed

several times with separating gel buffer, blotted with filter paper and a 10 or 20 well well-forming comb was inserted into the space above the gel. The separating gel was overlaid with deaerated 3% stacking gel solution (10 ml = 2.5 ml stacking gel buffer, 6.5 ml dH_2O, 1.0 ml acrylamide solution, 30 µl ammonium persulfate and 20 µl TEMED) which was allowed to polymerize 30 min at 23°C. The well-forming comb was carefully removed, the wells rinsed several times with running buffer and then the apparatus was placed in the electrophoresis tank with running buffer in the upper and lower reservoirs. The samples (5-30 µl/well for 20 wells) were applied with a micro syringe and cold tap water was circulated through the core of the apparatus. Electrophoresis was carried out at 35-40 ma per gel until the dye front had moved 7.5-8.5 cm through the separating gel (2.5 hr). The glass plates were then removed from the apparatus, separated and the gel removed. The dye front was marked with india ink and the gel stained as described below.

Sample preparation: Samples were dissolved in an equal volume of denaturing buffer (0.03 M Tris-HCl, pH 8.0, 40% sucrose, 2% SDS, 4% β-mercaptoethanol and 0.0025% bromophenol blue). In some cases, the samples were heated for 5 min at 100°C.

Staining of Gels for Protein

Gels were stained for protein following a modification of the method of Fairbanks *et al.* (84). Slab or tube gels were immersed overnight in a staining solution containing 0.025% Coomassie brilliant blue R-250,

25% isopropanol and 10% acetic acid. The gels were destained over 24-48 hr in several changes of 10% acetic acid. They were photographed on a light box using Kodak Panatomic-X film (ASA 32) and a #24 Wratten gelatin filter.

Molecular Weight Determinations from Gels

In all cases, standards were run on separate tube gels or in separate wells on slab gels at the same time as the samples. The relative mobility (R_f = distance migrated by protein band/distance migrated by dye front) was calculated for each standard and then plotted against the log of the molecular weight of the standard to give a straight line from which the molecular weight of an unknown protein band could be determined (128).

Staining of Gels for Carbohydrate

Carbohydrate was detected in gels using two different methods. The first method which was used in the early experiments is much less sensitive than the second but is adequate for large quantities of material.

Method I (84) - Periodic acid-Schiff base (PAS) stain

Reagents: Schiff reagent (500 ml dH_2O, 4g $K_2S_2O_5$ and 5.25 ml HCl were mixed well. 2g basic fuchsin were added and the solution stirred gently for 2 hr at 23°C. 2g Darco G-60 charcoal were added, the solution was stirred briefly and filtered. The colourless filtrate was stored at 4°C.)

Procedure: Longitudinal sections of slab gels were fixed overnight in 25% isopropanol, 10% acetic acid; washed overnight in 10% acetic acid; oxidized 2 hr in 0.5% (w/v) periodic acid in dH_2O; washed 60 min in 0.5% $NaAsO_2$ in 10% acetic acid, 2 x 20 min in 0.1% $NaAsO_2$ in 10% acetic acid, 20 min in 10% acetic acid; stained overnight in Schiff reagent; and washed in 0.01% $Na_2S_2O_5$ in 0.01 M HCl until the background was clear.

Method II (97)

Reagents: Dansyl hydrazine solution (2 mg dansyl hydrazine per ml of DMSO mixed with an equal volume of acidified DMSO (0.6 ml HCl/liter DMSO)).

Procedure: Longitudinal sections of slab gels were fixed overnight in 25% isopropanol, 10% acetic acid; oxidized 2 hr in 0.7% (w/v) periodic acid in 5% acetic acid; rinsed several times in dH_2O; washed in 0.5% $Na_2S_2O_5$ in 5% acetic acid until colourless (1-2 hr); stained 2 hr in a 60°C water bath with dansyl hydrazine solution; reduced 30 min at 23°C with 0.02% $NaBH_4$ in DMSO; and washed overnight in several changes of 1% acetic acid until the background was colourless.

Staining of Gels with Fluorescent Lectins

Longitudinal sections of slab gels were fixed overnight in 25% isopropanol, 10% acetic acid; washed in 25% isopropanol; fixed 2 hr in 0.25% glutaraldehyde in 25% isopropanol; washed overnight with PBS; incubated 2 hr in 0.1 M glycine in PBS; and washed several hours in PBS. In

the later experiments the 25% isopropanol wash, the second fixation and the incubation in glycine were found to be unnecessary and were omitted. After the PBS wash the gels were stained for 2-3 days at 4°C with Fl-lectin (0.2 mg/ml in PBS) in the absence and presence of a 0.2 M concentration of the appropriate inhibitor. To conserve material the staining was carried out by placing the gel slice around the inner edge of a small jar filled with 3-5 ml of Fl-lectin solution and then putting a smaller jar in the centre so as to displace the solution up around the gel. The gels were then washed for 2-3 days in PBS in the absence or presence of the inhibitor until the background was colourless. The gels were photographed under short wave uv light using Kodak Tri-X film (ASA 400) and a #15 or #61 Wratten gelatin filter. The #61 filter was found to give better pictures than the #15 filter and so was used in all the later experiments.

Hemagglutination

Hemagglutination was used as a measure of lectin activity. It was carried out in "V" bottom microtiter plates (Cooke) having 0.3 ml capacities. To each of the wells in a single row (12 wells/row, 9 rows/plate) was added 25 µl PBS. 25 µl of lectin reagent were then added to the first well and then 25 µl was serially diluted through the remaining 11 wells so that the total volume in each well was 25 µl. 25 µl of human red blood cells (RBC; usually type A) were then added to each well and after mixing, the plates were allowed to sit for 30 min at 23°C. Agglutinated RBC evenly covered the bottom of the wells whereas non-agglutinated cells formed a small, tight clump in the bottom of the "V". The titer was the last

dilution to give agglutination. The specificity of the reaction was checked by doing it in the presence of a 0.1 M concentration of the appropriate inhibitor.

Protein Assay

Protein was assayed by a modification of the method of Lowry *et al.* (88).

Reagents: Reagent D (2% Na_2CO_3, 0.02% $KNaC_4H_4O_6 \cdot 4H_2O$, 0.01% $Cu\ SO_4 \cdot 5\ H_2O$) and reagent E (1:1 dilution of Folin-Ciocalteau reagent with dH_2O).

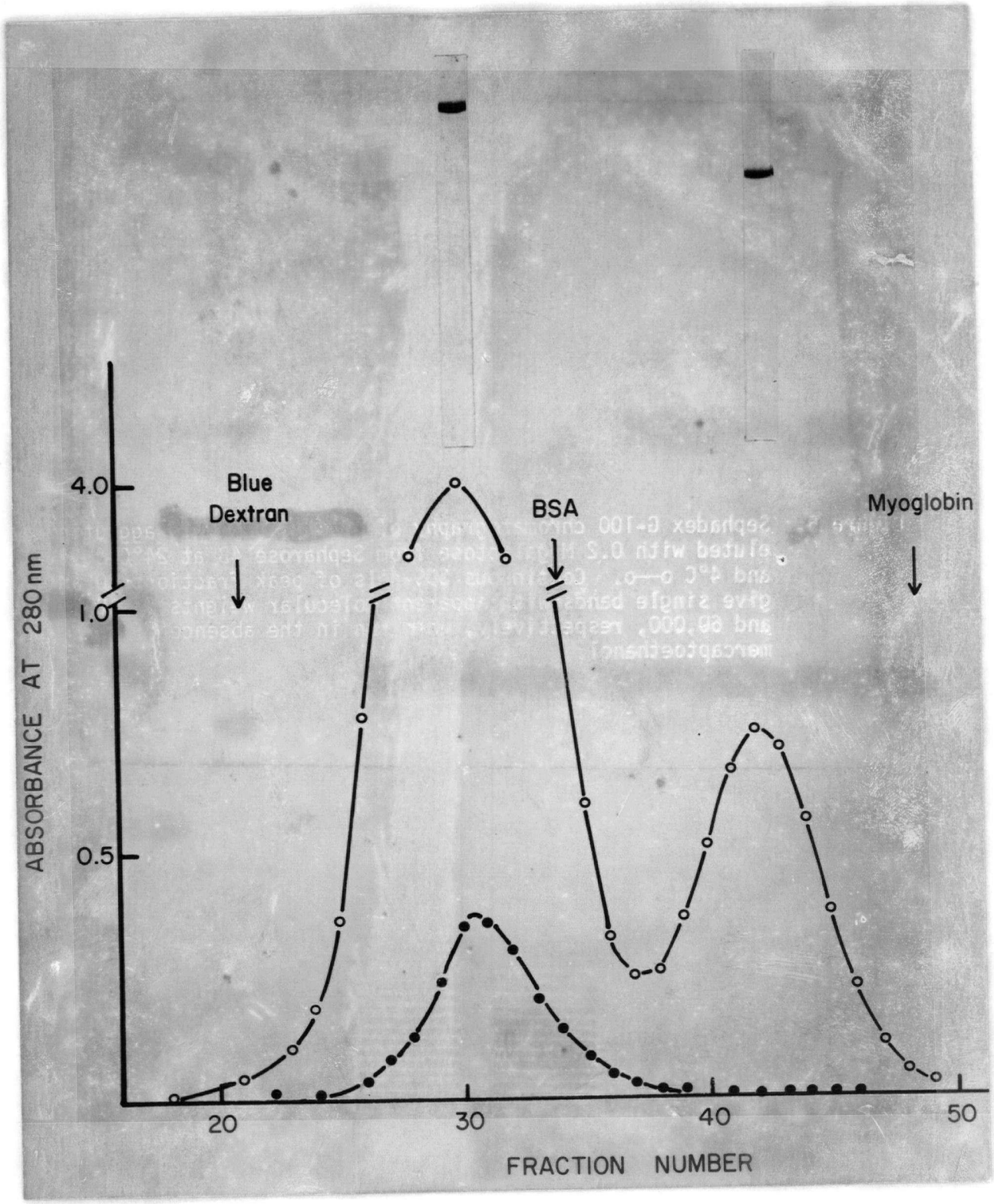
Procedure: To each 0.5 ml sample in 1 N NaOH was added 2.5 ml reagent D. After 10 min at 23°C, 0.25 ml of reagent E was added, the solution mixed vigorously and the colour allowed to develop for 30 min. The absorbance of the sample at 660 nm was then read. Bovine serum albumin (BSA; 0.25 mg/ml in 1N NaOH) was used to prepare the standard curve.

RESULTS

Lectin Preparation

During the purification of RCAI and RCAII, the effect of temperature on the binding of these lectins to Sepharose became apparent. As shown in Fig.5 , lectin which was specifically bound to Sepharose at 24°C and was eluted with 0.2 M galactose exhibited a single peak when chromatographed on Sephadex G-100. Analysis of this peak by SDS-gel electrophoresis

Figure 5. Sephadex G-100 chromatography of *Ricinus communis* agglutinins eluted with 0.2 M galactose from Sepharose 4B at 24°C ●—● and 4°C o—o. Continuous SDS-gels of peak fractions 30 and 42 give single bands with apparent molecular weights of 120,000 and 60,000, respectively, when run in the absence of β-mercaptoethanol.


Blue
Dextran
BSA
Myoglobin
4.0
1.0
0.5
ABSORBANCE AT 280 nm
20
30
40
50
FRACTION NUMBER

on 5.6% Fairbank's gels in the absence of sulfhydryl reducing agents revealed a single band with apparent molecular weight 120,000. When analyzed on 9% Laemmli gels in the absence of β-mercaptoethanol this peak gave three bands in the molecular weight range of 120,000-130,000. These bands were of unequal intensity, the 130,000 band staining much more heavily than the other two. The banding pattern and mobilities of this preparation were identical to those of RCAI obtained commercially.

When lectin was bound to Sepharose at 4°C and eluted with galactose, two peaks were obtained upon chromatography on Sephadex G-100 (Fig. 5). This result is in agreement with that reported by Nicolson and Blaustein (87). The relative size of the peaks differ, however, possibly due to differences in the castor beans. The major peak corresponded to RCAI. Analysis of the smaller peak on 5.6% Fairbank's gels in the absence of β-mercaptoethanol gave a single band with an apparent molecular weight of 60,000. Analysis of the peak on 9% Laemmli gels in the absence of β-mercaptoethanol gave two bands with molecular weights between 55,000 and 60,000. The peak appeared to be RCAII.

In the presence of β-mercaptoethanol RCAI exhibited two major bands of apparent molecular weights 29,000 and 36,000 and a minor band of 33,000 in both gel systems whereas RCAII had two bands of almost equal intensity with apparent molecular weights of 29,000 and 34,000 (see Fig. 7).

WGA came off in a single peak from ovomucoid-Sepharose and gave one band of apparent molecular weight 29,000 when analyzed in either gel system in the absence of β-mercaptoethanol. In the presence of this reducing agent, two bands of molecular weights 24,000 and 22,000 were seen using the Laemmli gel system.

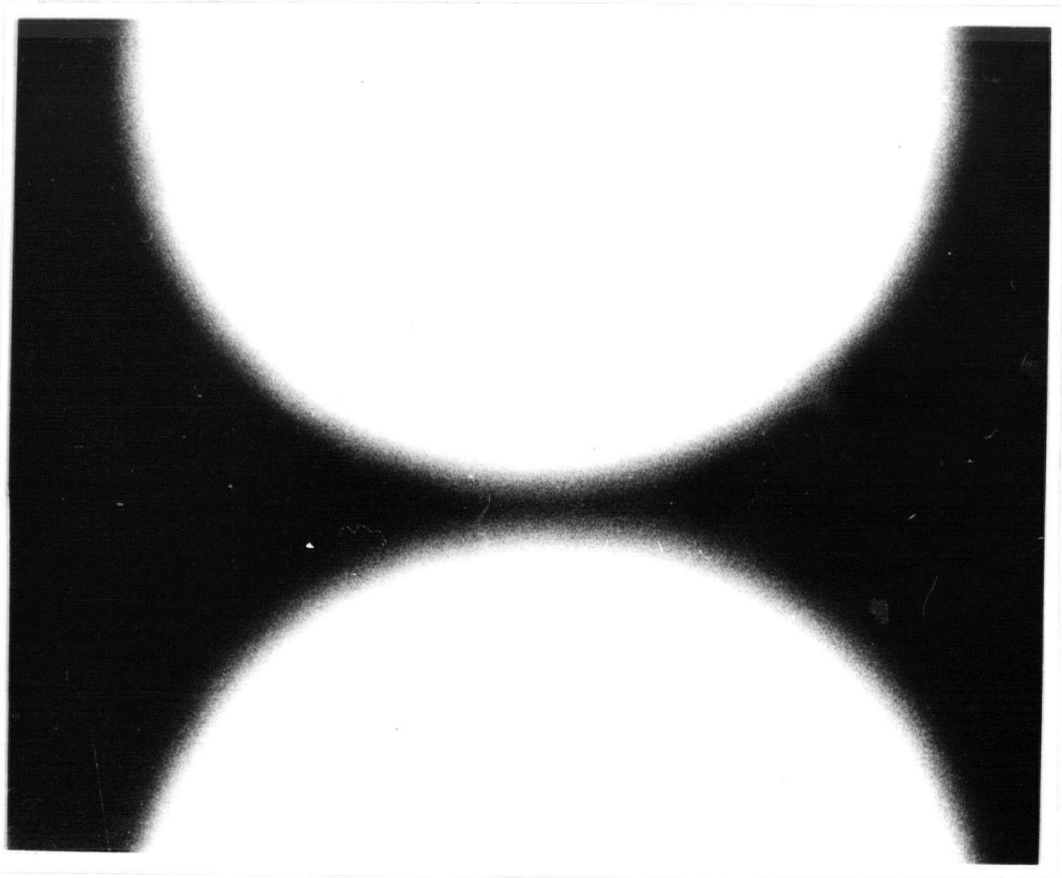


Figure 6. RCAI-coated Sepharose 4B beads labeled with Fl-ConA.

Binding of Fl-Lectins to Affinity Column Beads

Fl-lectins were found to specifically and heavily label their respective affinity column beads; i.e. Sepharose could be labeled with Fl-RCAI or RCAII, ovomucoid-Sepharose with Fl-WGA and Sephadex with Fl-ConA. Fl-RCAI and Fl-RCAII and Fl-WGA did not bind to Sephadex and Fl-ConA and Fl-WGA did not label Sepharose. All the lectins would bind to ovomucoid-Sepharose. In all cases the binding was inhibited by a 0.1 M concentration of the appropriate inhibitor.

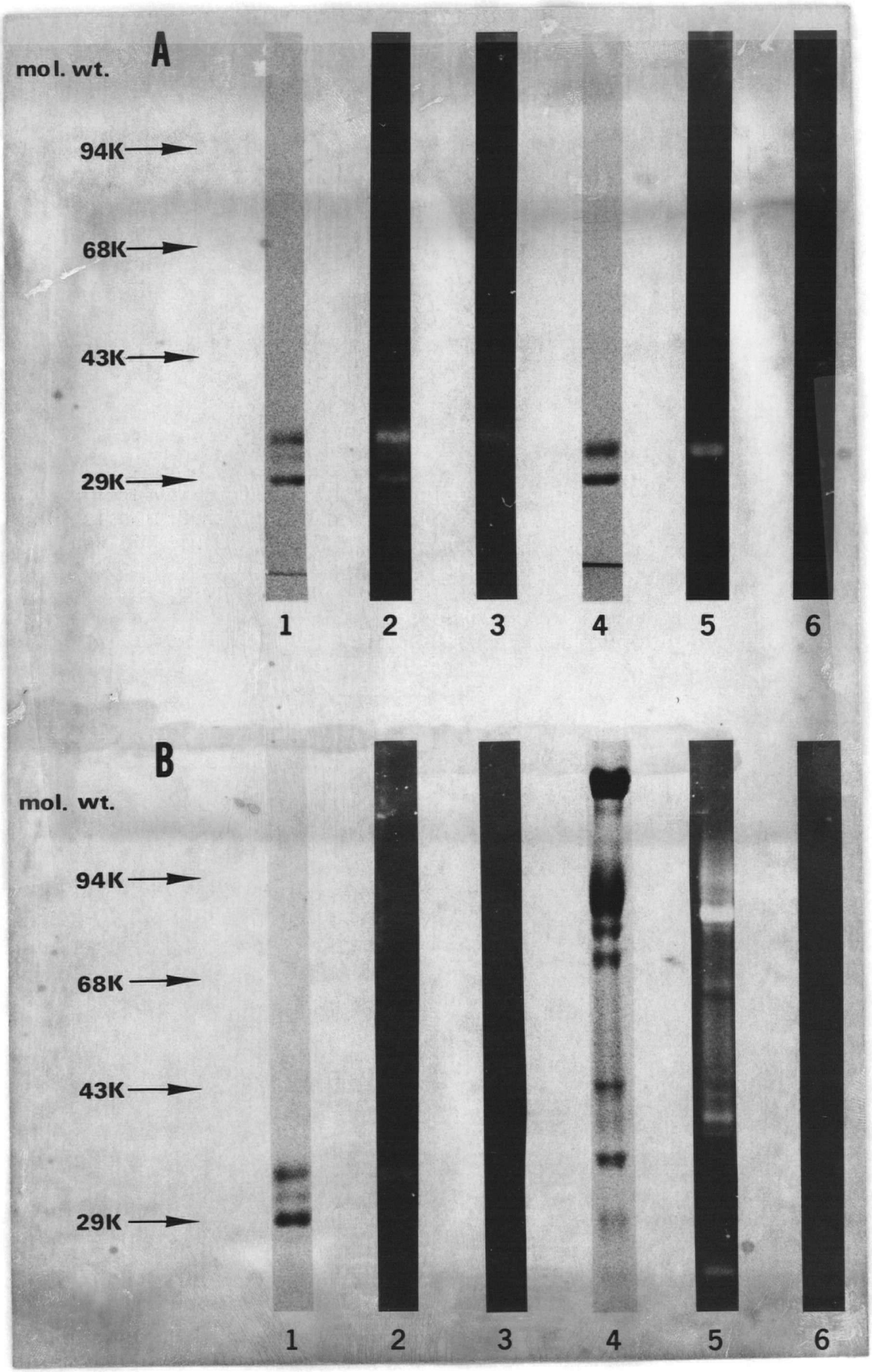
When RCAI- or RCAII-coated Sepharose was treated with Fl-ConA, strong labelling was observed as exemplified in Fig. 6. In control experiments in which 0.1 M α-methyl mannoside was present, no binding of Fl-ConA was seen. No binding of Fl-WGA to RCA-Sepharose was observed.

In the reverse experiments, Fl-RCAI was found to bind specifically to ConA-Sephadex. Again, no labelling was seen when Fl-WGA was used.

Binding of Fl-Lectins to RCA in Gels

The binding of ConA to the subunits of RCAI and RCAII was demonstrated using discontinuous SDS-gel electrophoresis in combination with fluorescent lectins. Initially, the gels were fixed with both acetic acid/isopropanol and glutaraldehyde before staining. However, the glutaraldehyde fixation was found to be unnecessary and, therefore, was omitted in later studies. As shown in Fig. 7a, when the individual subunits of RCAI were separated on 9% Laemmli gels they could all be stained with Fl-ConA, whereas; only the higher molecular weight (34,000) RCAII subunit showed strong ConA binding. A small amount of RCAI labelling appeared to be non-specific since binding

Figure 7. A. Discontinuous SDS gels of RCAI and RCAII run in the presence of β-mercaptoethanol. (1) RCAI stained with Coomassie blue; (2) RCAI stained with Fl-ConA; (3) RCAI treated with Fl-ConA and 0.2 M α-methyl mannoside; (4) RCAII stained with Coomassie blue; (5) RCAII stained with Fl-ConA; (6) RCAII treated with Fl-ConA and 0.2 M α-methyl mannoside.
B. Discontinuous SDS gels of RCAI and RBC membrane proteins run in the presence of β-mercaptoethanol. (1) RCAI stained with Coomassie blue; (2) RCAI treated with Fl-WGA; (3) RCAI treated with Fl-WGA and 0.01 M N-acetyl chitobiose; (4) RBC membranes stained with Coomassie blue; (5) RBC membranes treated with Fl-WGA; (6) RBC membranes treated with Fl-WGA and 0.01 M N-acetyl chitobiose.


A
mol. wt.
94K
68K
43K
29K
1
2
3
4
5
6
B
mol. wt.
94K
68K
43K
29K
1
2
3
4
5
6

was not completely inhibited by 0.2 M α-methyl mannoside. All the subunits of both RCAI and RCAII were lightly stained with PAS, confirming the presence of saccharide groups on each.

When gels were stained with Fl-WGA, only slight binding to RCAI subunits and no binding to RCAII subunits was observed (Fig. 7b). This binding was inhibited by N-acetyl-chitobiose. Fl-WGA, however, bound strongly and specifically to the PAS staining proteins of RBC membranes indicating that the lack of RCA staining was not due to an inactive reagent.

WGA showed no binding to ConA when this lectin was run on gels and stained with Fl-WGA.

Protein Synthesis Assay

To confirm that RCAI and RCAII were what they appeared to be from analysis on SDS-polyacrylamide gels their effects on protein synthesis in tissue culture cells was tested. The results are shown in Table II. RCAI, separated from RCAII on Sephadex G-100 did not inhibit protein synthesis, whereas RCAII, a known inhibitor of protein synthesis, inhibited it by 96% within 2 hours. RCAI, purified directly on Sepharose at 24°C, also had no effect on protein synthesis, confirming that RCAI obtained by this method was not contaminated with RCAII.

DISCUSSION

The use of affinity chromatography in lectin research provides both a rapid and simple method for obtaining highly purified lectin and an

Table II

Protein Synthesis Inhibition Assay

24°C Column

Sample	dpm ^{3}H-leucine incorporated/μg protein	% control
peak fractions	108.7	109
control	100.0	-

4°C Column

Sample	dpm ^{3}H-leucine incorporated/μg protein	% control
peak 1	114.3	114
peak 2	4.4	4.4
control	100.0	-

Cells were treated with aliquots (10-100 μg protein) of peak fractions from the Sephadex G-100 columns of *Ricinus communis* agglutinins (eluted with 0.2 M galactose from Sepharose 4B at 24°C or 4°C) for 1 hr at 37°C and the ability of the cells to incorporate ^{3}H-leucine into protein was determined.

excellent means for obtaining tagged lectins (e.g. fluorescent dyes, ^{125}I) which retain their biological activity.

The experiments on the purification of RCAI and RCAII showed that RCAI could be obtained free of RCAII in a single chromatographic step if the affinity column was run at 24°C. The purity of this RCAI was confirmed both by SDS-gel electrophoresis and a protein synthesis assay. Although the yield of RCAI was approximately one-fifth that obtained when the column was run at 4°C, this did provide a quick method for obtaining RCAI free of RCAII. It also indicated that if high yields of purified, tagged RCAI were desired the affinity column should be run at 4°C. These results are consistent with a study (89) which showed a lower binding stability for the RCAII-Sepharose complex at 18°C as compared with the RCAI-Sepharose complex.

The banding patterns and molecular weights obtained for RCAI and RCAII using the Fairbanks gel system agree with those previously reported (92, 93). Since RCAI is thought to consist of only two different subunits, the fainter 33,000 molecular weight subunit was thought to represent a slight contamination by RCAII (92). However, when the Laemmli gel patterns are considered, along with the protein synthesis assay data, it appears more probable that it is a true RCAI subunit. Thus, the intact lectin may be made up of a mixture of three isolectins: 2(37,000 • 29,000), (37,000 • 29,000) (33,000 • 29,000) and 2(33,000 • 29,000). The relative densities of the three bands seen on Laemmli gels agree with the observation that the 29,000 and 37,000 molecular weight subunits appear to be in much higher concentrations than the 33,000 molecular weight subunit. Although intact RCAII can also be resolved into more than one band on Laemmli gels, it

only appears to have two subunits when analyzed in the presence of β-mercaptoethanol.

The molecular weight found for WGA is consistent with the results reported by some researchers (52, 55) but not by others (53, 54, 55). The slight change in molecular weight and the appearance of a second band in the presence of β-mercaptoethanol in the Laemmli system suggest the lectin may actually be a mixture of several isolectins. This has been suggested by other groups as well (55).

The use of fluorescent lectins in combination with light microscopy and SDS-gel electrophoresis provided sensitive, specific and relatively simple methods for studying the binding of lectins to glycoproteins and their subunits.

Analysis of the interactions between lectins is especially important with respect to competition and multiple labelling studies in which glycoprotein lectins are used. For example, cells which are double labeled with RCAI and ConA may reflect the ConA-RCAI interaction rather than non-competitive binding to different cell surface receptors. A number of lectins besides RCAI and RCAII are known to be glycoproteins, including several which are commonly used in cell surface labelling studies (e.g. soybean agglutinin, *Dolichos biflorus* agglutinin, kidney bean agglutinin and lentil lectin). Hence, any kind of competition study must take the possibility of lectin-lectin interactions into account. This is the reason for the absence of competition experiments with ConA and RCA in the studies to be described in subsequent sections.

The presence of ConA binding sites on RCAI and RCAII is consistent with the report that both agglutinins are mannose-containing glycoproteins (90). Earlier studies, using indirect methods, have also shown that ConA

can bind to RCAI (91). However, the results from the gel labelling studies on the interaction of ConA with the subunits of RCAI and RCAII do not support the model proposed in the earlier work (91). This model suggested that only one of the RCAI subunits had ConA binding sites whereas the present studies indicate that ConA can bind to all three subunits. The model may be applicable to RCAII. In addition, the present results do not support the suggestion (92) that the lowest molecular weight subunit (29,000) of both RCAI and RCAII is the same. The gel labelling experiments showed that the 29,000 molecular weight RCAI subunit could bind ConA whereas the 29,000 molecular weight RCAII band could not.

Chapter 3

MATERIALS AND METHODS

Reagents

The sources of the reagents were: Diaminoheptane and cytochalasin B were purchased from Aldrich Chemical Co.; carrier-free ^{125}I (13-17 mCi/μg) from Amersham; Eagle's minimal essential medium, Eagle's minimal essential medium for suspension culture, fungizone, penicillin-streptomycin, fetal calf serum, and trypsin from Gibco; AG 1 X 10 and AG 50W X 12 from Bio-Rad Laboratories; formvar from Pelco; OsO_4 from Polysciences; colchicine, 5-bromodeoxyuridine (5-BrdU), dibutyryl cAMP (Bt_2cAMP), HEPES, fluorescein conjugated *Ulex europeus* lectin, lactoperoxidase, and 1-ethyl-3 (3-dimethyl-aminopropyl) carbodiimide from Sigma. All other chemicals were reagent grade.

Hepes-ConA buffer (H-CAB) contained 8.77g NaCl, 0.11g $CaCl_2$, 5.67g HEPES adjusted to pH 7.4 and 0.02g $MnCl_2$ per liter dH_2O. Balanced salt solution was composed of 8.0g NaCl, 0.4g KCl, 0.06g KH_2PO_4, 0.09g $Na_2HPO_4 \cdot H_2O$, 0.35g $NaHCO_3$ at pH 7.4 per liter of dH_2O.

Cell Cultures

Mouse neuroblastoma line neuro 2a cells (American Type Culture Collection) were routinely grown in Eagle's minimal essential medium (MEM)

supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (1.25 μg/ml) in a humidified incubator containing 5% CO_2 at 37°C. They were judged to be free of mycoplasma contamination by observation in the SEM (114, 115). Cells were routinely subcultured by harvesting confluent 100 mm Falcon tissue culture plates with 0.25% trypsin in Ca^{+2} and Mg^{+2} free balanced salt solution and seeding at a concentration of 1-2 x 10^5 cells/ml per 100 mm tissue culture dish. For labelling experiments 5 x 10^5- 1 x 10^6 cells were seeded in 60 mm Falcon tissue culture dishes containing alcohol-sterilized glass coverslips. To obtain differentiated cells, the cells were allowed to grow for two days after seeding in MEM containing 10% FCS. The medium was then removed and FCS-free MEM was added (62, 63). The cells were used between 24 and 48 hr later. In some cases, differentiation was brought about by incubating the cells for 48 hr in either 10^{-3} M Bt_2cAMP (61) or 10^{-5} M 5-BrdU (65) in MEM containing 10% FCS. To obtain undifferentiated cells, the cells were either grown on tissue culture dishes for three days in MEM containing 20% FCS (63) or in spinner culture at a concentration of 1-2 x 10^5 cells/ml in spinner MEM containing 10% FCS.

Lectin-Microsphere Conjugates

Preparation of fluorescent microspheres: Copolymer methacrylate microspheres approximately 40 nm and 80 nm in diameter were generously provided by S.P.S. Yen of the Jet Propulsion Laboratory, Pasadena, California (98). The spheres (8-15 mg/ml) were first purified by chromatography on a mixed-bed ion exchange column consisting of Bio-Rad AG 1 X 10 and AG 50W X 12.

They were then derivatized with diaminoheptane by the cyanogen bromide (CNBr) method (86). 10 ml of microspheres were adjusted to pH 10.5 with 1N NaOH. 0.1g of CNBr was added and the solution stirred for 20 min at 23°C with the pH maintained between 10 and 11. The spheres were chilled on ice, 1 M diaminoheptane in dH_2O was added to give a final concentration of 0.02M, and the solution was stirred 4 hr at 4°C. It was then extensively dialysed against 0.1 M NaCl (3 changes, 1 liter each at 4°C) and 0.1 M Na_2CO_3, pH 9.5 (2 changes, 1 liter each at 23°C). The microspheres were then tagged with fluorescein isothiocyanate (2 mg/ml spheres) or rhodamine isothiocyanate (0.25 mg/ml spheres) in 0.1 M Na_2CO_3, pH 9.5 at 23°C for 12-18 hr. Excess fluorescein was removed by extensive dialysis against 0.1 M NaCl at 23°C. Rhodamine was removed by passage through a Sepharose 6B column equilibrated with PBS followed by extensive dialysis against 0.1 M NaCl. The spheres were stored at 4°C or frozen until needed for conjugation to lectins.

Preparation of lectin-microsphere conjugates: 10 ml of microspheres were rederivatized with a final diaminoheptane concentration of 0.02M using 1-ethyl-3 (3-dimethyl aminopropyl) carbodiimide (5 mg/ml) as a coupling agent. The reaction was carried out for 4 hr at 4°C, pH 6.8-7.0 with stirring. The excess reagents were removed by dialysis against 0.1M NaCl, pH 7.0 (2 changes, 1 liter each at 4°C) followed by dialysis against 0.01M sodium phosphate buffer, pH 7.0 (2 changes, 1 liter each at 23°C). The microspheres were activated with a final concentration of 1.25% glutaraldehyde at 23°C with mixing on a platform shaker. Excess glutaraldehyde was removed by dialysis against 0.01 M sodium phosphate buffer, pH 7.0 (1 change, 1 liter at 4°C) and PBS or HEPES-ConA buffer (H-CAB; 2 changes,

1 liter each at 23°C). Lectins were coupled to the microspheres by adding 2 ml of activated spheres (12 mg/ml) in PBS containing 20 mM NaN_3 to 2ml WGA (1 mg/ml) or 0.8 ml RCA (3.4 mg/ml). The reaction was allowed to proceed for 15 hr at 23°C on a platform shaker. For the preparation of ConA-microsphere conjugates, 2 ml of activated spheres in H-CAB were added to 2 ml of ConA (4 mg/ml) in the same buffer, and the reaction was carried out as above. In some experiments, iodinated lectin was used. The unbound lectins were separated from the lectin-microsphere conjugates on discontinuous sucrose gradients. 2 ml of lectin-microsphere mixture were layered over 10 ml 20% sucrose (w/w) in 0.1 M glycine, 0.01 M Tris-HCl, pH 8.0 and 1.5 ml 60% sucrose in the same buffer in Beckman SW41 rotor tubes. The gradients were centrifuged at 153,400 x g_{max} for 3 hr at 15°C. The lectin-microsphere conjugate, which banded sharply at the 20%/60% interface, was carefully removed in a minimum volume of solution and extensively dialysed against PBS for WGA and RCA conjugates or T-CAB for ConA conjugates. All samples were stored at 4°C or frozen until needed. The dilutions of lectin-microsphere conjugates (initial concentration 6-8 mg/ml) which still resulted in detectable agglutination of human type A RBC were 1/4096 for RCAI, 1/1024 for WGA and 1/64 for ConA. In all cases, concentrations of microspheres in solution were based on dry weight analysis. A small volume (0.25-0.50 ml) of microspheres was dried to constant weight in a preweighed glass vial.

Preparation of ^{125}I microspheres: For quantitative studies, Fl-microspheres were tagged with carrier free ^{125}I by the chloramine T method (99).

Preparation of ^{125}I Lectins

Lectins were tagged with ^{125}I using the lactoperoxidase method (100). 10 mg of lectin in 2-4 ml PBS or T-CAB were mixed with lactoperoxidase (20 µg/ml) and carrier-free ^{125}I (250 µCi/ml). 0.03% H_2O_2 (5 µl/ml) was then added at three minute intervals over a period of 30 min at 23°C. The reaction was stopped by the addition of 1M NaN_3 (5 µl/ml) 3 min after the last H_2O_2 addition. The iodinated lectins were separated from ^{125}I and inactive lectin on the appropriate affinity column as described for the preparation of fluorescent lectins. Lectin concentrations were determined from the absorbance at 280 nm. Typical specific activities of the iodinated lectins were: 1.8×10^5 cpm/µg for ^{125}I-WGA, 6.7×10^4 cpm/µg for ^{125}I-ConA and 1.5×10^5 cpm/µg for RCAI. The efficiency of coupling was 60%-70% for ConA and RCAI and 10%-20% for WGA. Analysis of the iodinated lectins on 9% Laemmli gels indicated they were unaltered by the iodination.

Cell Labelling

A. Quantitation using ^{125}I-lectins or lectin-^{125}I-microsphere conjugates.

Method I: Monolayer cells were harvested with 0.02% EDTA in PBS and washed in DPBS. For each assay, 2.5×10^5 cells were incubated with ^{125}I-lectin or lectin-^{125}I-microsphere conjugates in a total volume of 100 µl in 10 x 75 mm disposable culture tubes for 30 min at 24°C. (For time course studies the labelling period varied from 5 to 60 min.) The cells were washed three times in DPBS, suspended in 200 µl DPBS, transferred to fresh 10 x 75 mm tubes and counted in a gamma counter. To determine

specific binding, the appropriate saccharide inhibitor was included in the assay mixture (0.1 M galactose for RCAI, 0.1 M α-methyl mannoside for ConA and 0.01 M N-acetylchitobiose or 1 x WGA inhibitor (50 mM N-acetyl-D-glucosamine, 5 mg/ml ovomucoid for WGA (116))).

Method II: Cells were harvested, washed and labeled as in Method I. 75 µl of the sample was then carefully layered over 300 µl of 5% BSA in PBS in a 500 µl Eppendorf microfuge tube and the cells pelleted for 4 min in a Eppendorf microfuge (#5413). The top of the tube was pierced to release pressure and the bottom containing the cell pellet sliced off with a red-hot scalpel blade, placed in a 10 x 75 mm tube and counted in a gamma counter. Controls were run with the appropriate saccharide inhibitor in both the assay mix and the BSA solution. All assays were done in duplicate.

Competition studies: Cells were harvested and washed as described for ^{125}I-lectin labelling. For each assay, 2.5-3 x 10^5 cells were incubated for 15 min at 24°C with varying concentrations of unlabeled lectin in a volume of 90 µl. ^{125}I-lectin (10 µl) was then added and the assay continued for 30 min. The labeled cells were then washed, collected and counted by Method II as described. Controls were run simultaneously in the presence of the specific inhibitory sugar of the ^{125}I-lectin. All assays were done in duplicate. In some experiments, the labeled and unlabeled lectins were added simultaneously to the cells and the assay carried out for 30 min at 24°C.

B. Visualization using fluorescent lectins or lectin-microsphere conjugates

a. Fixed cells. Cells on coverslips were rinsed by repeated immersion in DPBS and fixed for 10-15 min at 24°C in 1.25% glutaraldehyde. Glutaraldehyde was rinsed off in DPBS and the coverslip was suspended over a 100 µl drop of lectin reagent (fluorescent lectin or lectin-microsphere conjugate) on a piece of Parafilm in a glass petri dish for 10-15 min at 24°C. The cells were rinsed well and immediately examined under the light microscope to limit glutaraldehyde-induced fluorescence which developed with time. This was done by inverting the coverslip, cell side down, over a drop of DPBS on a glass slide. Controls were run simultaneously in the presence of the specific inhibitory sugar (0.1 M galactose for RCAI, 0.1 M α-methyl mannoside for ConA and 0.01 M N-acetylchitobiose or 1 x WGA inhibitor for WGA).

b. Unfixed cells continuously labeled. Cells on coverslips were rinsed in DPBS and suspended over 100 µl of lectin reagent for 5 to 60 min at 37°C. Unbound lectin was rinsed off in DPBS and the cells either fixed for 5 min at 37°C in 1.25% glutaraldehyde before examination or examined immediately.

c. Unfixed cells discontinuously labeled. Cells on coverslips were rinsed in DPBS and suspended over 100 µl of the lectin reagent for 5-15 min at 37°C. Unbound lectin was rinsed off and the cells were either immediately examined under the light microscope or incubated in DPBS at 37°C for the specified time. Reversibility of lectin binding was tested by treating labeled cells with excess lectin inhibitor (0.25 M α-methyl mannoside and D-galactose and 2.5 x WGA inhibitor) for 15 min at 37°C. In some cases, the experiments were carried out at 4°C or 24°C.

d. Multiple labelling of cells. (1) Fixed cells; double labeled. Cells were fixed and labeled as described in (a) but with saturating concentrations of fluorescent lectin (0.5-1.0 mg/ml) or lectin-microsphere conjugates (1-5 mg/ml). After rinsing off unbound lectin the cells were immediately relabeled for 10 min at 24°C with lectin coupled to a dye or a dyemicrosphere conjugate of the other colour (usually, Fl-lectin followed by Rh-lectin), rinsed and examined for both dyes under the fluorescent microscope. (2) Unfixed cells; double labeled. Cells were labeled as described in (c) but with saturating concentrations of the lectin reagents. In some experiments, after the rinse the cells were immediately relabeled for 10 min at 37°C with a different lectin reagent. In other cases, the coverslips were suspended over buffer for 30-120 min at 37°C and then relabeled for 10 min at 37°C with the second reagent.

e. Studies with drugs. Cells on coverslips were suspended in 10^{-6} M colchicine in DPBS or in 5 µg/ml cytochalasin B containing 0.5% DMSO in DPBS for 30 min at 37°C and subsequently labeled as described in section (b), (c), (d) or (f) in the presence of the same concentration of the drug. In parallel control experiments cells were treated with 10^{-6} M lumicolchicine (117, prepared by uv-irradiation of colchicine) or 0.5% DMSO.

Visualization of the Same Cell by Fluorescent Light and Scanning Electron Microscopy

For examination of the same cell under the fluorescent light and scanning electron microscopes, the cells were grown on H-2 gold finder

grids (Ernest F. Fullam, Schenectady, N.Y.) attached to glass coverslips as described (101). The grids were boiled for 10 min in concentrated HNO_3, rinsed well with dH_2O, sandwiched between a glass coverslip and a layer of formvar (0.7% formvar in ethylene dichloride), coated with a thin layer of carbon and sterilized for 2 hr in shortwave uv light. Differentiated cells were discontinuously labeled with RCA I conjugated to Fl-microspheres. The grids were examined under the fluorescent light microscope while immersed in buffer, and individual cells were located. The grids on the coverslips were then prepared for scanning electron microscopy as described below. After critical point drying, the coverslips with the grids were coated with carbon, mounted on stubs, coated with gold and examined in the SEM.

Scanning Electron Microscopy

For examination in the SEM, the cells were fixed for 1 hr at 24°C in 1.25% glutaraldehyde in DPBS, rinsed in PBS, dehydrated through ethanol (50%, 60%, 70%, 80%, 90% and 4 x 100%; 10 min each), critical point dried from CO_2 (102) in a Polaron bomb and sputter-coated with 200 nm of gold-palladium in a Technics Hummer V. The samples were examined in a Cambridge S-4 Steroscan scanning electron microscope. In some cases, the cells were post-fixed for 15 min in 1% OsO_4 in 0.3 M cacodylate or 90 min in 1.33% OsO_4 in collidine. Fixation in 2% glutaraldehyde in 0.1M cacodylate with 0.1 M sucrose followed by post-fixation for 90 min in 1.33% OsO_4 in collidine (103) was also tried.

Double Labelling with ^{125}I-Lectin and Fl-Lectin

Cells on coverslips were labelled in duplicate for 15 min at 37°C with ^{125}I-ConA (495 µg/ml, 5 µg/ml, 2.5 µg/ml). One set was then rinsed, incubated in buffer for 60-120 min at 37°C, treated with Fl-ConA for 10 min at 37°C and examined in the fluorescent light microscope. Immediately after labelling, the cells on the other set of coverslips were solubilized with 1.5 ml 1N NaOH and scraped into test tubes. Lowry protein assays were run in duplicate on 250 µl aliguots and the protein concentration per ml converted to cells per ml using a prepared plot of cell number versus protein concentration. 500 µl aliquots of the same sample were counted in a gamma counter. The number of molecules of ^{125}I ConA bound per cell at each concentration and the per cent saturation of the ConA binding sites could thus be determined and compared to the amount of relabelling seen with Fl-ConA.

Cell Viability

Viability of cells was determined using trypan blue (104). Trypan blue was made up as a stock solution (0.2% trypan blue in dH_2O) and immediately before use four parts of this stock were mixed with one part of a NaCl solution (42.5 g/liter). Cells were immersed in this solution for 3 min, rinsed thoroughly in DPBS and examined in the light microscope for uptake of the blue dye.

RESULTS

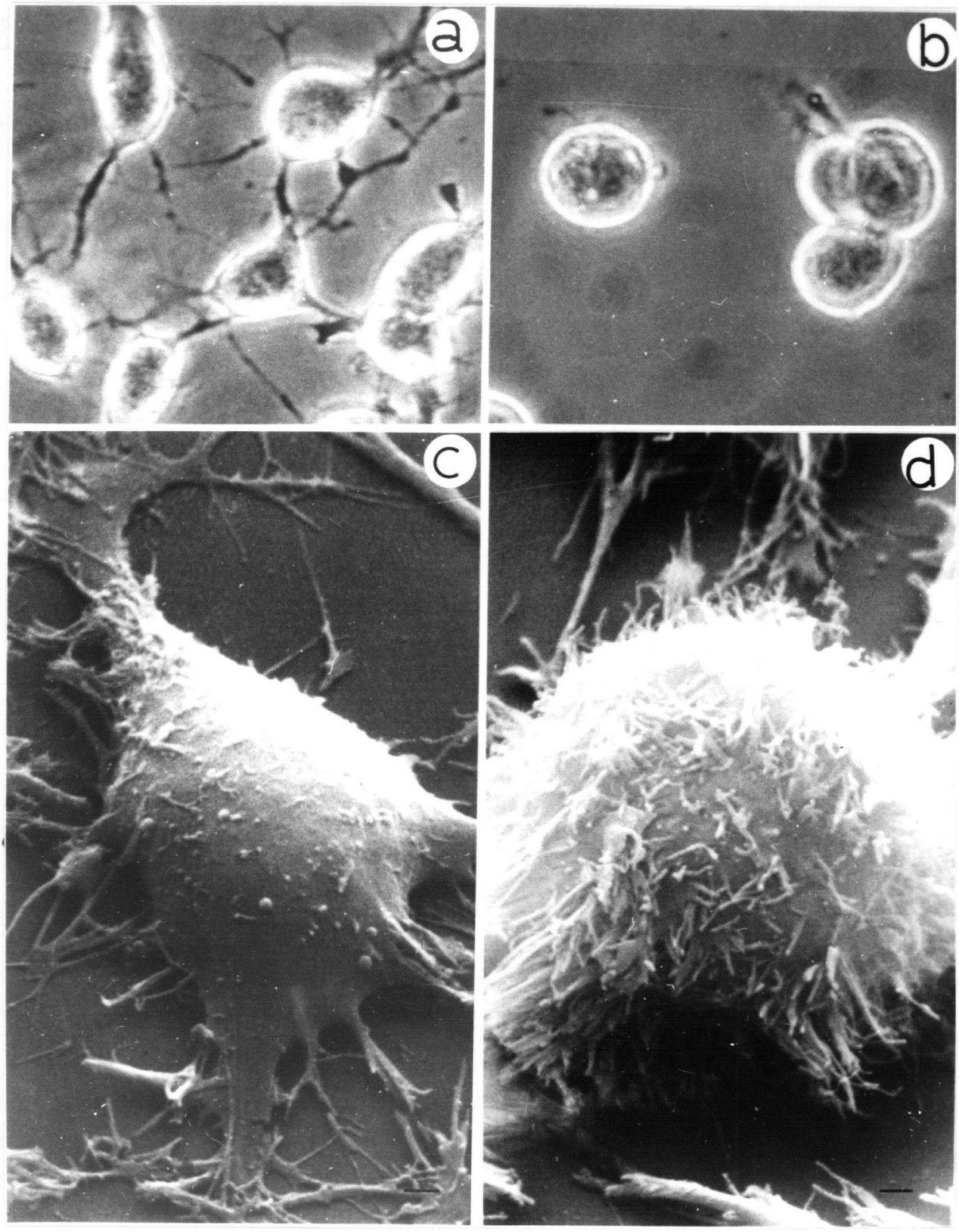
Lectin-Microsphere Conjugates

The covalent coupling of lectins to glutaraldehyde-activated microspheres was demonstrated in experiments where the lectins were mixed with the activated microspheres in the presence of 0.1 M glycine. If covalent coupling of the lectin to the microspheres was essential for the preparation of active lectin-microsphere conjugates, then the presence of glycine should result in no active conjugate. However, if the main interaction between lectins and microspheres was through nonspecific adsorption of the lectin to the microspheres, conjugates prepared in the presence of glycine should be as active as those prepared in its absence. It was found that lectin-microsphere conjugates prepared in the presence of glycine did not agglutinate RBC and RBC treated with these preparations showed no fluorescence.

Morphology of Cells

At the level of the light microscope, undifferentiated cells appeared to be of two types (Fig. 8b): (1) small, round cells with many short, hair-like projections and (2) larger, round cells with a few longer hair-like projections extending from a single area of the cell surface. Under the SEM (Fig. 8 d), they were also round in shape with numerous microvilli and varying numbers of short, spine-like projections on their surfaces. Cells which had been induced to differentiate with serum depletion were oval with large nuclei and 2-3 long (750 μm) neurites (Fig. 8 a). The neurites were covered with small hairs and often ended in bulbous projections.

Figure 8. (a) Light micrograph of differentiated neuroblastoma cells;
(b) Light micrograph of undifferentiated neuroblastoma cells;
(c) SEM micrograph of a differentiated neuroblastoma cell;
(d) SEM micrograph of an undifferentiated neuroblastoma cell.
Bar = 1 μm in all SEM micrographs; all fluorescent and light micrographs = 280 x magnification and the bar = 100 μm.


a
b
c
d

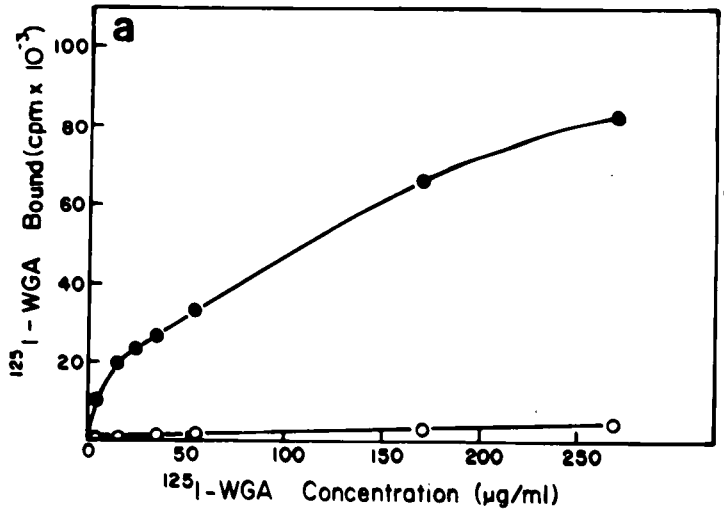
Under the SEM, the differentiated cells appeared flattened out (Fig. 8c) with only a few surface microvilli in comparison with the undifferentiated cells. The neurites were covered wtih many small spines and branched much more extensively than was apparent in the light microscope. Cells whose differentiation had been brought about by Bt_2cAMP or 5-BrdU appeared similar under the light microscope except that Bt_2cAMP-induced cell neurites were shorter and thicker. Preparation of the cells for the SEM by any of the alternative procedures described in Methods did not alter the morphology of the cells as seen in the SEM.

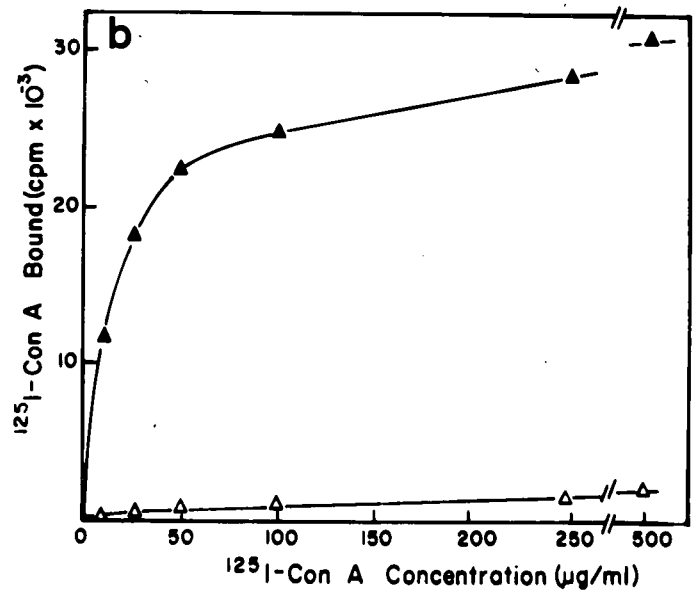
Quantitation of Lectin Binding Sites

The binding of ^{125}I-WGA, ^{125}I-ConA and ^{125}I-RCAI to neuroblastoma cells is shown in Fig. 9 a,b,c. Relatively high (>200 µg/ml) concentrations of lectins were required to reach saturation with all three lectins. Labelling of cells with the lectins was reduced by greater than 90% in the presence of the appropriate saccharide inhibitors. Scatchard analyses (105) of the binding data indicate (Fig.10 a,b,c) that there are at least two classes of binding sites for RCAI, WGA and ConA on the neuroblastoma cells. As indicated in Table III, there are over 1.5×10^7 high affinity sites (Kd = $1\text{-}3 \times 10^{-7}$ M) for the three lectins. These values were determined using molecular weights (52) of 55,000 for ConA, 23,000 for WGA and 120,000 for RCAI. The high affinity ConA binding sites determined with ^{125}I-ConA are in close agreement with the results of Rosenberg and Charalampous (105) using ^{3}H-ConA.

Method I was used in most of the early labelling experiments (quantitation of ConA and WGA binding sites) but Method II proved faster

Figure 9. Binding of ^{125}I-lectins to differentiated neuroblastoma cells. Cells (2-3 x 10^5) suspended in DPBS were incubated with increasing concentrations of lectin in a total volume of 100 µl for 30 min at 24°C. The cells were then washed and counted. (a) ^{125}I-WGA binding in the absence (●) and presence (○) of 0.01 M N-acetyl chitobiose. (b) ^{125}I-ConA binding in the absence (▲) and presence (△) of 0.1 M α-methyl-mannoside. (3) ^{125}I-RCAI binding in the absence (■) and presence (□) of 0.1 M D-galactose.


a
^{125}I-WGA Bound (cpm x 10^{-3})
100
80
60
40
20
0
50
100
150
200
250
^{125}I-WGA Concentration (µg/ml)


b
^{125}I-Con A Bound (cpm x 10^{-3})
30
20
10
0
50
100
150
200
250
500
^{125}I-Con A Concentration (µg/ml)

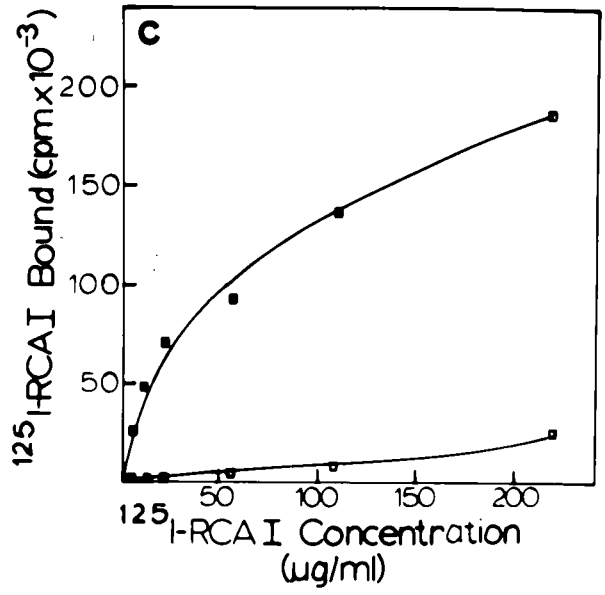
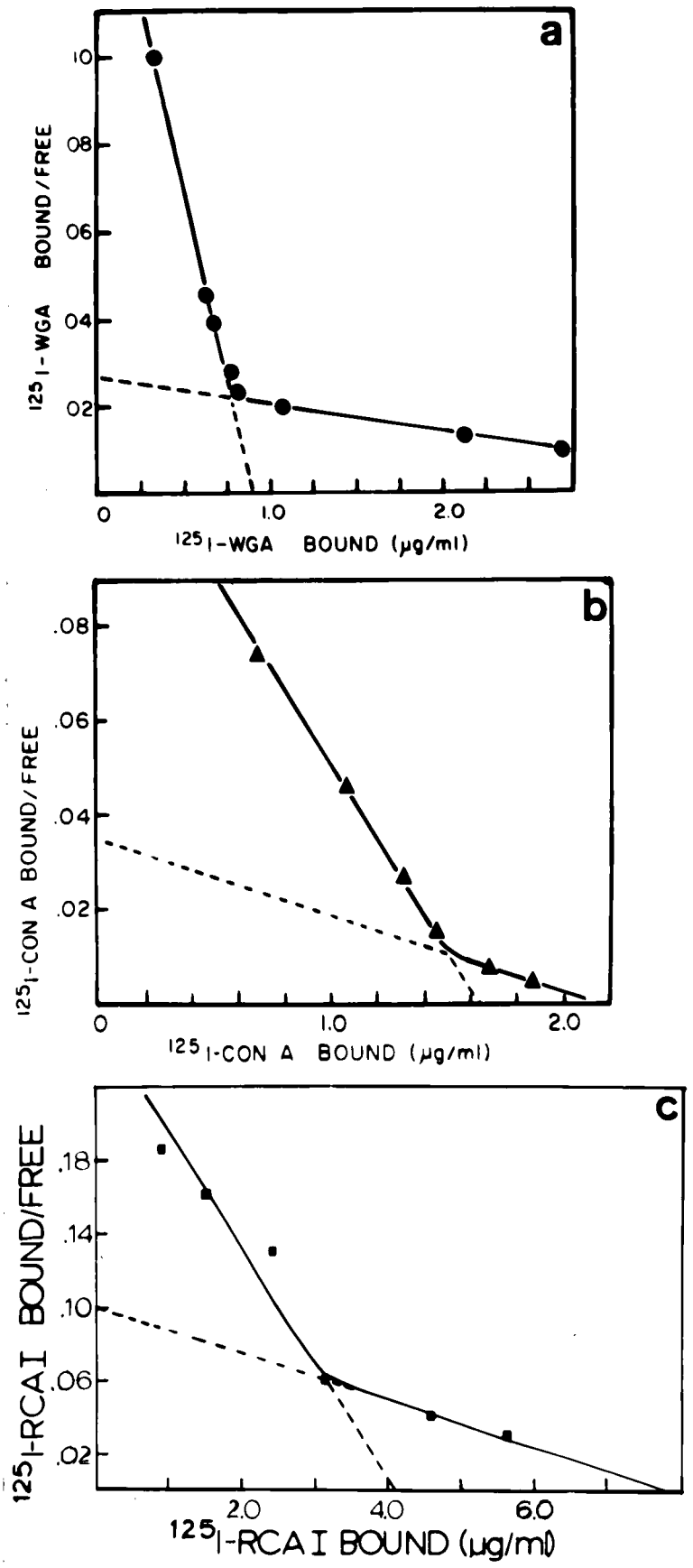

c
^{125}I-RCA I Bound (cpm x 10^{-3})
200
150
100
50
50
100
150
200
^{125}I-RCA I Concentration
(µg/ml)

Figure 10. Scatchard analysis of the binding curves of Fig. 9.(a) ^{125}I-WGA; (b) ^{125}I-ConA; (c) ^{125}I-RCAI.


a
^{125}I-WGA BOUND/FREE
.10
.08
.06
.04
.02
0
1.0
2.0
^{125}I-WGA BOUND (μg/ml)
b
^{125}I-CON A BOUND/FREE
.08
.06
.04
.02
0
1.0
2.0
^{125}I-CON A BOUND (μg/ml)
c
^{125}I-RCAI BOUND/FREE
.18
.14
.10
.06
.02
2.0
4.0
6.0
^{125}I-RCAI BOUND (μg/ml)

and less subject to cell loss during the washing process and so was adopted for all further experiments. However, when the early quantitation studies were repeated using Method II the results were very similar indicating that results obtained by either method are comparable.

The validity of a 30 min labelling period at 24°C for the binding studies was confirmed using both ^{125}I-WGA and ^{125}I-ConA. The results are shown in Fig.11. Both lectins achieve at least 95% of their maximal binding within 30 min. At this time essentially all the label can be removed by a 15 min treatment with excess lectin inhibitor indicating that it is still localized to the cell surface. With longer labelling periods, less of the iodinated lectin can be removed by this treatment suggesting that it has become internalized by the cells.

When cells were labeled with WGA conjugated to ^{125}I-microspheres having an average diameter of 80 nm, the binding leveled off at 1×10^5 microspheres per cell. By coupling ^{125}I-WGA to microspheres it was found that an average of 28 lectin molecules are bound to each microsphere. Due to the geometric constraints of the microspheres, at most half of these lectin molecules could be bound to a cell at one time. Thus, when microspheres are seen to coat the surface of neuroblastoma cells, less than 2.8×10^6 sites, or 10% of the available high affinity sites, are actually labeled.

Distribution of Lectin Binding Sites on Fixed Cells

When glutaraldehyde-fixed neuroblastoma cells in either the undifferentiated or differentiated state were labeled with Fl-ConA, Fl-WGA,

Figure 11. Time course of ^{125}I-ConA binding to cells. Cells were labeled for 5-60 min with ^{125}I-ConA, washed and counted (▲) or labeled, treated for 15 min with 0.25 M α-methyl mannoside, washed and counted (Δ). Similar results were seen using ^{125}I-WGA.

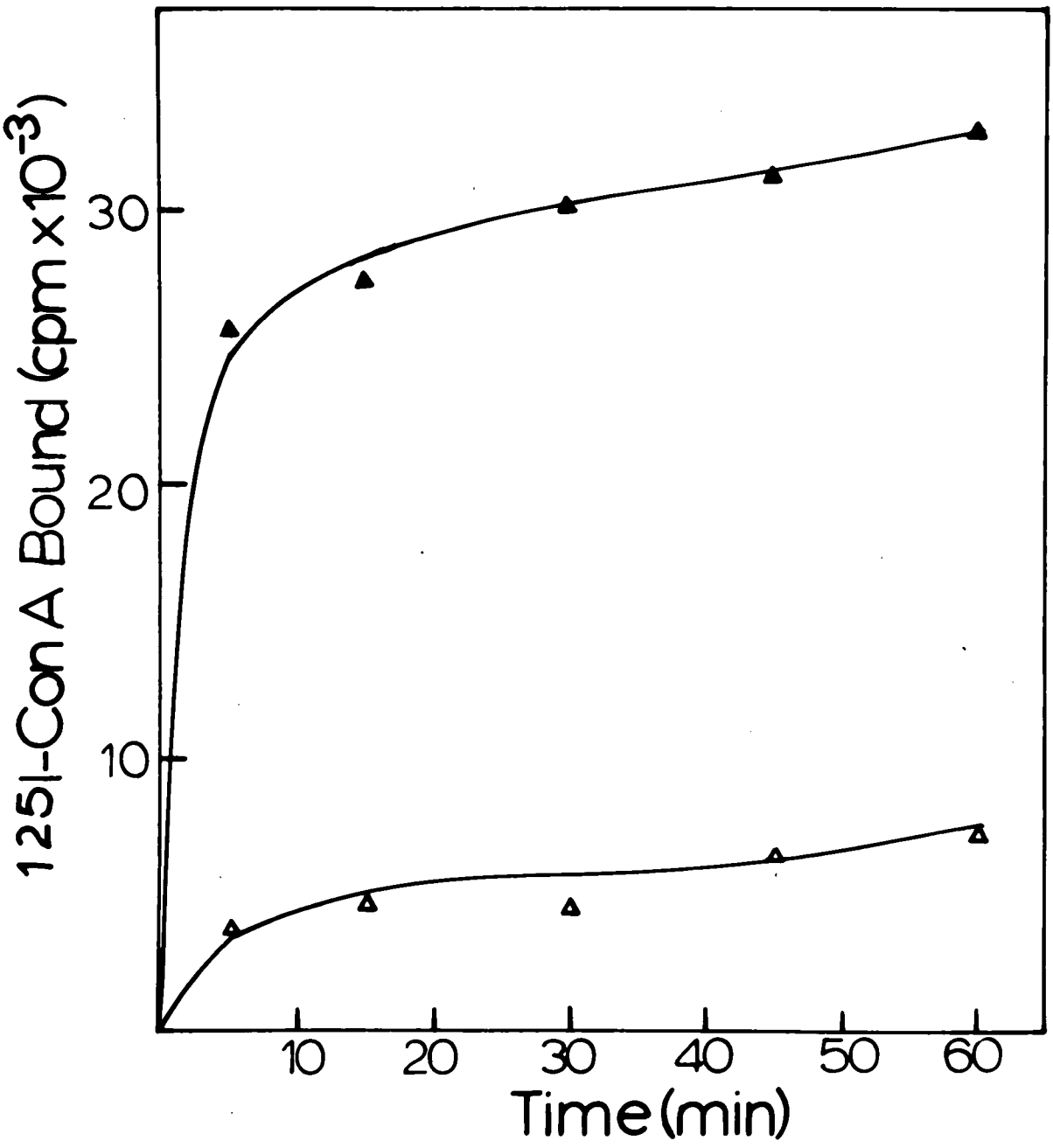

125I-Con A Bound (cpm x10-3)
30
20
10
10
20
30
40
50
60
Time (min)

Table III

Lectin Binding Sites on Neuroblastoma Cells

Lectin	Sites/cell	K_{diss}(M)
^{125}I-ConA		
Class A	1.8×10^{7}	2.4×10^{-7}
Class B	0.5×10^{7}	1.2×10^{-6}
^{125}I-WGA		
Class A	2.5×10^{7}	2.7×10^{-7}
Class B	5.6×10^{7}	2.0×10^{-6}
^{125}I-RCAI		
Class A	2.1×10^{7}	1.6×10^{-7}
Class B	1.9×10^{7}	6.3×10^{-7}
WGA-^{125}I-microspheres	1.0×10^{5}	

Fl-RCAI, or Fl-RCAII or the corresponding lectin-Fl-microsphere conjugates, a uniform fluorescence was observed along the periphery of the cell body and neurite projections (Fig.12 a,b). Under the SEM, lectin-microsphere conjugates were seen to be densely packed over the entire cell surface (Fig.12 c). A similar uniform labelling pattern was observed when unfixed cells were labeled at 4°C. Cells treated with Fl-*Ulex europeus* lectin did not show any fluorescence at any concentration tested although Type O human red blood cells did.

In control experiments, cells treated with Fl-lectins in the presence of the appropriate saccharide inhibitor showed no appreciable fluorescent staining if viewed directly after labelling. Glutaraldehyde-fixed cells, however, gradually developed some autofluorescence that was independent of labelling. The specificity of labelling was confirmed by SEM. Only a small number of lectin-microsphere conjugates were obsevered to bind to the cells in the presence of the lectin inhibitor (Fig.12d).

Lectin-Induced Redistribution

Neuroblastoma cells which were labeled for 5 min at 37°C with 50-100 µg/ml Fl-WGA and subsequently washed free of excess reagent, initially displayed a uniform ring of fluorescence (Fig.13a). It proved possible to reverse this labelling by the subsequent addition of N-acetyl chitobiose or WGA inhibitor (Fig.13b). If Fl-WGA-labeled cells were incubated for 60 min at 37°C, redistribution of the label into a central spot in the extranuclear region was observed (Fig.13c). Some fluorescence was still visible on the cell periphery but this staining diminished on prolonged

Figure 12. (a) Fluorescent micrograph of a fixed, differentiated neuroblastoma cell labeled with Fl-RCAI.
(b) Fluorescent micrograph of fixed, undifferentiated neuroblastoma cells treated with Fl-WGA.
(c) SEM micrograph of a fixed cell labeled with ConA-Fl-microspheres; inset shows a neurite of a cell treated in the same way.
(d) SEM micrograph of a fixed cell treated with ConA-Fl-microspheres in the presence of 0.1 M α-methylmannoside.

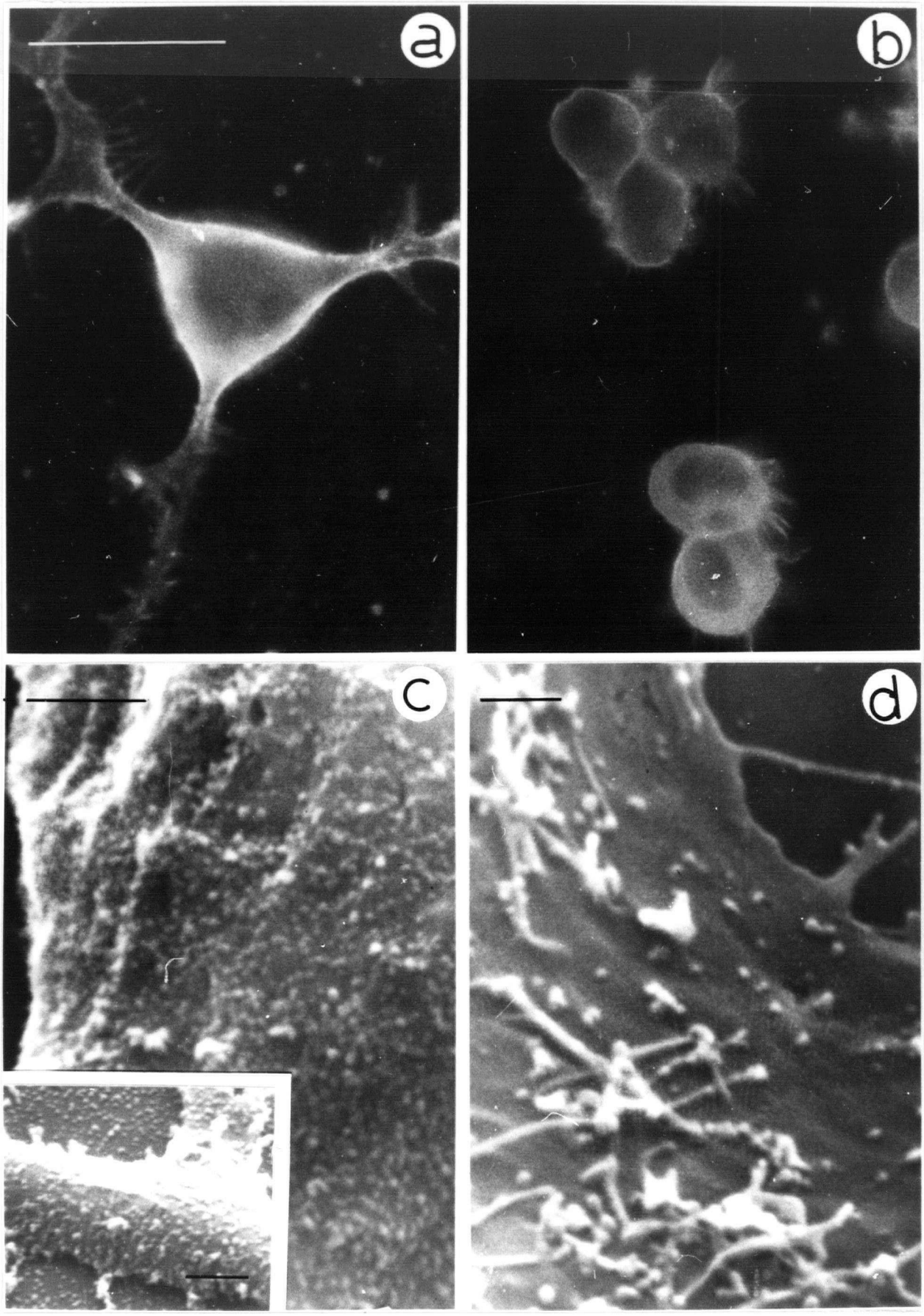

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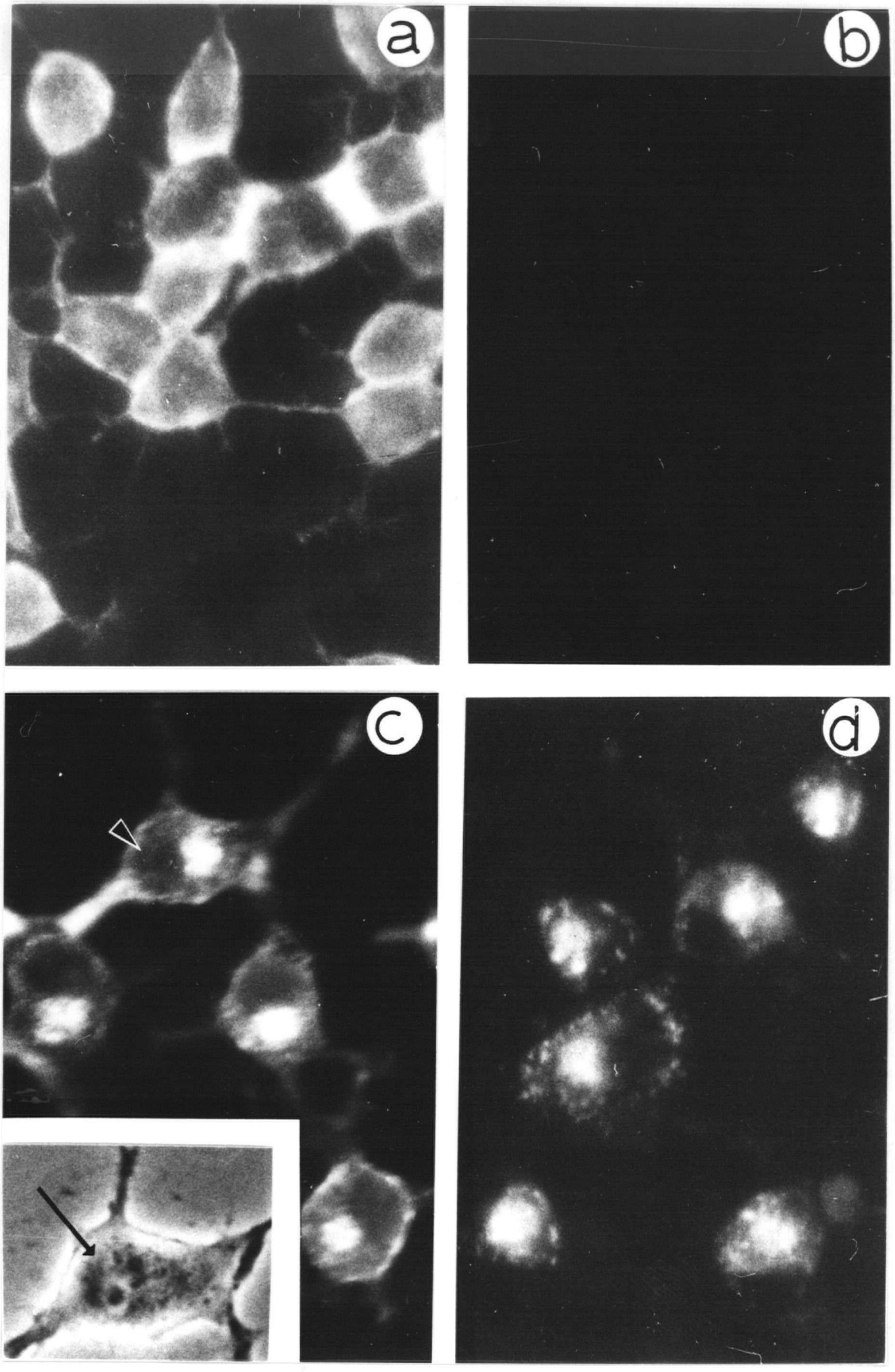
Figure 13. Fluorescent micrographs of unfixed cells labeled with Fl-WGA.
(a) with 5 min label;
(b) 5 min label followed by a 15 min incubation in 0.025 M N-acetyl chitobiose;
(c) 5 min label followed by rinse and 60 min incubation in buffer at 37°C; inset shows phase picture of top cell and arrows indicate the nuclear region;
(d) treated as in (c) and then incubated in 0.025 M N-acetyl chitobiose for 15 min.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incubation of the cells at 37°C. Addition of inhibitor resulted in a loss of the peripheral label but not the central spot (Fig.13d). A similar redistribution of label was observed with both Fl-RCAI and Fl-RCAII.

ConA binding sites also appeared to be initially distributed in a uniform pattern over the cell surface as revealed by reversible labelling studies (Fig. 14a,b). After maintaining the cells for 60 min at 37°C, however, the label had redistributed into an extremely patchy arrangement (Fig.14c). This staining was only partially removed by the addition of 0.25 M α-methyl mannoside (Fig.14d).

In all cases, spot formation was inhibited by the presence of 10 mM sodium azide. Similar results were found using undifferentiated cells. The formation of the fluorescent spot was also seen at 24°C, but it took 90-120 min before this was apparent. After 2 hr at 4°C, however, the fluorescence was still uniform.

When the labelling was viewed in the SEM using RCA or WGA-Fl-microsphere conjugates, the redistribution of the lectin at 37°C became evident at earlier times. Cells prepared for the SEM immediately after treatment with the lectin showed uniform arrays of microspheres on their surfaces (Fig.15a) which were completely removed by the addition of excess lectin inhibitor (Fig.15b). This was consistent with the results seen with the fluorescent microscope. Labeled cells washed free of excess reagents and maintained in buffer for 30 min exhibited patches (> 200 microspheres/patch) of microspheres (Fig.16a) interspersed with areas that were free of label. The clear areas had increased in size after a 45 min wash (Fig.16b) whereas the patches seemed to have shrunk somewhat (< 200 microspheres/patch). After a 60 min wash, the microspheres appeared to be

Figure 14. Fluorescent micrographs of unfixed cells labeled with Fl-ConA.
(a) with 5 min label;
(b) 5 min label followed by a 15 min incubation in 0.25 M α-methyl mannoside;
(c) 5 min label followed by rinse and 60 min incubation in buffer at 37°C;
(d) treated as in (c) and then incubated for 15 min with 0.25 M α-methyl mannoside.

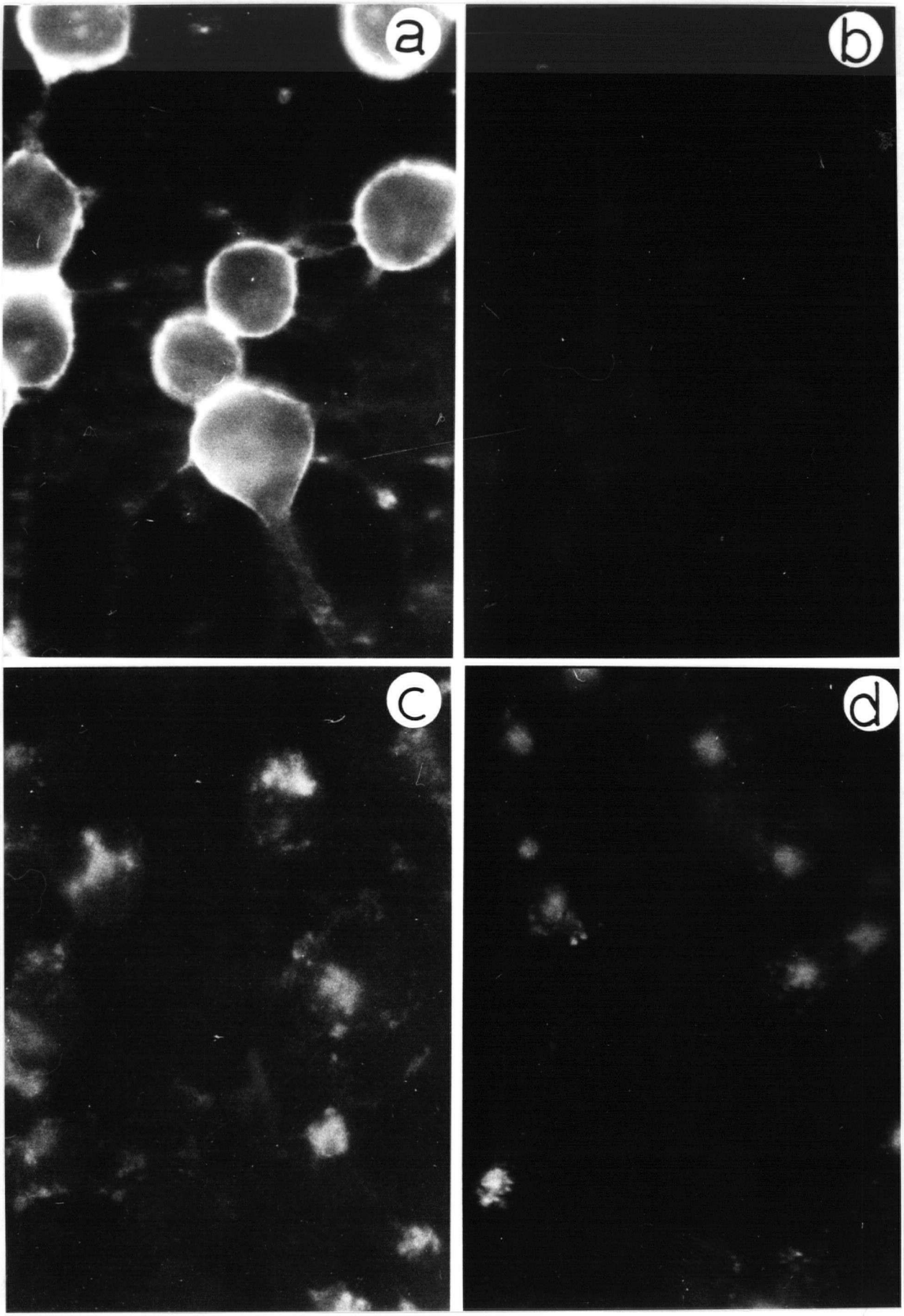
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Figure 15. SEM micrographs of unfixed cells labeled with WGA-Fl-microspheres (80 nm).
(a) with 5 min label;
(b) 5 min label followed by a 15 min incubation in 0.025 M N-acetylchitobiose;
(c) 5 min label followed by rinse and 60 min incubation in buffer at 37°C;
(d) treated as in (c) and then incubated in 0.025 M N-acetylchitobiose for 15 min.

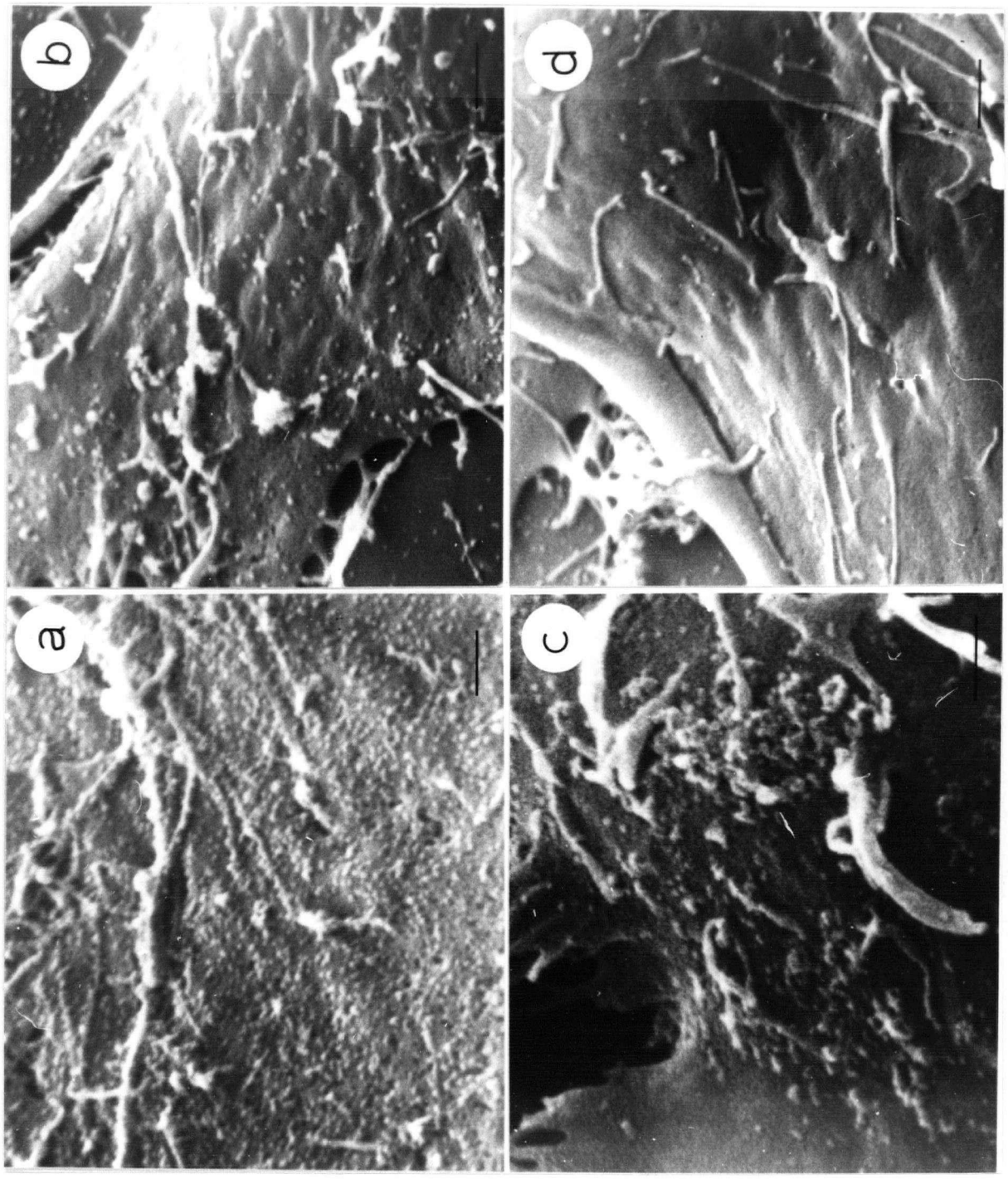
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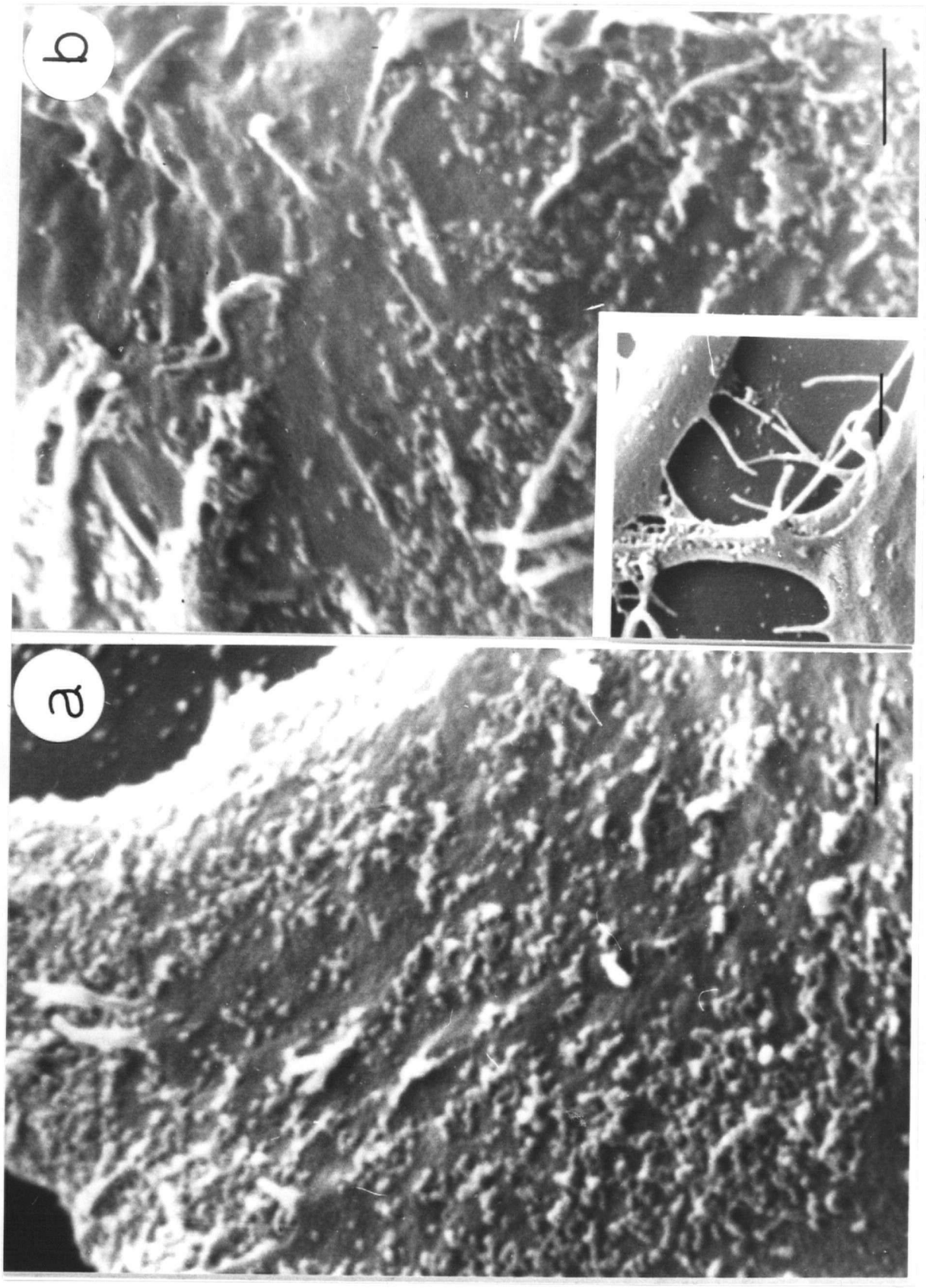
Figure 16. SEM micrographs of unfixed cells labeled with ConA-microspheres (80 nm).
(a) 5 min label followed by rinse and 30 min incubation in buffer at 37°C;
(b) 5 min label followed by rinse and 45 min incubation in buffer at 37°C. Inset shows a neurite of a cell treated in the same way.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almost completely cleared from the surface of the cells (Fig.15c). The remaining label could be removed by excess lectin inhibitor (Fig.15d).

To confirm that the clearing of label from the surface of the cells was due to an internalization of the label by the cells, the same cells were examined under both the light and scanning electron microscopes (Fig.17). In this way, fluorescent patterns could be precisely correlated with the distribution of microspheres on the cell surface. In all the cells which had formed a spot of fluorescence as visualized with the fluorescent light microscope (Fig.17a), the SEM showed few microspheres on their surfaces (Fig. 17a,b). Furthermore, the majority of these remaining microspheres could be correlated with a small amount of fluorescence along the edges of the cell bodies and neurites on differentiated cells. In addition, these cells often had a number of ruffle-like membrane extensions which were not present on unlabeled cells or cells treated for only brief time periods with the lectins. Controls showed little labelling.

Difference in the Redistribution of Lectin Binding Sites

Continuous labelling with lectin-Fl-microsphere conjugates.

Neuroblastoma cells treated for 5 min with WGA-or RCAI-Fl-microspheres exhibited a characteristic ring of fluorescence (Fig.18 a). After 60 min of continuous labelling, however, an intense fluorescence was spread throughout much of the cell (Fig.18b). Visualization by SEM indicated that WGA- and RCA- microspheres were densely packed over the cell surface throughout the duration of labelling (Fig. 18c). In contrast, ConA-Fl-microspheres which were displayed in a fluorescent ring on the cell after

Figure 17. Light and SEM micrographs of the same unfixed neuroblastoma cell labeled for 5 min at 37°C with RCAI-Fl-microspheres (80 nm), rinsed and incubated in buffer for 60 min.
(a) SEM micrograph of the whole cell. Inset shows a fluorescent micrograph of the same cell. The area indicated by the arrow is outlined in the SEM photo.
(b) Enlarged view of the same cell as seen in the SEM. Outlined area is the same as in (a).

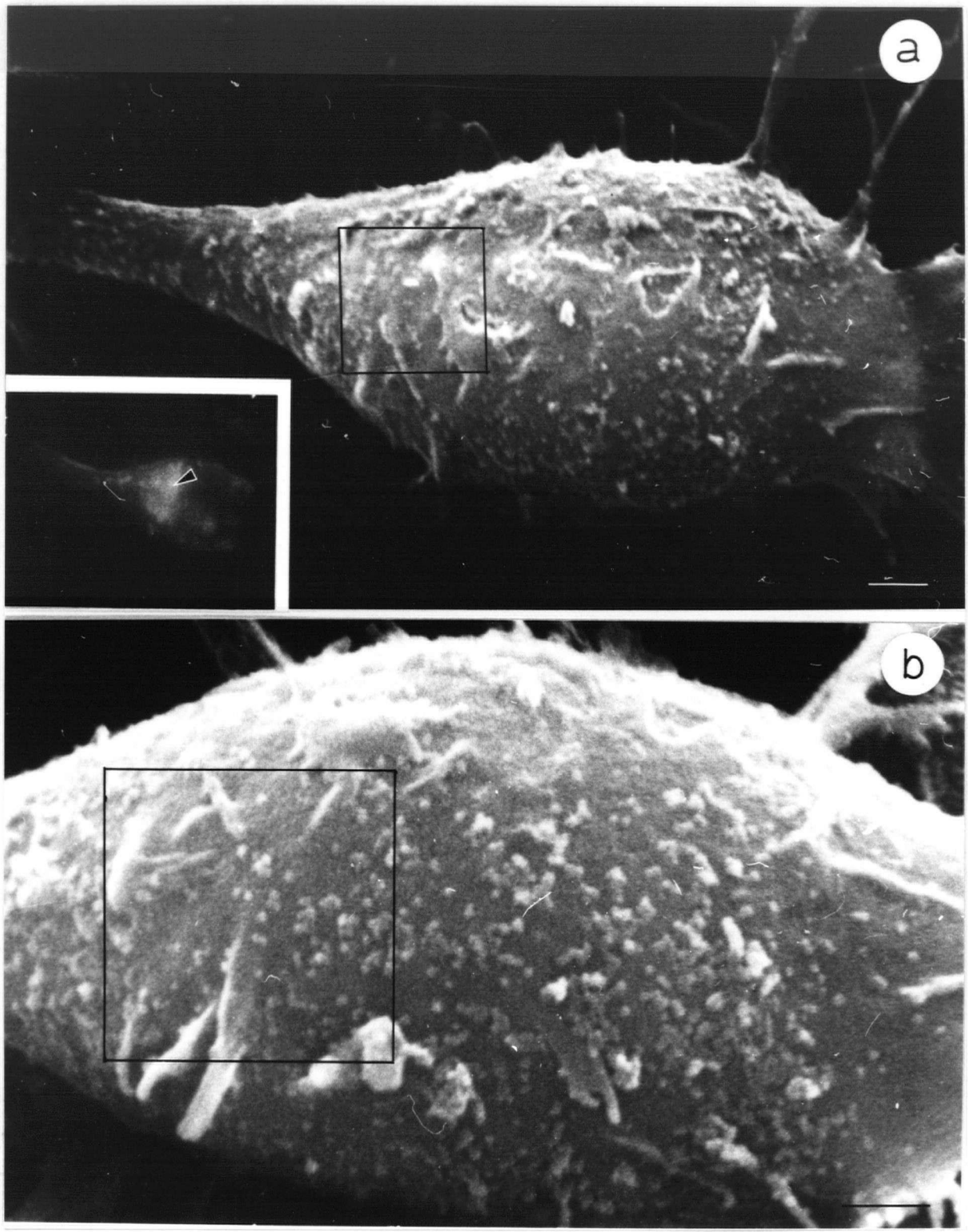
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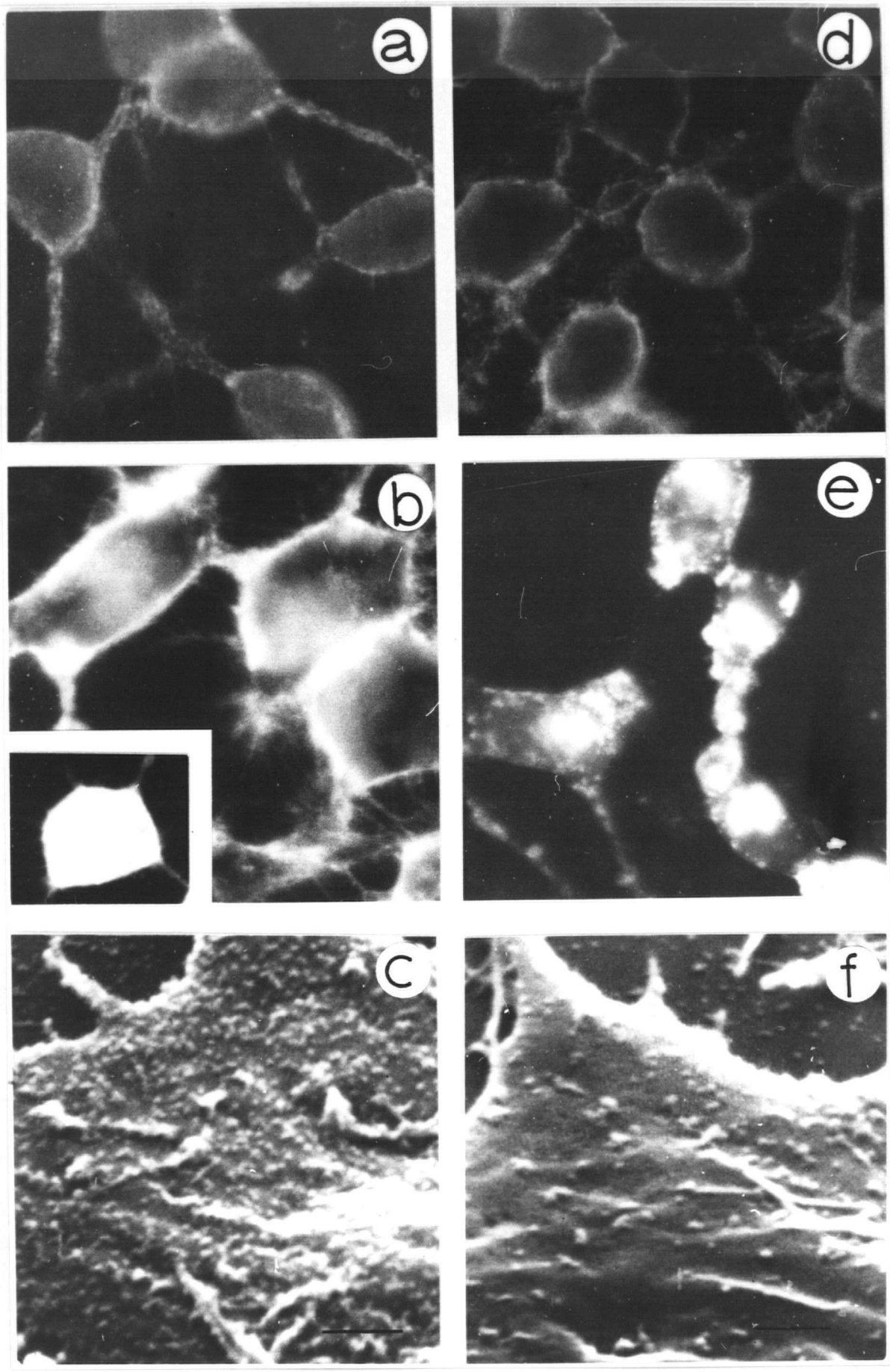
Figure 18. Continuous labelling of cells with lectin-microsphere conjugates. Fluorescent micrographs of cells labeled 5 min (a) and 60 min (b) with WGA-Fl-microspheres. Inset shows a cell labeled for 60 min with RCA-Fl-microspheres. SEM micrograph of a cell treated for 60 min (c) with WGA-Fl-microspheres. Fluorescent micrographs of cells incubated 5 min (d) and 60 min (e) with ConA-Fl-microspheres. SEM micrograph of a cell labeled 60 min (f) with ConA-Fl-microspheres.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a 5 min labelling period (Fig. 18d) redistributed into large clusters after 60 min of continuous labelling (Fig. 18e). The sparse display of microspheres on the cell surface as visualized by SEM (Fig. 18f) after a 60 min labelling indicated that most of the ConA binding sites had been internalized under these conditions.

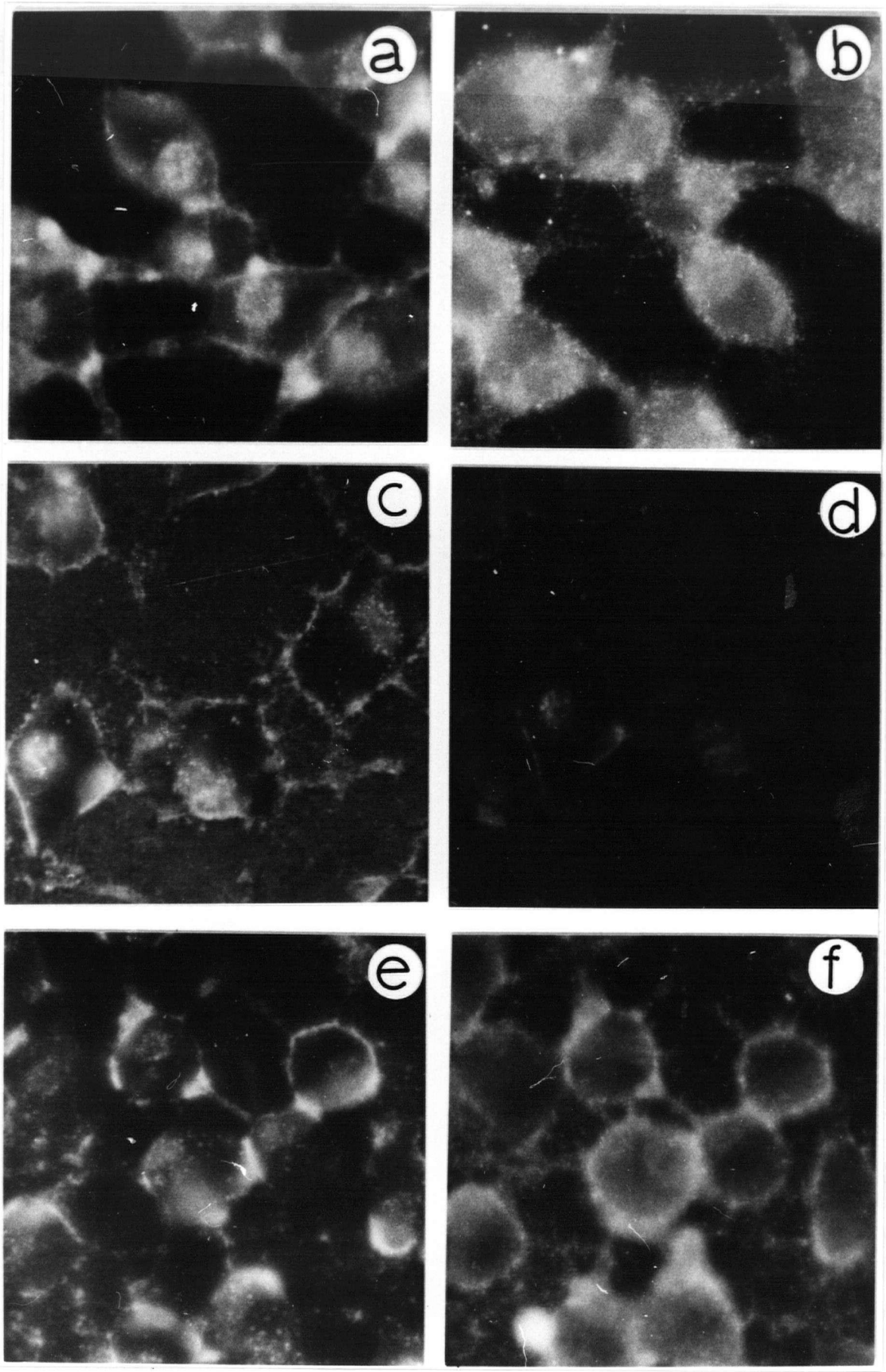
Double labelling. A difference in the mode of redistribution between ConA and WGA binding sites was also revealed in double labelling experiments. Cells which were coated with WGA-Fl-microspheres could be relabeled with Rh-WGA or WGA-Rh-microspheres if the cells were induced to redistribute and internalize the initial label, i.e., if the labeled cells were incubated at 37°C for 60-120 min prior to treating with Rh-lectins (Fig. 19a,b). A similar result was obtained if RCAI was used in place of WGA in both labelling steps.

On the other hand, cells treated with ConA-Fl-microspheres under identical conditions could not be relabeled with either Rh-ConA or ConA-Rh-microspheres (Fig. 19c,d) even after redistribution had occurred. Similarly, cells initially treated with ^{125}I-ConA at concentrations (2.5-10 μg/ml) which only labeled 8-15% of the available high affinity sites could not be relabeled with Fl-ConA after the cells were incubated at 37°C for 60-120 min.

Effect of Cytochalasin B and Colchicine on Redistribution

Cytochalasin B altered the mode of redistribution of ConA-labeled sites and retarded the clearing of label from the cell surface. Cells which were pretreated with cytochalasin B in 0.5% DMSO and then labeled for

Figure 19. Fluorescent micrographs of cells double-labeled with lectin conjugates. Cells were treated for 5 min with the fluorescein label, rinsed, incubated in buffer 60 min, relabeled for 5 min with the rhodamine label, and subsequently examined for both Fl and Rh staining. Fl staining (a) and Rh staining (b) of cells treated with WGA-Fl-microspheres/WGA-Rh-microspheres. Fl (c) and Rh (d) staining of cells incubated with ConA-Fl-microspheres/Rh-ConA (low degree of staining in (d) is due to Fl fluorescence (green) which is not filtered out). Fl (e) and Rh (f) staining of cells treated with ConA-Fl-microspheres/WGA-Rh-microspheres. (Similar results were seen if Fl-ConA/Rh-WGA were used instead of their respective microsphere counterparts.)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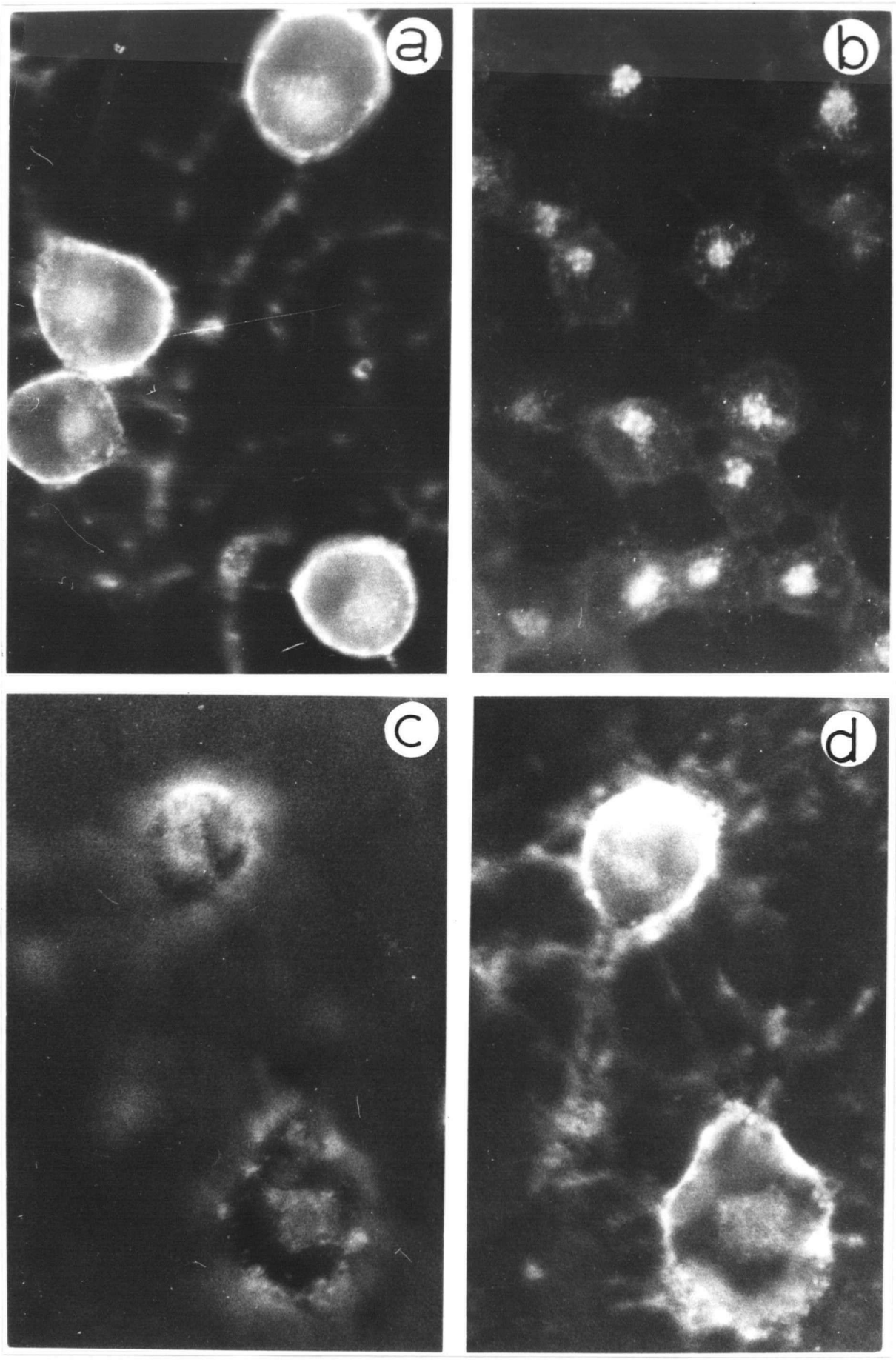
60-120 min with ConA-Fl-microspheres displayed a uniform ring of fluorescence around their periphery as well as a fluorescent central spot (Fig. 20a). α-Methyl mannoside was seen to displace the peripheral label, but not the central fluorescent spot (Fig. 20b). Furthermore, cytochalasin B-treated cells which were labeled briefly with ConA-Fl-microspheres, rinsed and incubated in buffer for 120 min could be relabeled with Rh-ConA (Fig. 20c,d). This is in contrast to untreated or DMSO-treated cells which showed a patchy surface distribution of fluorescence and could not be relabeled with Rh-ConA after 60-120 min (see Fig. 19c,d). Cytochalasin B did not seem to alter the pattern of redistribution for WGA receptors but did retard their clearing. 0.5% DMSO did not change the redistribution pattern of ConA receptors or retard their clearing. Cytochalasin B had no effect on the morphology of the cells or their viability, as judged by their ability to exclude trypan blue.

Cells pretreated with 10^{-6} M colchicine for 30 min at 37°C rounded up. When they were subsequently labeled with Fl-ConA or Con-A-Fl-microspheres for 5 min, washed and incubated in buffer for 60 min, they displayed a patchy distribution of ConA-labeled sites as seen by both fluorescent microscopy and SEM (Fig. 21a,b). Although the central spot was not formed, label could be only partially removed by addition of α-methyl mannoside. Similar results were seen with WGA and RCAI. Colchicine had no effect on the viability of the cells. Lumicolchicine had no visible effect on either the cell morphology or the redistribution of lectin-labeled sites.

Figure 20. Fluorescent micrographs showing the effect of cytochalasin B on the redistribution of ConA binding sites. All cells were pretreated 30 min at 37°C with 5 μg/ml cytochalasin B and labeled and washed in the presence of cytochalasin B. (a) cells continuously labeled with ConA-Fl-microspheres; (b) labeled as (a) and then treated 15 min with 0.25 M α-methyl mannoside; Fl (c) and Rh (d) staining of cells double-labeled with the fluorescein label, rinsed, incubated in buffer for 120 min, and relabeled for 5 min with the rhodamine label.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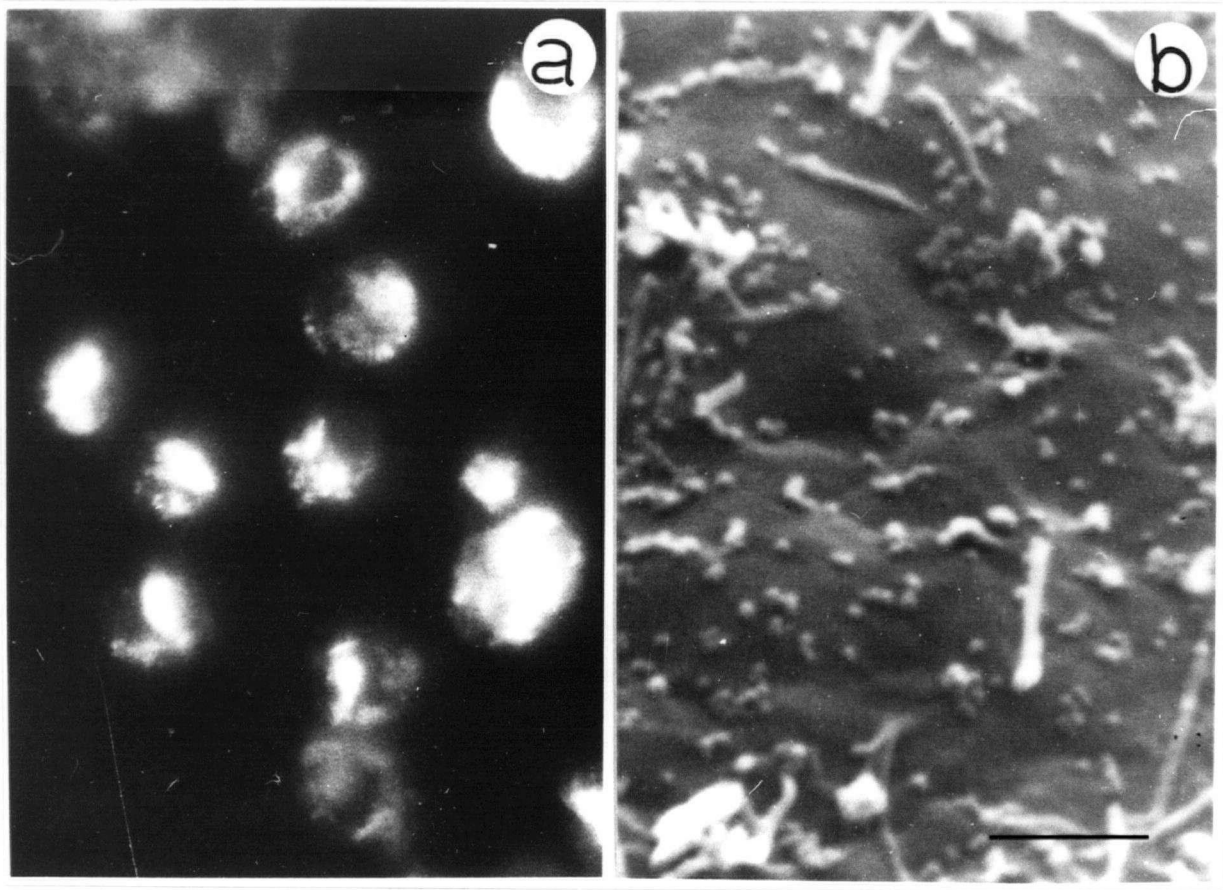

Figure 21. The effect of colchicine on ConA redistribution. All cells were pretreated 30 min at 37°C with 10^{-6} M colchicine and labeled and washed in the presence of colchicine.
(a) Fluorescent micrograph of cells labeled 5 min with Fl-ConA, rinsed and incubated in buffer 60 min;
(b) SEM micrograph of a cell treated 5 min with ConA-Fl-microspheres, rinsed, and maintained for 60 min in buffer.

Double Labelling Studies

Fixed cells. When glutaraldehyde-fixed neuroblastoma cells were labeled with saturating concentrations of Fl-lectin and then treated with the same lectin conjugated to rhodamine, no rhodamine fluorescence was observed in the fluorescent microscope (Fig. 22 a,b). It generally took longer (10-15 min) to saturate all the ConA binding sites with Fl-ConA than it did to label all the WGA or RCAI binding sites.

When different lectins were used for each step, a variety of results was seen. Fixed cells initially labeled with Fl-WGA could be re-labeled with either Rh-ConA or Rh-RCAI (Fig. 22 c,d). Similarly, fixed cells labeled with Fl-ConA relabeled with Rh-WGA. However, when fixed cells were first labeled with Fl-RCAI, they relabeled only poorly or not at all with Rh-WGA (Fig. 22 e,f). Studies with RCAI and ConA were not carried out because of the interaction between these two lectins described in Chapter 2. The results were identical if the labelling was done on unfixed cells at 37°C for 10 min each step. In all experiments controls run simultaneously showed little labelling in either step.

Unfixed cells, discontinuously labeled. If cells were treated for 10-15 min with saturating concentrations of Fl-lectin and then incubated in buffer for 60 min at 37°C so as to clear all the lectin receptors from the cell surface and allow formation of the fluorescent spot (Fig. 23 a,b), they could not be relabeled with the same lectin conjugated to rhodamine or rhodamine-microspheres. When the same type of experiment was carried out using Fl-WGA as the first label, the cells could still bind Rh-ConA and reduced amounts of Rh-RCAI (Fig. 23 c,d) after the 60 min wash. However, clearance of all RCA receptors prevented the relabelling

Figure 22. Fluorescent micrographs of fixed cells double labeled with fluorescent lectins. Fixed cells were treated for 10 min with the fluorescein label, rinsed, incubated for 10 min with the rhodamine label, and examined for both Fl and Rh staining. Fl staining (a) and Rh staining (b) of cells treated with Fl-RCAI/Rh-RCAI. Fl (c) and Rh (d) staining of cells incubated with Fl-WGA/RCAI-Rh-microspheres. Fl (e) and Rh (f) staining of cells treated with Fl-RCAI/Rh-WGA.

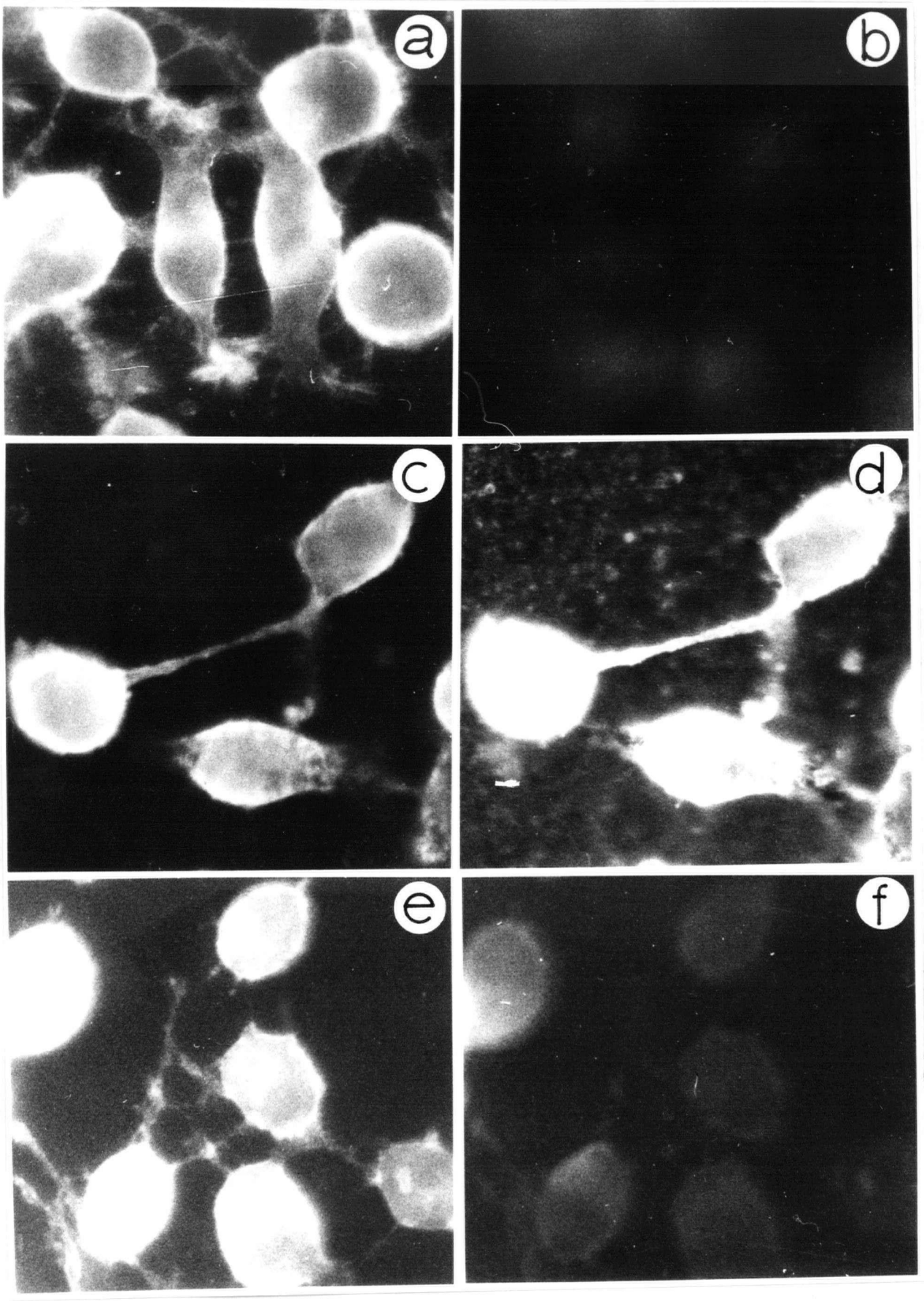
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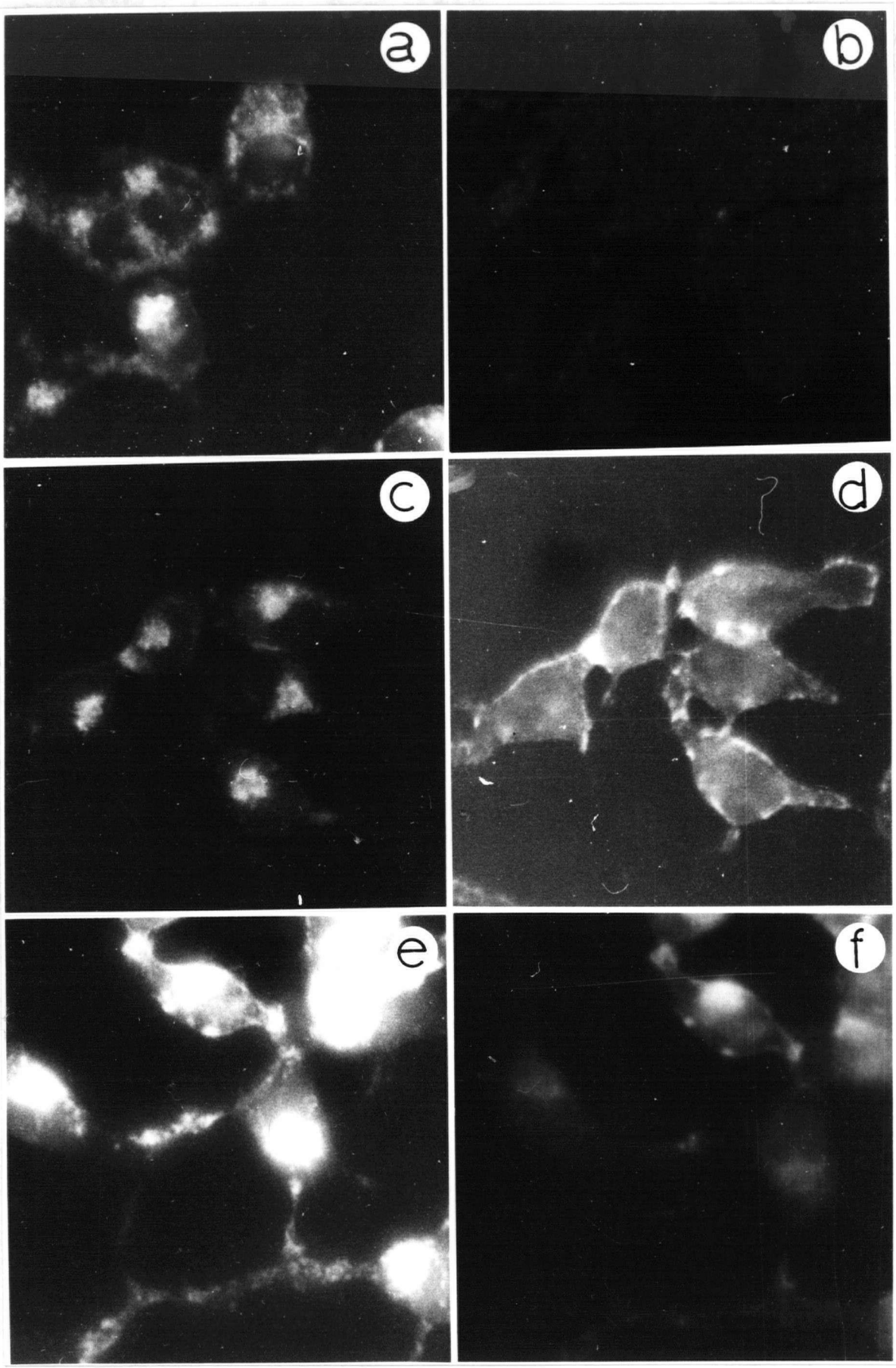
Figure 23. Fluorescent micrographs of unfixed cells double labeled with fluorescent lectins. Cells were labeled for 10 min with the fluorescein label, rinsed, incubated in buffer for 60 min, treated for 10 min with the rhodamine label and examined for both Fl and Rh staining. Fl staining (a) and Rh staining (b) of cells treated with Fl-WGA/Rh-WGA. Fl (c) and Rh (d) staining of cells incubated with Fl-WGA/Rh-RCAI. Fl (e) and Rh (f) staining of cells treated with Fl-RCAI/Rh-WGA.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of the cells with Rh-WGA (Fig. 23 e,f) as well as Rh-RCA. If RCA conjugated to Fl-microspheres was used as the first label instead of Fl-RCA, the cells did relabel with both Fl-RCA and Fl-WGA. Cells initially labeled with Fl-ConA or ConA-Fl-microspheres could be relabeled with Rh-WGA after clearance of the all ConA receptors into the fluorescent spot (Fig. 19 e,f).

Competition studies with ^{125}I-lectins. All three lectins were able to reduce the binding of their ^{125}I-labeled counterparts to the neuroblastoma cells by 80-90% (Fig.24). This is in close agreement with the reduction of lectin binding seen in the presence of the specific saccharide inhibitors (Fig. 9). ConA had no effect on the binding of ^{125}I-WGA (Fig. 24c) to the cells in agreement with the results seen with the fluorescent lectins. Similarly, WGA had no effect on the labelling of the cells with ^{125}I-ConA (Fig.24 a). However, WGA was able to reduce the binding of ^{125}I-RCA by over 80% (Fig.24 b). In the reverse experiment (Fig. 24c), RCAI brought about a similar reduction in the binding of ^{125}I-WGA to the cells. In these studies, more reproducible results were obtained if the cells were first pretreated for 15 min with the unlabeled lectin before incubation with the ^{125}I-lectin so this protocol was used in all the experiments. Although the ^{125}I-lectin concentrations were chosen so that they would fall in the plateau regions of the binding curves, these concentrations were such that only the high affinity (Kd ~ 10^{-7} M) binding sites could be saturated.

Microsphere-conjugated lectins and fixed cells. In order to determine the relative positions of the different lectin receptors on the neuroblastoma cell surface, lectins conjugated to 80 nm Fl-microspheres were used to label fixed cells. The results are shown in Table IV.

Figure 24. Competition studies with ^{125}I lectins. Cell suspended in DBPS were incubated for 15 min at 24°C with increasing concentrations of lectin in a total volume of 90 µl. 10 µl of ^{125}I-lectin was added and the incubation was continued for 30 min. The cells were then washed and counted. (a) ^{125}I-ConA binding in the presence of ConA (●) and WGA (o). (b) ^{125}I-RCAI binding in the presence of RCAI (●) and WGA (o). (c) ^{125}I-WGA binding in the presence of WGA (●), RCAI (▲) and ConA (o).

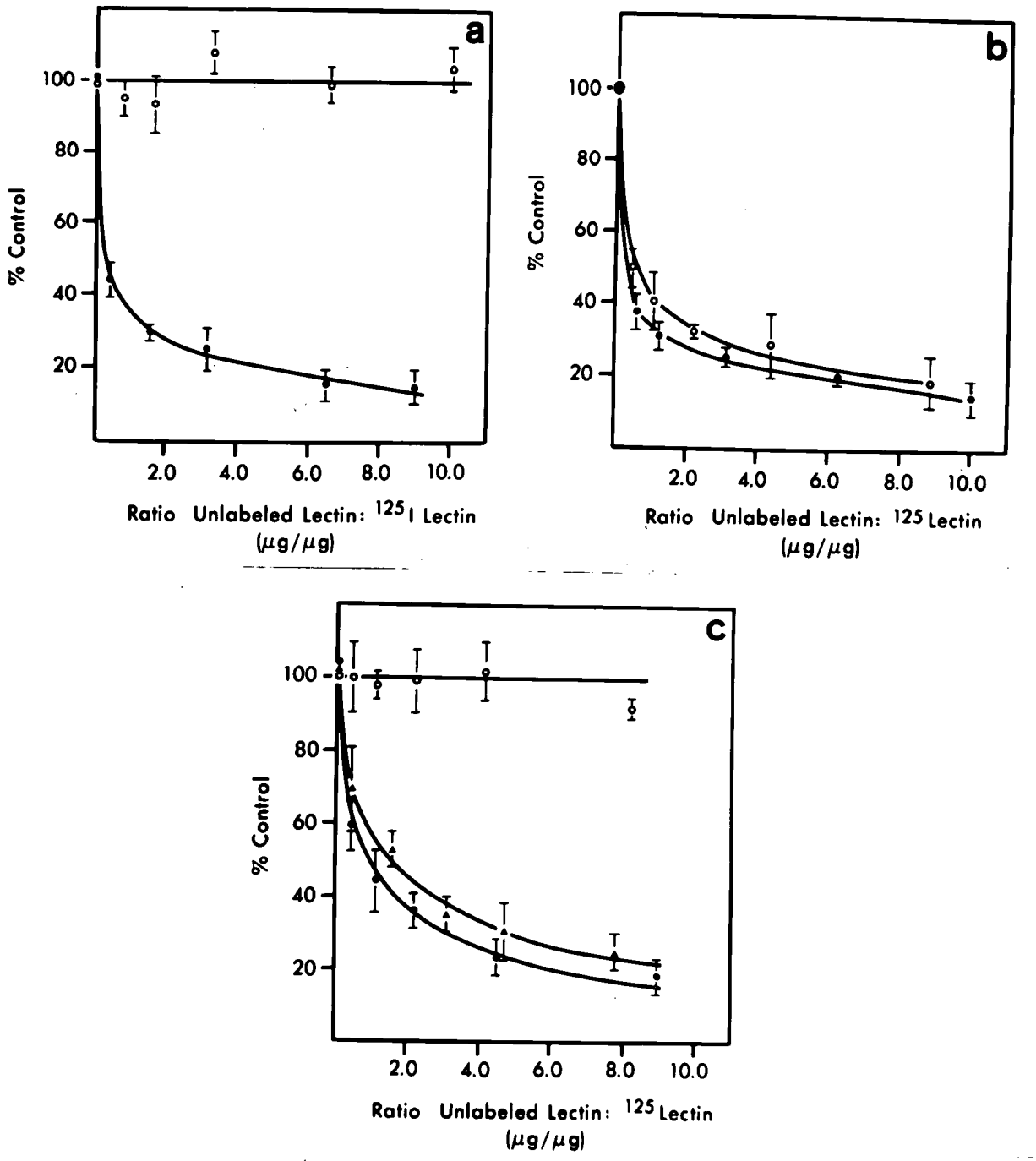

a
b
c
% Control
100
80
60
40
20
2.0
4.0
6.0
8.0
10.0
Ratio Unlabeled Lectin: 125I Lectin
(μg/μg)
Ratio Unlabeled Lectin: 125 Lectin
(μg/μg)

Table IV

Double Labelling of Fixed Cells

Second Label (Rh-conjugate)		First Label (lectin-Fl-microsphere)			
			RCAI	WGA	ConA
RCAI	microsphere		Fl/-	Fl/Rh	
	free		Fl/Rh	Fl/Rh	
WGA	microsphere		Fl/-	Fl/-	Fl/-
	free		Fl/Rh	Fl/Rh	Fl/Rh
ConA	microsphere			Fl/Rh	Fl/-
	free			Fl/Rh	

Cells were fixed, labeled for 10 min with lectin-Fl-microspheres, rinsed, incubated for 10 min with the rhodamine label (Rh-lectin or lectin-Rh-microspheres); and examined for both Fl and Rh staining. (—) indicates that relabelling could not be seen under the fluorescent microscope.

When the first label was with lectin-Fl-microspheres and the second treatment was with the same lectin, the cells could be relabeled with Rh-lectin but not lectin-Rh-microspheres. Although WGA-microspheres had no effect on the subsequent labelling of the cells with either free or microsphere-conjugated ConA and RCAI, both ConA-microspheres and RCAI-microspheres blocked the labelling of the cells with microsphere-conjugated but not free, WGA.

DISCUSSION

The morphology of both the undifferentiated and the differentiated neuroblastoma cells observed in the light microscope is essentially identical to that reported previously (62, 63, 72). The appearance of the cells in the SEM is consistent with their morphology in the light microscope and the TEM (72, 73).

Quantitative studies using ^{125}I-lectins indicate that there are over 10^7 high affinity binding sites for ConA, WGA and RCAI on mouse neuroblastoma cells. Labelling studies on glutaraldehyde-fixed cells and cells maintained at 4°C indicate that on both undifferentiated and differentiated neuroblastoma cells these sites are densely and randomly distributed over the cell surface and neurites as visualized by fluorescent and scanning electron microscopy. A similar "native" distribution of lectin binding sites was observed on the surface of cells induced to differentiate with Bt_2cAMP and 5-BrdU, as well as serum withdrawal. Using the horseradish peroxidase technique in conjunction with TEM, Graham *et al.* (72) have noted some differences in the patterns of ConA binding at

4°C on unfixed differentiated and undifferentiated cells. The differences were abolished if the cells were fixed before labelling. Some differences in the membrane glycoproteins, however, have been reported (78, 79, 80, 81) but their carbohydrate composition and lectin binding properties were not studied.

An energy-dependent redistribution of the lectin binding sites occurs when the cells are labeled with free lectins or lectin-microsphere conjugates at 37°C. This phenomenon is common to both developmental states. The label initially forms patches which are particularly evident under the SEM when lectin-microspheres are used as markers. After 60 min most of the label has been cleared from the cell surface and under the fluorescent microscope appears as an intense spot centralized in the extranuclear region of the cell. This staining pattern appears to reflect an internalization of label since addition of a high concentration of the appropriate saccharide inhibitor cannot remove this localized staining. The view that the fluorescent spot represents internalized label is supported by results in which cells induced to redistribute lectin-Fl-microspheres into a fluorescent spot do not exhibit a localized concentration of microsphere markers on the cell surface as visualized by SEM. Gonatas *et al.* (107) have also observed the internalization of ricin-labeled sites on cultured embryonal neurons using peroxidase markers in conjunction with TEM.

Neither microfilament- nor microtubule-disrupting drugs could prevent the energy-dependent internalization of the lectin receptors. Colchicine, however, altered the morphology of differentiated cells and and appeared to affect the intracellular movement of internalized label.

Cytochalasin B had no effect on cellular morphology but did retard the clearing of label from the cell surface so that it took 2 hr to observe the same degree of redistribution normally seen within 1 hr. These results are in agreement with studies by Gonatas *et al.* (107) who found that both 5-10 µg/ml cytochalasin B and 10^{-6} M colchicine did not prevent endocytosis of lectin sites on embryonal neurons.

Although ConA, WGA and RCAI cell surface binding sites can be induced to redistribute by means of labelling with lectins or lectin-microsphere conjugates, the mode of rearrangement of ConA binding sites differs markedly from that of WGA or RCAI sites. Whereas labeled WGA and RCAI sites redistribute independently of their unlabeled counterparts, both labeled and unlabeled ConA binding sites redistribute together. This is documented in both continuous and double labelling experiments. When unfixed cells are continuously labeled with WGA- or RCAI- microspheres for up to 60 min a dense, uniform pattern of marker is visualized in the SEM. Under the fluorescent microscope, however, an increase and extension of fluorescent staining towards the cell centre is seen as labelling proceeds. This pattern suggests that redistribution and internalization of RCAI and WGA receptors is constantly occurring so that previously unlabeled receptors are becoming accessible for labelling. Hence, a dense pattern of label on the cell surface is maintained. On the other hand, ConA receptors continuously labeled with ConA-microspheres form patches on the cell surface as seen under both the fluorescent and scanning electron microscopes. This suggests that with ConA, previously unlabeled ConA receptors do not become accessible to label during the redistribution and internalization of the labeled receptors.

Double labelling studies indicate that when less than 10% of the total number of high-affinity binding sites are initially labeled using WGA or RCA and are induced to clear from the cell surface additional sites become available for relabelling with the same lectin. In contrast, when cells are labeled with subsaturating quantities of ConA under similar conditions, no relabelling of the cells with ConA is observed.

This difference in the pattern of redistribution of ConA binding sites and WGA or RCAI binding sites is not unique to neuroblastoma cells. When *Dictyostelim discoideum* cells are continuously labeled with ConA-microsphere conjugates, ConA markers redistribute into patches and caps as visualized by SEM; cells cannot be relabeled with ConA (108). WGA microspheres, however, are maintained in a dense, uniform array on the cell surface. Brown and Revel (109) have also reported that when cultured mouse L cells were continuously labeled with ricin-hemocyanin markers, a homogeneous pattern of label was observed; cells continuously labeled with ConA-hemocyanin displayed a heterogeneous labelling pattern. In addition, Jacobson and Hou (118) have found in photobleaching studies on the mobility of Fl-WGA-receptor complexes that the percentage of mobile WGA-receptor complexes is much higher than the fraction of mobile ConA-receptor complexes on the same cell type. Both lectin-receptor complexes had similar average lateral diffusion coefficients.

The co-ordinate redistribution of labeled and unlabeled ConA binding sites may reflect the presence of multiple ConA sites on the same or tightly associated membrane components. West and McMahon (110), however, have reported that there are over 35 different ConA binding polypeptides in plasma membranes of *D. discoideum*. It would appear unlikely that all

or most of these polypeptides would be complexed togther in the membrane. The result reported here that the microfilament-disrupting agent cytochalasin B uncouples the redistribution of labeled ConA sites from unlabeled sites suggests that microfilaments may co-ordinate the movement of ConA sites on the cells. Microtubules, however, do not appear to play a role since colchicine had no effect on the redistribution pattern of ConA receptors.

Differences in the turnover rates of the receptors for ConA and WGA could be used to explain the differences in the redistribution patterns of the receptors for these lectins. However, studies (111) have shown the mean half-life for total proteins of plasma membranes from several cell types to be 30-60 hr. Furthermore, in neuroblastoma cells, most of the membrane proteins only reach a steady-state radioactivity after 18-20 hr (112) which is significantly longer than the time period for the reported experiments (1-2 hr).

In addition to differences in redistribution, ConA and WGA or RCAI have differential effects on other cellular properties. ConA induces a mitogenic response in some classes of lymphocytes (113) and alters the morphology and differentiation pattern of a number of cell types. In particular, it has been reported that ConA reverses the differentiation in neuroblastoma cells (75), causes a rounding up and clustering of microvilli on *D. discoideum* cells (108) and inhibits ruffling activity on L cells (109). WGA and/or RCA, however, have no apparent effect. It is not known whether the effect of ConA on cellular morphology is related to the co-ordinate redistribution of labeled and unlabeled ConA binding sites. It is tempting to speculate, however, that both these effects may be

controlled by microfilaments (11) or related cytoskeletal structures and reflect an efficient mechanism for transmitting a signal initiated at one region on the cell surface to other parts of the cell.

The results of double labelling studies on unfixed cells were used to determine direct and indirect associations between the binding sites for the different lectins. In all cases, when cells were labeled with saturating concentrations of Fl-lectin and the label was allowed to concentrate into an internal fluorescent spot, the cells could not be relabeled with the same lectin indicating that under these conditions all the detectable receptors for the lectin were internalized by the cells. Since unfixed cells treated with saturating concentrations of Fl-RCAI and incubated for 60 min in buffer also could not be relabled with WGA, WGA receptors must redistribute and internalize along with those for RCAI. This suggests that most, if not all, the WGA binding sites which are visible in the fluorescent microscope must be associated with RCA binding sites in the cell membrane. However, since cells initially labeled with WGA will relabel at a reduced level with RCAI under the same conditions, some of the RCAI binding sites must be independent of those for WGA. Similar experiments using both free and microsphere conjugated ConA in the first step indicate that most, if not all, the receptors for WGA must reside on different membrane components than those for ConA. This, in turn, suggests that many of the RCAI binding sites must also be separate from those for ConA. These results are consistent with the differences in redistribution patterns observed between ConA and WGA or RCAI which were discussed earlier.

Further information on the arrangement of the lectin receptors and their interactions in the cell membrane was obtained from the

competition studies with ^{125}I-lectins and the double labelling experiments on fixed cells. Since no interference was observed in the binding of Fl- or ^{125}I-WGA to cells labeled with ConA, the binding sites for these two lectins must not only be on different membrane components but must be on the average greater than 45 Å apart, or roughly the radius of a ConA molecule (54). On the other hand, WGA and RCA binding sites appear to be in close proximity, indicating that they must be located on the same or two different but directly-associated membrane components. The double labelling experiments showed that Fl-RCA could greatly reduce the binding of Rh-WGA but that Fl-WGA had only a slight effect on the labelling of the cells with Rh-RCA. The competition assays, however, indicated that both lectins were equally effective in blocking each other's binding. This apparent contradiction can be resolved by considering the labelling conditions used for the two different studies .

Although the first labelling period in the double labelling experiments was shorter than in the competition assays, it was still sufficient to saturate all the visible receptors as shown by the inability of the cells to relabel with Rh-WGA when the cells were initially treated with Fl-WGA. The lectin concentrations used in the two different experiments were also not the same. The fluorescent lectins were used at concentrations (0.5-1.0 mg/ml) that did not permit relabelling with the same lectin in the fluorescent microscope and that should have saturated all the available binding sites according to the binding curves in Figure 9. However, the ^{125}I-lectins were used at concentrations (0.1-0.15 mg/ml) that could completely saturate only the high affinity lectin sites. Thus, in the competition studies only the high affinity WGA and RCAI binding sites

should be important whereas in the double labelling experiments, both the high and low affinity binding sites should play a role. Thus, it is the high affinity WGA and RCA binding sites which are on the same or directly-associated membrane components and which are internalized simultaneously. The low affinity RCA binding sites do not appear to be associated with these high affinity sites since they seem to label under conditions where the high affinity sites are not available; i.e. after internalization of all the WGA receptors. This hypothesis could be tested by treating cells first with WGA so as to clear all the WGA binding sites from the cell surface and then labelling the cells with ^{125}I-RCAI. If the low affinity RCAI sites are available then Scatchard analysis of the binding data should reveal a single line representing these sites.

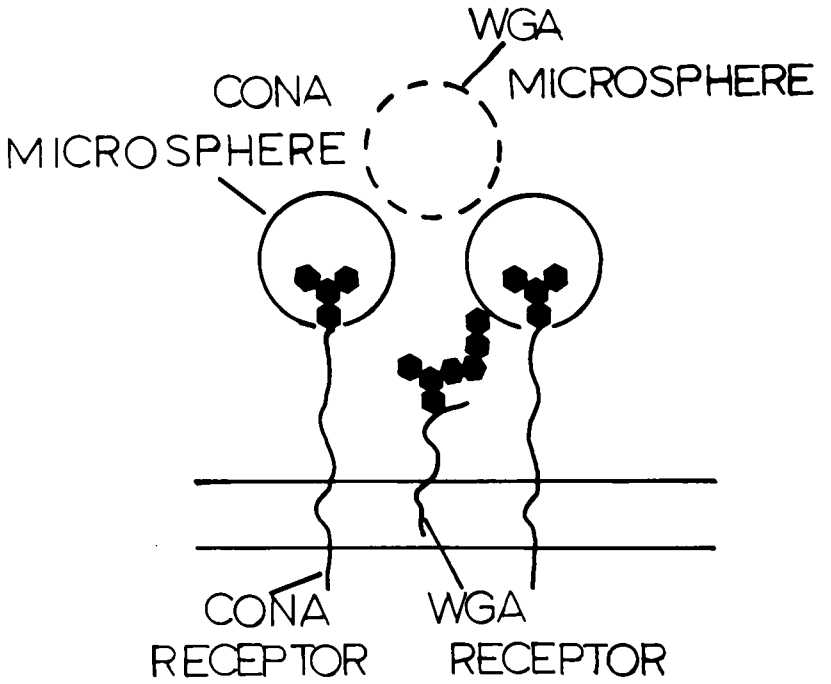
WGA also appears to have a large number of low affinity binding sites as indicated by Scatchard analysis of the binding data for ^{125}I-WGA (Fig. 10). However, unlike the RCAI sites, these WGA sites were not evident in the double labelling experiments. There are a number of possible explanations for this difference. The simplest one is that both the high and low affinity WGA binding sites are located on the same protein molecule whereas RCAI sites are separate. This hypothesis could be tested in a manner similar to that described for the low affinity RCAI sites. A second alternative is that the apparent lack of labelling of the low affinity WGA sites with fluorescent WGA is due to their low association constants for the lectin (K_a = 5.0 x 10^5 M^{-1}). RCAI has a substantially higher affinity for its low affinity sites (K_a = 2.8 x 10^6 M^{-1}). A third possibility is that the low affinity WGA sites are inaccessible to fluorescent

WGA, although it seems unlikely that fluorescent WGA is more sterically hindered than ^{125}I-WGA. Alternatively, fluorescent WGA could have a much lower affinity for the low affinity sites than ^{125}I-WGA.

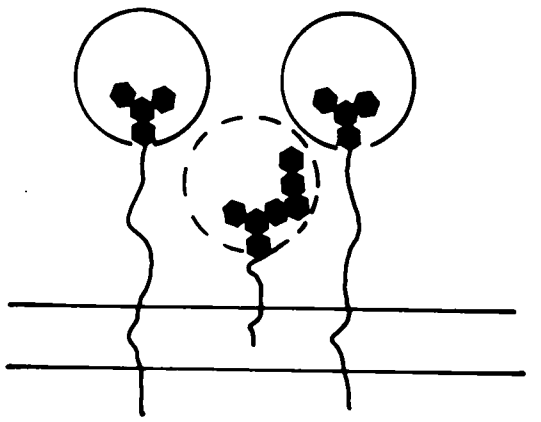
Labelling studies using the microspheres as markers on fixed cells were done in order to obtain information on the relative accessibilities of the different lectin receptors. With all three lectins, labelling of the cells with high concentrations of lectin-microsphere conjugates blocked relabelling with the same microsphere-conjugated but not free lectin. This substantiates the earlier finding that when cells are heavily coated with lectin-microsphere conjugates as seen in the SEM only a small percentage of the lectin receptors are actually labeled. The observation that both ConA and RCA conjugated to Fl-microspheres prevented relabelling of the cells with WGA-Rh-microspheres but not Rh-WGA indicates that both RCA and ConA binding sites are more accessible than those for WGA. This suggests that they may project farther from the cell surface than the binding sites for WGA. For this to be possible, the ConA and RCAI binding sites would have to extend over 80 nm from the cell surface. The cell surface carbohydrate coat of a number of mammalian cells has been determined to be between 50 and 100 nm in thickness (157). A diagram showing a possible arrangement of WGA and ConA receptors is given (Fig. 25).

Figure 25. Hypothetical arrangement of ConA and WGA receptors on the neuroblastoma cell surface. In (1.) is shown the result when fixed cells are first labeled with ConA-microspheres and then treated with WGA-microspheres. The initial labelling of the ConA receptors with the microsphere conjugates makes the WGA receptors inaccessible to the WGA-microspheres. In (2.) is shown the result when fixed cells are first labeled with WGA-microspheres and then treated with ConA-microspheres. The arrangement of the receptors for the two lectins allows the cells to be labeled with both the WGA- and ConA-microspheres under these conditions.

1.


WGA
CONA
MICROSPHERE
MICROSPHERE
CONA
RECEPTOR
WGA
RECEPTOR

2.



Chapter 4

MATERIALS AND METHODS

Reagents

The sources of the reagents were: Uridine diphospho-D-[U-^{14}C] galactose (UDP-galactose), lithium salt (347 mCi/mmol) was purchased from Amersham; Bio-lyte pH 3-10 ampholine and Bio-Rad protein assay reagent from Bio-Rad Laboratories; urea, ultra pure from Schwarz-Mann; adenosine 5'-triphosphate, Tris salt (Tris-ATP), bovine gamma globulin, calf thymus DNA, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), p-nitrophenyl phosphate, and phenylmethylsulfonyl fluoride (PMSF) from Sigma. All other chemicals were of reagent grade.

Buffer A contained 0.58g NaCl, 1.22g Tris, 1.02g $MgCl_2 \cdot 6H_2O$ and 0.11g $CaCl_2$ at pH 8.5 per liter of dH_2O. Buffer B contained one-third of these concentrations. Sucrose solution buffer was composed of 6.06g Tris, 1.02g $MgCl_2 \cdot 6H_2O$ and 0.11g $CaCl_2$ at pH 7.5 per liter of dH_2O.

Plasma Membrane Purification

Neuroblastoma cell plasma membranes were purified following a modification of the method of Garvican and Brown (81). Differentiated monolayer cells were removed from 20 100mm tissue culture dishes by

treatment with 0.02% EDTA in PBS for 30 min at 37°C. Cells were collected at 1000 x g for 4 min and resuspended in PBS.

All subsequent operations were carried out at 0-4°C. The cells were collected as above and then washed once in buffer A and once in buffer B. They were resuspended in buffer B (20 ml per 10^8 cells), allowed to swell for 12-15 min on ice and disrupted by two gentle strokes with the "B" pestle in a glass Dounce homogenizer (clearance = 0.5 mm). This broke up more than 95% of the cells as judged by examination of the homogenate in the light microscope. The nuclei were stabilized by the addition of an equal volume of 30% (w/w) sucrose and collected by centrifugation at 480 x g for 30 sec. The supernatant was gently layered onto discontinuous sucrose gradients (4 per 10^8 cells) consisting of 5.0 ml 42% (w/w) sucrose and 15 ml 30% (w/w) sucrose. The gradients were centrifuged for 30 min at 5800 x g_{max} in a SW 27 Beckman rotor. The material at the 42%/30% interface was harvested with a 16 gauge needle, diluted with buffer A and collected at 2000 x g for 30 min. The pellet was washed once and resuspended in 1 ml buffer A to which was added an equal volume of 30% (w/w) sucrose. The crude membrane was then layered onto a 42%-30% linear sucrose gradient (12 ml total) which had been formed over 1.5 ml 43% (w/w) sucrose and 1 ml 42% (w/w) sucrose in Beckman SW 27.1 rotor tubes. The gradients were centrifuged overnight (12-16 hr) at 130,000 x g_{max}.

The 42%-35% sucrose fraction was carefully removed with a 16 gauge needle, diluted with buffer A and collected at 2000 x g for 30 min. The pellet was resuspended in buffer 1 and 30% sucrose (w/w) mixed in a 1:1 ratio and stored at -20°C until needed.

In some experiments PMSF, a protease inhibitor, was included in all the solutions at a concentration of 4 mM.

Internalization of Con A Receptors

Monolayer cells in 100 mm tissue culture dishes were rinsed 2 times in Tris-Con A buffer (T-CAB) and incubated for 1 hr at 37°C in 2.0 ml T-CAB containing 100-200 µg/ml Con A or ^{125}I-Con A. The Con A solution was then removed, the cells were rinsed 2 times with T-CAB and incubated for 1 hr at 37°C in 5 ml T-CAB. This treatment permitted complete internalization of all visible Con A receptors as discussed in Results. The cells were then scraped off the plates and a homogenate prepared as described above. The homogenate was layered onto continuous 42% (w/w)-30% (w/w) sucrose gradients formed on top of 2.0 ml 43% (w/w) sucrose and 1.0 ml 42% (w/w) sucrose in SW 27 (27.1) rotor tubes and centrifuged at 5800 x g for 30 min. The SW 27.1 gradients were then fractionated into 30 drop fractions. The 42%/30% interface and upper band were removed from the SW 27 gradients with a 16 gauge needle, collected at 2000 x g and 27, 000 x g, respectively for 30 min and resuspended in 1 ml buffer A and 1 ml 30% (w/w) sucrose. The samples were prepared and run on SDS-polyacrylamide gels as described below.

Enzyme Assays

Na^+, K^+ ATPase. Na^+, K^+ ATPase was assayed by a modification of the method of Costantino-Ceccarini *et al.* (119). 200µl samples (50-100µg protein) were incubated for 2 min at 37°C in a total volume of 1.0 ml consisting of 50 µmoles of Tris-HCl, pH 7.4: 1.0 µmole EDTA; 100 µmoles NaCl; 20 µmoles

KCl and 3.0 μmoles $MgCl_2$. The samples were chilled on ice and then 3.0 μmoles of Tris-ATP were added and the samples mixed and incubated for 15 min at 37°C. (The reaction was linear for up to 45 min at 37°C.) The reaction was terminated by the addition of 0.3 ml of 30% (w/w) trichloroacetic acid (TCA). The samples were chilled on ice for 5-10 min and then centrifuged at 3000 x g for 5 min to pellet the precipitate. 0.5 ml of each sample was then assayed for inorganic phosphate as described below. To determine Na^+, K^+ ATPase activity the reaction was carried out in the absence of Na^+ and K^+ (120, 121, 122). The Na^+, K^+ ATPase was calculated as the difference in specific activity in the presence and absence of Na^+ and K^+.

NADPH cytochrome C reductase. NADPH cytochrome C reductase activity was measured by the method of Ragnotti *et al.* (123). To a 50 μl sample (10-50 μg protein) in a 3 ml cuvette was added: 0.05 μmoles KCN; 100 μmoles KCl; 66 μmoles potassium phosphate, pH 7.6; and 0.075 μmoles cytochrome C in a total volume of 1.47 ml. After zeroing the spectrophotometer, 30 μl of 3 mM NADPH was added and the increase in absorbance at 550 nm was recorded for 4 min. The specific activity was calculated using a molar extinction coefficient of 18.7×10^3 M^{-1} cm^{-1} for cytochrome C (reduced - oxidized) at 550 nm.

Succinate cytochrome C reductase. Succinate cytochrome C reductase activity was determined by the method of King (124). To a 20 μl sample (10-50 μg protein) in a spectrophotometer cuvette was added: 2.5 μmoles KCN; 0.3 μmoles EDTA, pH 7.4: 0.1 μmole cytochrome C; and 100 μmoles

potassium phosphate, pH 7.4 in a total volume of 0.97 ml. The spectrophotometer was zeroed, 30 µl of 0.6 M succinate, pH 7.4 were added and the increase in absorbance at 550 nm was recorded for 3 min. The specific activity was calculated using a molar extinction coefficient at 18.7×10^3 M^{-1} cm^{-1} for cytochrome C (reduced-oxidized) at 550 nm.

UDP-galactose: N-acetylglucosamine galactosyl transferase. UDP-galactose: N-acetylgalactosyl transferase activity was measured according to the method of Morré *et al.* (125). The sample (0.15-0.30 mg protein dissolved in 0.5% Triton X-100) was added to the reaction mixture consisting of: 2.0 µmoles Tris-HCl, pH 7.5; 1.0 µmole $MgCl_2$; 0.5 µmoles $MnCl_2$; 5 µmoles β-mercaptoethanol, 0.45 µmoles N-acetyl glucosamine and 32.2 nmoles UDP-D-galactose-^{14}C in a total volume of 0.2 ml. The samples were incubated at 37°C for 10 min and then layered over 1 ml of Bio-Rad AG-1X10 resin (Cl^- form) prepared in dH_2O and packed into a pasteur pipet plugged with glass wool. N-acetyl lactosamine and galactose were eluted with 3 x 0.4 ml washes with dH_2O, collected in scintillation vials and counted in 10 ml ACS scintillation fluid. Controls were run in the absence of N-acetylglucosamine. The specific activity was calculated as the difference in specific activity in the presence and absence of N-acetylglucosamine.

Acid phosphatase. Acid phosphatase activity was measured as described by Barrett (133). 0.15 ml of sample (25-100 µg protein) were mixed with 0.6 ml 0.2 M acetate buffer, pH 5.0 and incubated at 37°C for 10 min. 0.5 ml of 32 mM p-nitrophenyl phosphate were added and the assay

was carried out for 30 min at 37°C. The reaction was terminated by the addition of 1.0 ml of ice-cold 1 M Tris-HCl, 0.4 M K_2HPO_4, pH 8.5. The absorbance at 420 nm was taken and the moles of p-nitrophenyl phosphate hydrolyzed calculated using a molar extinction coefficient of 17.6×10^3 M^{-1} cm^{-1} for p-nitrophenol.

Other Assays

Protein. Protein concentrations were determined by the method of Lowry *et al.* (88) described in Chapter 2 or by the Bio-Rad protein assay (128). For this procedure, 50 µl of sample were mixed with 2.5 ml of Bio-Rad dye reagent (diluted 1:4 with dH_2O and filtered) and incubated for 5-30 min at 23°C. The absorbance at 595 nm was taken and the protein concentration determined from a standard curve prepared simultaneously using bovine gamma globulin (1.4 mg/ml in PBS).

DNA. DNA was measured by the method of Burton (126). Samples (0.25-0.5 mg protein/ml) were suspended in 0.5 N perchloric acid and heated for 20 min at 70°C to remove protein. 1.0 ml aliquots were then mixed with 2.0 ml diphenylamine reagent and incubated for 18-20 hr at 30°C. The absorbance of the samples at 550 nm was taken and the DNA concentration determined from a standard curve prepared simultaneously using perchloric acid-treated calf thymus DNA (0.5 mg/ml). The diphenylamine reagent contained 1.5 g diphenylamine dissolved in 100 ml acetic acid to which was added 1.5 ml H_2SO_4 and, just before use, 0.1 ml of 1.6% aqueous acetaldehyde per 20 ml of reagent.

Phosphate. Inorganic phosphate was determined by the method of Banting *et al.* (120). To each 0.5 ml sample in a new 10 x 75 mm test tube was added 0.5 ml of phosphate reagent. The tubes were mixed and the absorbance at 700 nm determined after 15 min at 23°C. Phosphate concentrations were calculated from a standard curve run simultaneously using inorganic phosphate. The phosphate reagent contained 1 g $(NH_4)_6\ Mo_7O_{24} \cdot 4\ H_2O$ dissolved in 100 ml 0.5 M H_2SO_4 to which was added $FeSO_4 \cdot 7H_2O$ (0.5 g/ 10 ml) shortly before use.

^{125}I. 100 µl aliquots of samples were precipitated with 10% TCA and kept on ice for 1 hr. The samples were centrifuged for 10 min at 4000 x g and the pellets counted in a gamma counter.

SDS polyacrylamide gel electrophoresis of plasma membranes. Plasma membrane samples were collected at 2000 x g for 30 min and then dissolved in denaturing buffer (0.015 M Tris-HCl, pH 8.0, 20% sucrose, 5% SDS, 10% β-mercaptoethanol and 0.0025% bromomphenol blue) to give a concentration of 5.6 µg protein per µl of solution. The samples were then applied to Laemmli slab gels and run as described in Chapter 2. The gels were stained with Coomassie blue for protein, with dansyl hydrazine for carbohydrate or with fluorescent lectins.

Isoelectric focussing of plasma membranes. (A modification of the method of O'Farrell (129) and Ames and Nikaido (130).

Reagents: Acrylamide solution (28.4% (w/w) acrylamide and 1.6% (w/w) Bis in dH_2O), lysis buffer (9.5 M urea, 5% β-mercaptoethanol, 8% (w/v) Nonidet P-40 (NP-40)), 10% (w/v) NP-40, ampholine pH 3-10, 10% (w/v) ammonium persulfate and TEMED.

Procedure: 3.0 ml acrylamide solution, 4.5 ml NP-40, 1.13 ml ampholine pH 3-10, 12.38 g urea and 4.4 ml H_2O were mixed, warmed briefly at 37°C to dissolve the urea and deaerated. 15 µl TEMED and 30 µl ammonium persulfate were added, the solution was pipeted into 10 (11 x 5 mm) glass tubes sealed at one end with Parafilm, overlaid with d H_2O and allowed to polymerize for 1 hr at 23°C. After the gels had polymerized they were overlaid with lysis buffer and allowed to sit for another hour. The Parafilm was removed and the tubes were placed in a Savant tube gel apparatus. The upper reservoir was filled with fresh, deaerated 0.2 M NaOH, and the bottom reservoir with 0.01 M H_3PO_4 and the gels were pre-run 15 min at 200 volts, 30 min at 300 volts and 30 min at 400 volts at23°C. The upper reservoir was replaced with fresh 0.02 M NaOH and samples (10-30 µl) were applied with a microsyringe. Electrophoresis was carried out at 23°C for 16 hr at 450 volts and 1 hr at 800 volts. The gels were then removed from the glass tubes, fixed for 24-36 hr in 25% isopropanol/10% acetic acid to remove ampholine and stained with Coomassie blue for protein or with lectins as described in Chapter 2.

One gel was run without a sample and was used to determine the pH gradient. The gel was frozen, cut into 0.5 mm sections, each section was placed in a vial with 1.0 ml deaerated d H_2O and incubated several hours at 23°C with mixing. The pH of each sample was then determined.

Sample preparation:(130): 1.0 mg of membrane protein was suspended in 100 µl of 0.7% SDS, 0.5 mM $MgCl_2$, 0.05 M Tris-HCl, pH 6.8 and heated for 30 min at 70°C. The sample was centrifuged for 45 min at 27,000 x g and the supernatant removed and stored at 5°C until just before use.

The supernatant was then mixed with an equal volume of lysis buffer and applied to the gels as described above.

Iodination of Cells

Monolayer cells were iodinated by a modification of the method of Truding *et al.* (80). Cells on 100 mm tissue culture dishes were rinsed 3 times with DPBS and overlaid with 2 ml DPBS. Lactoperoxidase (40 µg) and ^{125}I (250 µCi) were added and the reaction was initiated by the addition of 20 µl of 88 µM H_2O_2. 20 µl aliquots of H_2O_2 were then added every 15 sec for the next 10 min (41 times). The cells were rinsed twice with DPBS, scraped off into test tubes and washed by centrifugation 3 times with DPBS. Controls were run in the absence of lactoperoxidase. This procedure gave 1.5-2 x 10^6 cpm per 2.5 x 10^6 cells in the presence of lactoperoxidase and 0.1%-0.5% of this value in the absence of lactoperoxidase.

Samples were dissolved and run on SDS-polyacrylamide gels as described for plasma membranes. After electrophoresis the gels were either stained for protein with Coomassie blue or fixed overnight in 25% isopropanol/10% acetic acid and then dried under vacuum in preparation for autoradiography.

Autoradiography

Dried gels having 10^5-4 x 10^4 cpm per well were placed in contact with pre-flashed Kodak X-Omat R x-ray film for 24-36 hr at -70°C in a cassette containing two DuPont Lightning-Plus intensifying screens.

RESULTS

Plasma Membrane Preparation

The fractionation of the neuroblastoma cell membranes was monitored by following the distribution of markers for the different subcellular components: Na^+, K^+ ATPase (122, 131, 132) and acid-precipitable ^{125}I (131) for the plasma membrane; NADPH cytochrome C reductase (123, 132) for endoplasmic reticulum; succinate cytochrome C reductase (126, 132) for mitochodria; acid phosphatase (135) for lysosomes; UDP-galactose: N-acetylglucosamine galactosyl transferase (123, 125) for golgi apparatus and DNA (127) for nuclei. Tables V and VI show the total and specific activities of each of these markers for each step of the membrane isolation as well as their enrichment and recovery.

Both plasma membrane markers showed considerable increases in specific activity in the membrane fraction. The Na^+, K^+ ATPase was enriched by a factor of 10 relative to the homogenate, a value which agrees with that reported by Charalampous (132) for his membrane preparation from the same neuroblastoma cell line. The increase in acid-precipitable ^{125}I specific radioactivity in the membrane fraction and the recovery of radioactivity in each fraction are very similar to the values for the Na^+, K^+ ATPase. Indeed, the peaks of both Na^+, K^+ ATPase activity and radioactivity were found in the same fractions when either the discontinuous or continuous sucrose gradients were fractionated (Table VII).

The small fractional enrichment factor for NADPH cytochrome C reductase, the endoplasmic reticulum marker, suggests there is only slight contamination of the plasma membrane preparation by this organelle. The

Table V

Distribution of Markers in Various Fractions Recovered During Membrane Isolation

Fraction	Protein		Na^+,K^+ ATPase				Acid-Precipitable ^{125}I				NADPH Cytochrome C Reductase				Succinate Cytochrome C Reductase			
	Total (mg)	% R	TA	% R	SA	PF	TA	% R	SA	PF	TA	% R	SA	PF	TA	% R	SA	PF
homogenate	55.2	100	258.4	100	4.6	1.0	6.2	100	113.1	1.0	520.0	100	9.4	1.0	1115.0	100	20.2	1.0
480 x g pellet	16.4	29.7	78.8	30.5	4.8	1.0	1.7	26.6	101.2	0.9	67.2	12.9	4.1	0.4	64.0	5.7	3.9	0.2
top	25.1	45.5	46.2	17.9	1.8	0.4	8.4	13.5	33.5	0.3	374.0	71.9	14.9	1.6	401.6	36.0	16.0	0.8
interface	1.88	3.4	82.0	31.7	43.6	9.5	1.7	26.4	877.7	7.8	34.6	6.6	18.4	2.0	57.3	5.1	30.5	1.5
membrane	0.84	1.5	39.4	15.3	47.0	10.2	7.9	14.2	939.3	8.3	3.2	0.6	3.8	0.4	0.0	0.0	0.0	0.0

The isolation of the membranes and various assays were performed as described in Material and Methods. The top fraction is the top of the discontinuous sucrose gradient and the interface fraction is the 30%/42% interface of the same gradient. For the enzymes: TA = total activity (nmoles per min); % R = % recovery with respect to the homogenate; SA = specific activity (nmoles per min per mg protein); PF = purification factor with respect to the homogenate. For the acid-precipitable ^{125}I: TA = total radioactivity (cpm x 10^{-6}); SA = Specific radioactivity (cpm per µg protein); % R and PF are the same as for the enzyme activities.

Table VI

Distribution of Markers in Various Fractions Recovered During Membrane Isolation

Fraction	Acid Phosphatase				Galactosyl Tranferase				DNA			
	TA	% R	SA	PF	TA	% R	SA	PF	TA	% R	SA	PF
homogenate	767.3	100	13.9	1.0	5.52	100	0.10	1.0	4.3	100	0.06	1.0
480 x g pellet	55.8	7.3	3.4	0.2					3.1	72	0.19	3.2
top	115.5	15.1	4.6	0.3								
interface	15.4	2.0	8.2	0.6								
membrane	7.6	1.0	9.0	0.7	0.06	1.1	0.07	0.7	0.0	0.0	0.0	0.0

The fractions are the same as in Table I. For the enzymes: TA = total activity (nmoles per min); % R = % recovery; SA = specific activity (nmoles per min per mg protein); PF = purification factor. For DNA: TA = total amount (mg DNA); SA = specific amount (mg DNA per mg protein); % R and PF are the same as for the enzyme activities.

Table VII

Fractionation of a Continuous Sucrose Gradient

Fraction Number	% Sucrose	Protein (μg/fraction)	^{125}I (cpm/fraction)	Na^+,K^+ ATPase	NADPH cytochrome C reductase	succinate cytochrome C reductase
1	43.0	24.2	1364	1.76	0.00	0.00
2	42.0	24.2	4136	4.84	0.00	0.00
3	41.5	48.4	5577	6.38	1.76	0.00
4	40.0	72.6	3619	3.74	0.00	0.00
5	39.0	24.2	2596	1.76	0.00	0.00
6	38.0	48.4	3762	2.42	0.00	0.00
7	37.0	24.2	4829	3.08	0.00	0.00
8	35.5	72.6	4917	5.50	1.76	0.00
9	34.0	48.4	3454	2.64	0.00	0.00
10	32.5	48.4	1727	1.10	0.00	0.00
11	31.0	24.2	1254	0.00	0.00	0.00
12	29.7	48.4	385	0.00	0.00	0.00
13	27.5	48.4	352	0.00	0.00	0.00
14	24.0	48.4	627	1.32	0.00	0.00
15	19.3	48.4	528	0.00	0.00	0.00
16	19.3	72.6	253	0.00	3.52	0.00
17	13.8	48.4	286	0.00	0.00	0.00

A continuous sucrose gradient was separated into 17 1.1 ml fractions and each fraction was assayed for protein, acid-precipitable ^{125}I, Na^+, K^+ ATPase, NADPH cytochrome C reductase and succinate cytochrome C reductase as described in Materials and Methods. % sucrose was determined using a refractometer. All enzyme activities are in nmoles per minute per fraction.

endoplasmic reticulum appeared to collect predominantly in the upper part of the discontinuous sucrose gradient. The lack of detectable succinate cytochrome C reductase activity in the plasma membrane fraction indicates that mitochondrial contamination is very low. The highest recovery of this organelle was also in the upper part of the discontinuous sucrose gradient.

The DNA assay indicates little or no contamination of the purified plasma membrane by nuclei. The nuclei are heavily concentrated in the 480 x g pellet. The lack of nuclear contamination of the plasma membrane is substantiated by the absence of nuclear proteins on SDS-polyacrylamide gels of the isolated membrane (nuclear proteins = several dark bands of 10,000-20,000 molecular weight) and the absence of nuclei when isolated membranes were viewed in the light microscope.

There appears to be some contamination of the plasma membrane preparation by both lysosomes and golgi apparatus based on assays for the marker enzymes for these organelles.

The average yield of membrane protein reported here (0.84 mg from 10^8 cells) is similar to that previously reported (81) for a similar membrane preparation from the same cell line. This is 1.5% of the protein present in the homogenate.

Staining of Gels for Protein

When isolated differentiated neuroblastoma cell plasma membranes were dissociated in SDS and run on 10% Laemmli slab gels from 40-50 bands could be seen when the gels were stained for protein with Coomassie blue

(see Fig. 29). Over 80% of these polypeptides had molecular weights between 30,000 and 120,000 daltons. When the same preparations were run on 6% Laemmli slab gels only 6-8 polypetides with molecular weights greater than 120,000 daltons were observed (Fig. 26). Most of these only stained faintly with Coomassie blue. The addition of PMSF to all the solutions used in the membrane preparation had no effect on the Coomassie blue staining pattern of the membrane polypeptides indicating that no proteolysis was occurring during the preparation of the membranes.

Staining of Gels for Carbohydrate

If the dissociated plasma membranes were run on 10% Laemmli gels and then stained for carbohydrate using the periodic acid-Schiff base reaction (Method I) the only staining observed was of a broad band located below the dye marker. However, when the same membrane preparation was run on gels and stained for carbohydrate using the periodic acid-dansyl hydrazine-sodium borohydride reaction (Method II) 8-9 fluorescent bands, including a broad one below the dye marker, were seen (Fig. 27b). All the fluorescent bands located above the dye marker appeared to stain with Coomassie blue as well. Control gels, which were treated with dansyl hydrazine but not with periodic acid, had no fluorescent bands (Fig. 27c).

^{125}I Labelling of Plasma Membrane Polypeptides

Intact monolayer cells labeled with ^{125}I by the lactoperoxidase method showed 7-8 bands on autoradiograms made from 10% Laemmli slab gels run with the cells dissociated in SDS (Fig. 28b). An identical banding pattern was seen when plasma membranes were isolated from iodinated cells,

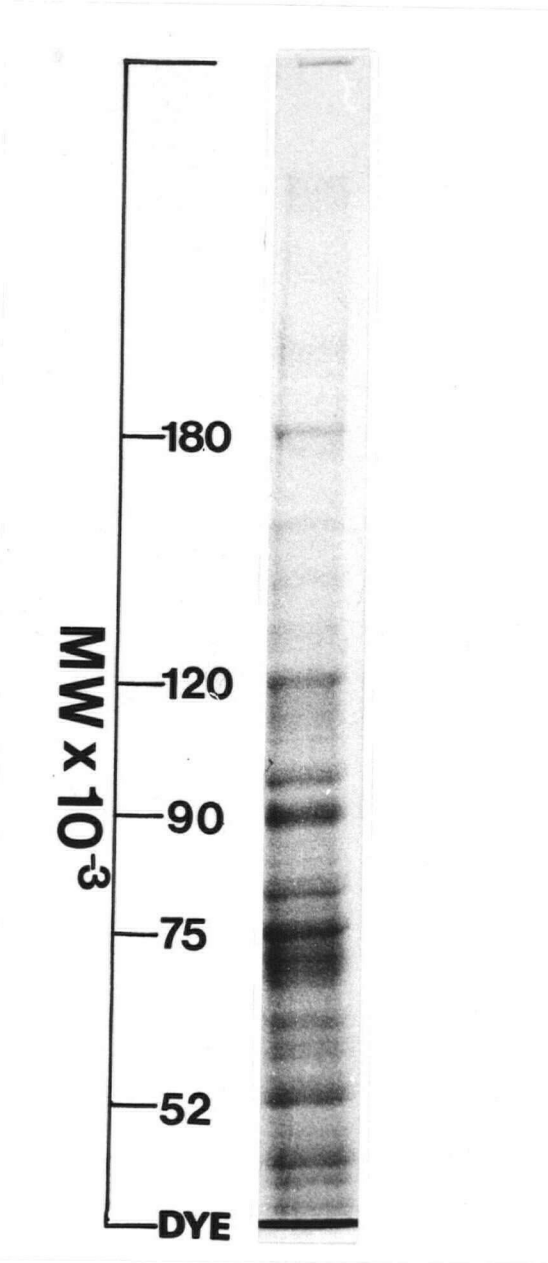
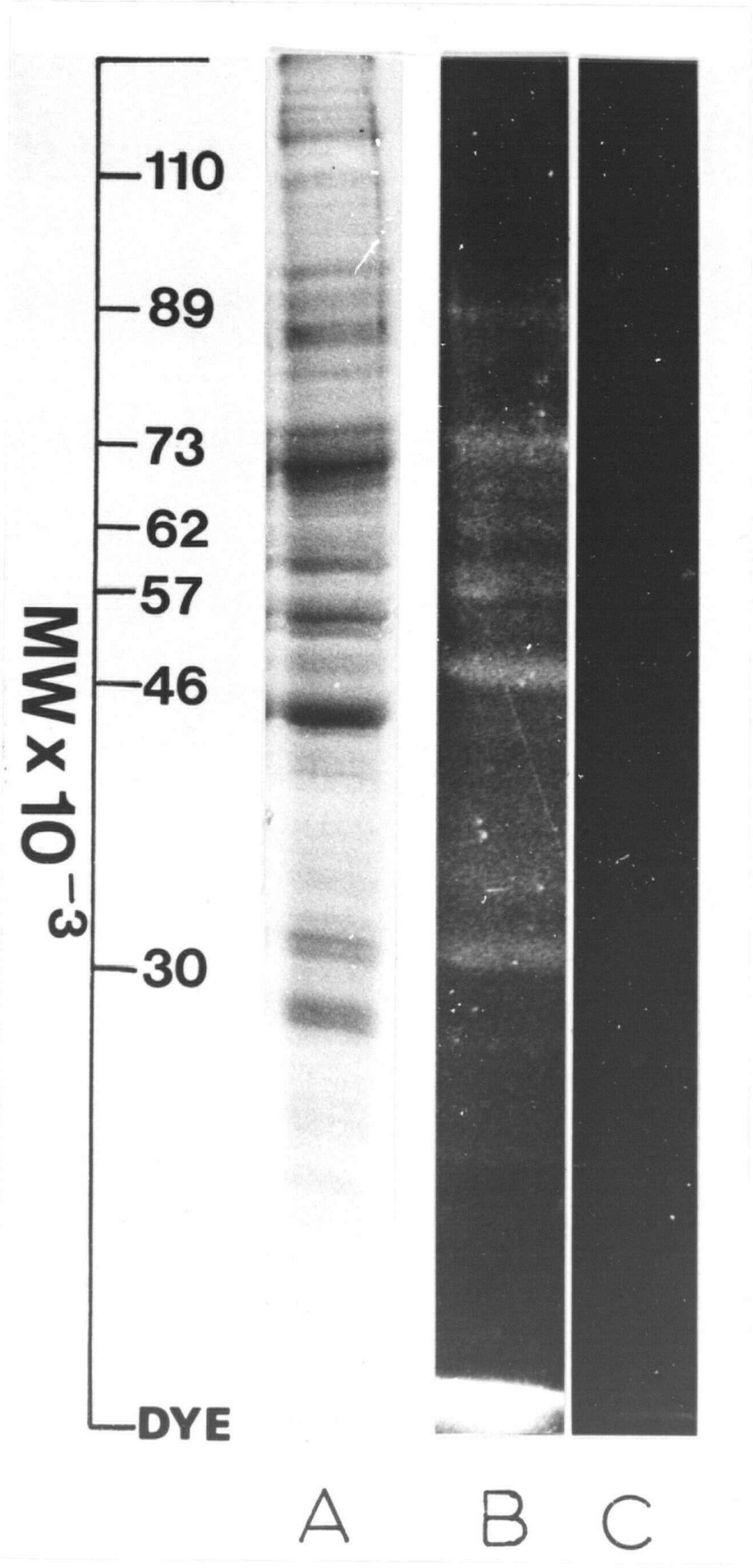


Figure 26. SDS-polyacrylamide gel (6%) of 135 μg isolated neuroblastoma cell plasma membrane protein stained for protein with Coomassie blue.

Figure 27. Identification of periodate-reactive membrane polypeptides. SDS-polyacrylamide gels (10%) of 135 μg isolated plasma membrane protein treated with: A, Coomassie blue; B, periodic acid followed by dansyl hydrazine; C, dansyl hydrazine.


110
89
73
62
57
46
30
DYE
MW x 10⁻³
A
B
C

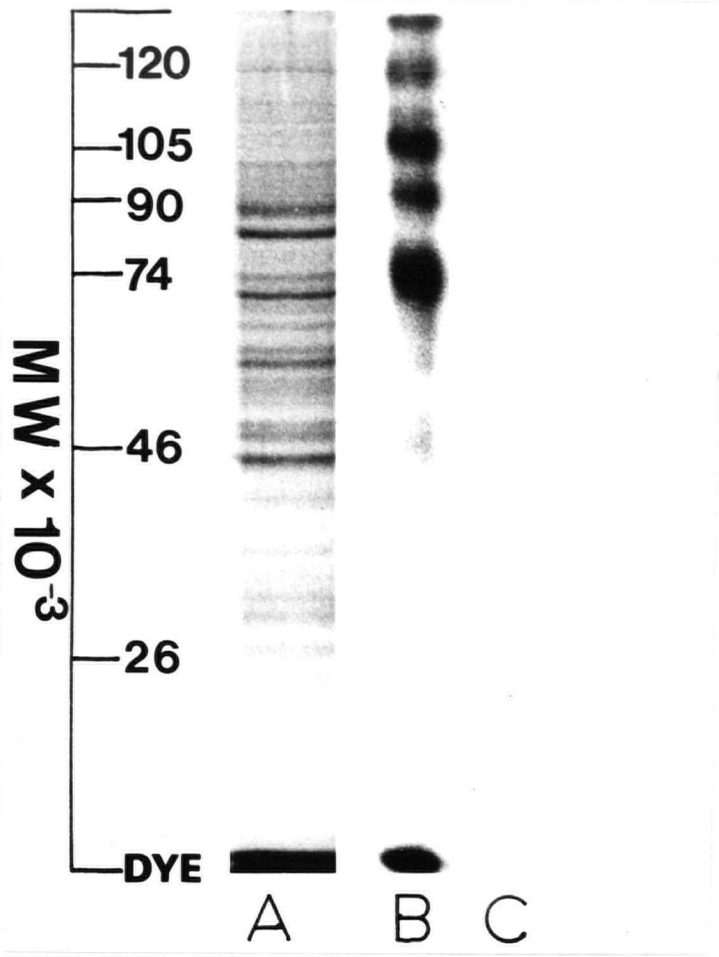


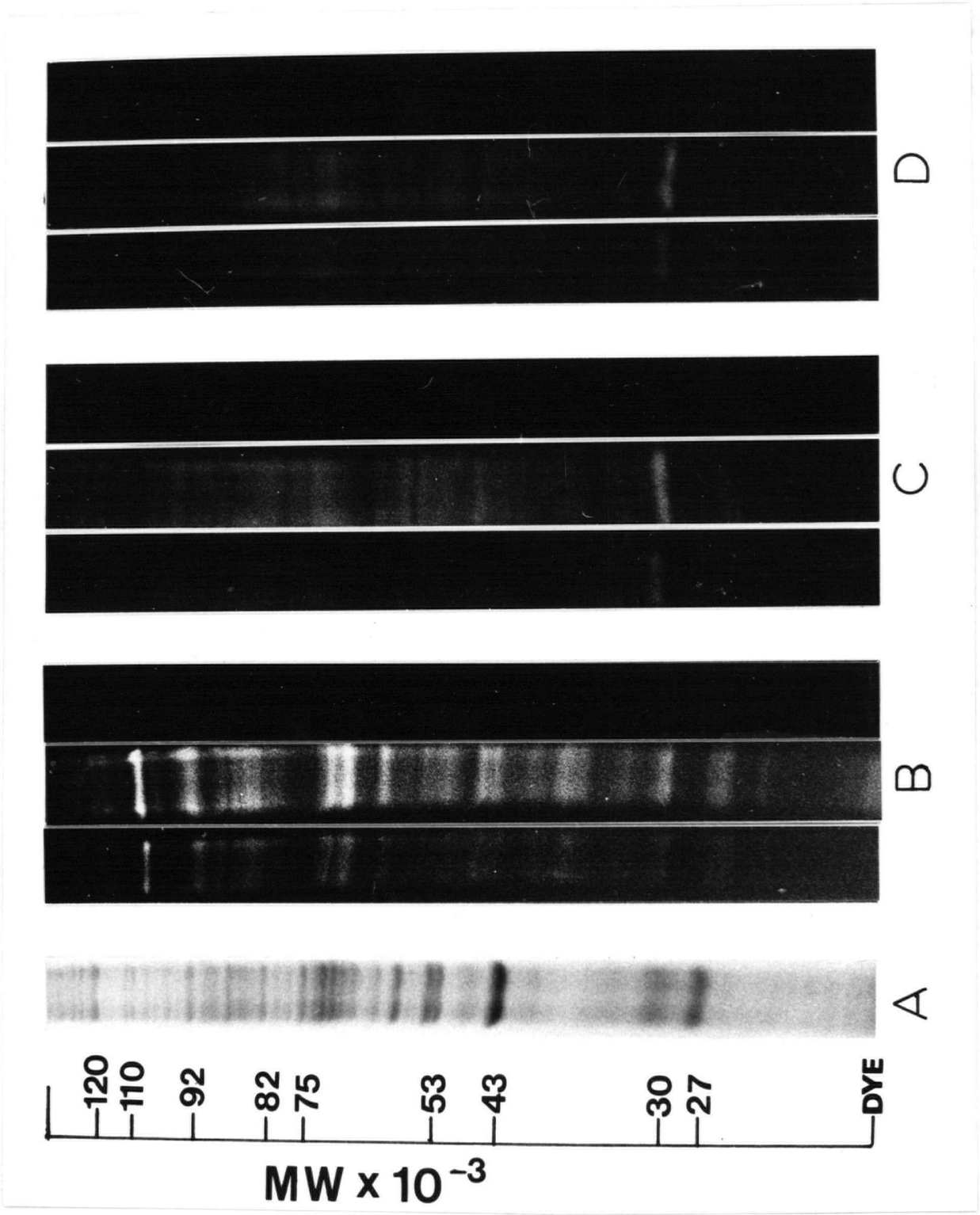
Figure 28. Identification of membrane polypeptides labeled with ^{125}I in the presence of lactoperoxidase. SDS-polyacrylamide gels (10%) of labeled cells (~120 μg protein) are shown. A, cells stained with Coomassie blue; B, autoradiogram of ^{125}I-labeled cells; C, autoradiogram of cells treated with ^{125}I in the absence of lactoperoxidase.

dissociated and run on gels. All the polypeptides revealed using ^{125}I also appeared to stain for protein with Coomassie blue. However, there was no correlation between the amounts of Coomassie blue and ^{125}I bound to each polypeptide. Cells which were treated with ^{125}I in the absence of lactoperoxidase gave no bands when gels of these samples were autoradiographed (Fig. 28c).

Lectin Labelling of Plasma Membrane Polypeptides

Laemmli gels. Plasma membranes dissociated in SDS, run on 10% Laemmli slab gels and stained with Fl-lectins as described in Chapter 2 showed a variety of labelling patterns (Fig. 29). When the gels were treated with Fl-ConA over 20 polypeptide bands were found to specifically bind the lectin (Fig. 29b). The majority of these polypeptides had molecular weights greater than 50,000 daltons. All the ConA staining-bands also appeared to stain with Coomassie blue to some extent. Lentil lectin, which has a saccharide specificity very similar to that of ConA, gave an identical pattern when gels were treated with Fl-lentil lectin. Both Fl-WGA and Fl-RCAI bound almost exclusively to a single polypeptide band of 30,000 molecular weight (Fig. 29c,d). This band also stained quite heavily with Coomassie blue. At the higher plasma membrane protein concentration several other polypeptides appeared to label with Fl-WGA and/or Fl-RCAI. RCAII gave an identical staining pattern to RCAI at both membrane protein concentrations. Fl-*Ulex europeus* lectin did not stain gels of plasma membranes, in agreement with the finding that neuroblastoma cells could not be labeled with this lectin. Control gels, run in the presence of a 0.2 M concentration

Figure 29. Identification of lectin-binding membrane polypeptides. SDS-polyacrylamide gels (10%) of purified plasma membranes infiltrated with fluorescent lectins in the absence and presence of the appropriate inhibitor. A, 45 µg membrane protein stained with Coomassie blue. In B to D staining of 45 µg of membrane protein with Fl-lectin is on the left, staining of 135 µg of membrane protein with Fl-lectin is in the centre and staining of 135 µg of membrane protein with Fl-lectin in the presence of inhibitor is on the right. B, Fl-ConA ± 0.2 M α-methyl mannoside; C, Fl-RCAI ± 0.2 M D-galactose and D, Fl-WGA ± 2 x WGA inhibitor.


D
C
B
A
120
110
92
82
75
53
43
30
27
DYE
MW x 10 -3

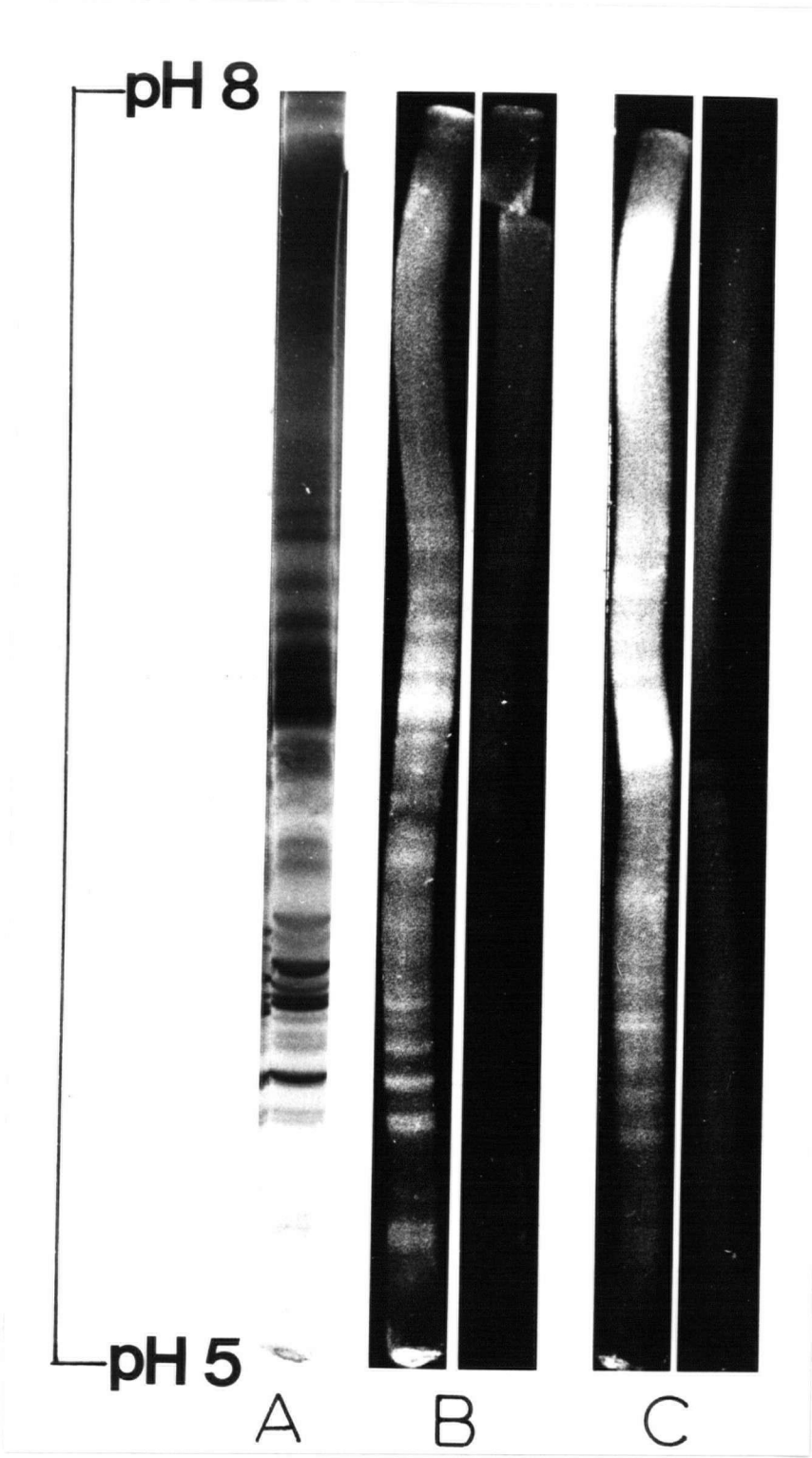
of the appropriate inhibitor, showed no fluorescence in any experiment. In addition, there was never any staining below the dye marker.

Isoelectric focussing gels. Plasma membranes prepared as described and run on isoelectric focussing gels gave over 20 sharp bands and a braod smear when stained for protein with Coomassie blue (Fig. 30a). The majority of these bands were in the pH range of 6.0-7.0. When similar gels were stained with Fl-WGA a number of fluorescent bands were revealed (Fig. 30b). The same bands were seen if gels were stained with Fl-RCAI. Control gels showed some diffuse fluorescence.

Internalization of ConA Receptors

When monolayer cells were treated with ConA in order to internalize all their ConA receptors and subsequently labeled with Fl-ConA, no labelling of the cells was seen. When the cells were treated with ^{125}I-ConA, allowed to internalize all their ConA receptors and a cell homogenate prepared and run on a sucrose gradient, a single peak of radioactivity and several peaks of Na^+, K^+ ATPase activity were seen (Fig. 31). The pattern of Na^+, K^+ ATPase activity was very similar to that seen when untreated cells were homogenized and run on a similar gradient except near the top of the gradient. The major peak of Na^+, K^+ ATPase activity near the bottom of the gradient, which corresponded to the plasma membrane fraction, contained little or no radioactivity whereas the peak of Na^+, K^+ ATPase activity not seen with untreated cells corresponded to the major peak of radioactivity.

Figure 30. Analysis of membrane polypeptides on isoelectric focussing gels. Isolated membranes were prepared for isoelectric focussing as described in Materials and Methods and 150 µg of protein were applied to each tube gel. The gels were run and stained for protein or with Fl-lectins as described in Materials and Methods. The pH gradient was determined using a blank gel. A, isoelectric focussing gel stained with Coomassie blue. In B and C staining of membrane protein with Fl-lectin in the absence (on left) and presence (on right) of the appropriate inhibitor. B, Fl-RCAI ± 0.2 M D-galactose and C, Fl-WGA ± 2 x WGA inhibitor.


pH 8
pH 5
A
B
C

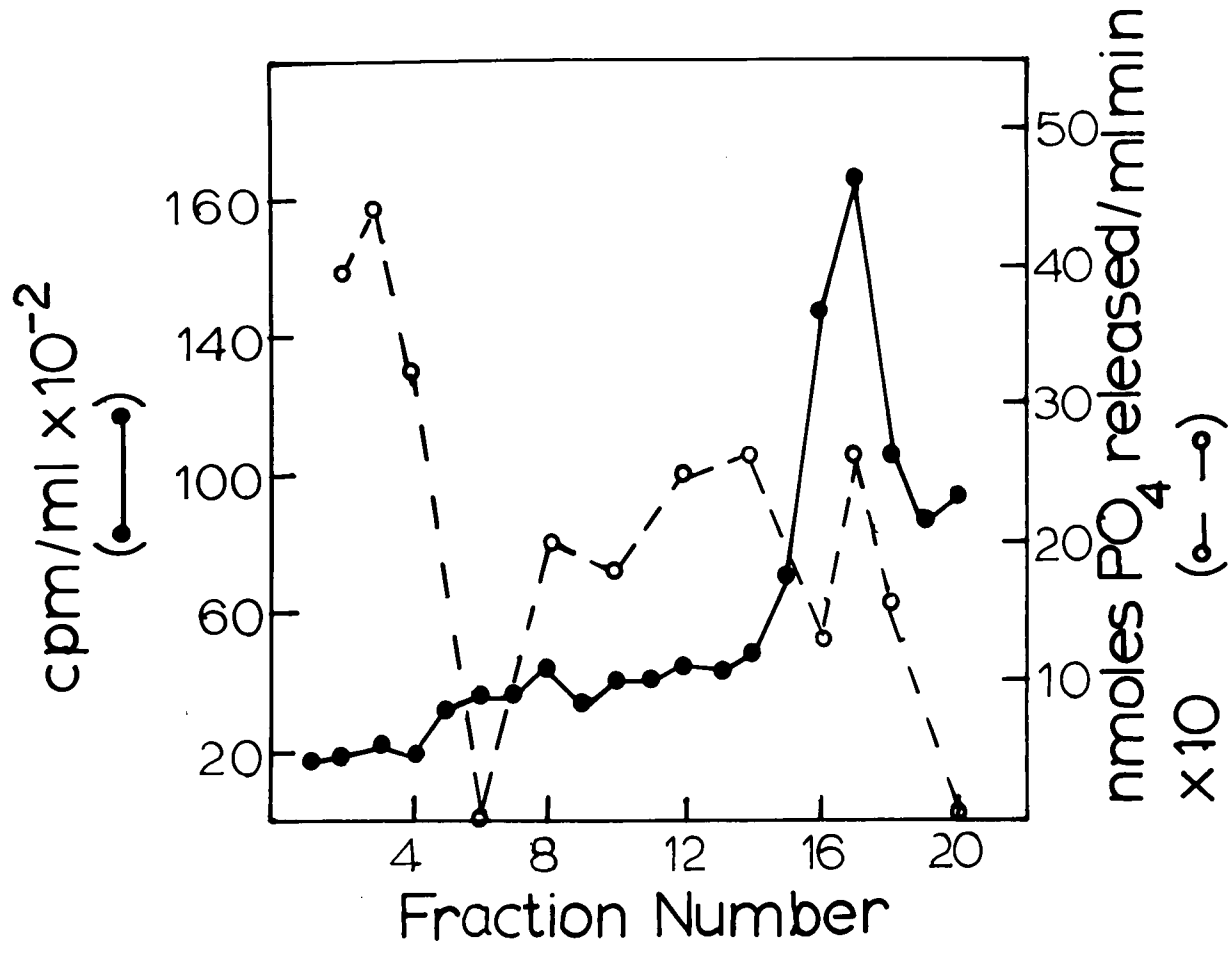


Figure 31. Analysis of a continuous sucrose gradient containing material from cells made to internalize their ConA receptors. Cells were labeled for 1 hr at 37°C with ^{125}I-ConA and treated for a second hour at 37°C with buffer. A cell homogenate was prepared as described in Materials and Methods and run on the continuous sucrose gradient. The gradient was separated into 21 fractions (0.9 ml each) and aliquots of these were assayed for Na^+, K^+ ATPase (o--o) and counted for ^{125}I (●—●).

Analysis of ConA Treated Plasma Membranes on Gels

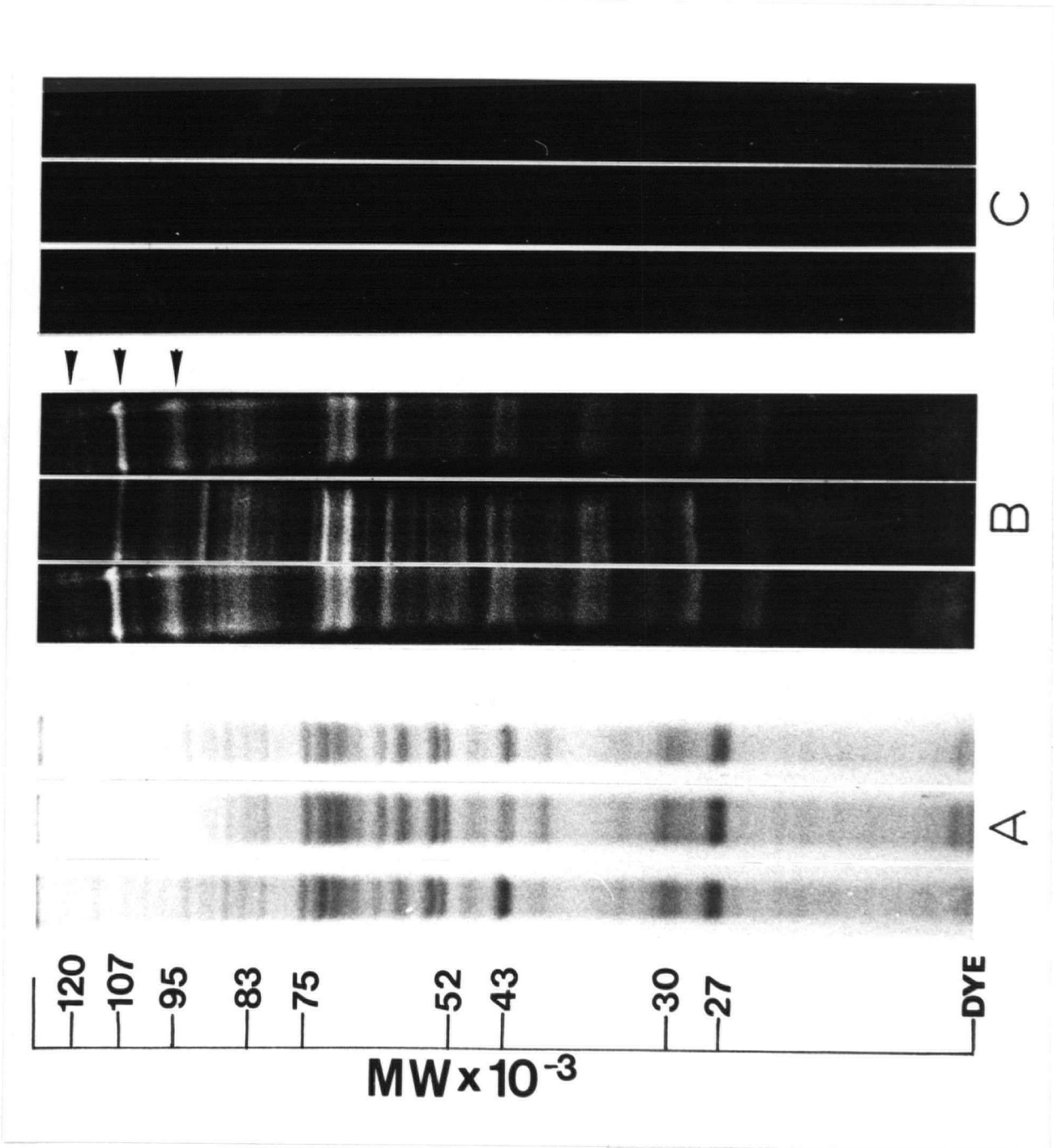
When gels were run of the upper and plasma membrane bands from the continuous sucrose gradient discussed above as well as of untreated plasma membranes and subsequently stained with Coomassie blue or ConA a number of differences were revealed. Several high molecular weight Coomassie blue-staining bands were absent in the gels of the plasma membrane fraction from ConA-treated cells (Fig. 32a). Some of these bands were present in gels of the upper band.

When the gels of ConA-treated membranes were stained with Fl-ConA four high molecular weight ConA binding polypeptides showed little or no fluorescence as compared to the untreated membranes. These ConA-binding polypeptides were present in gels of the upper band. Gels of both the ConA-treated plasma membranes and the upper band had more ConA-staining material which did not move into the gel than gels of untreated plasma membranes. The remainder of the Fl-ConA staining patterns were very similar for all three samples. Controls showed no fluorescence.

DISCUSSION

The preparation of plasma membranes can be estimated to be 80-90% pure based on the data obtained from the assays for the different subcellular component markers. This value is similar to that reported for the same membrane isolation procedure and a similar neuroblastoma cell line (81) as well as for membrane preparations from a variety of different types of cells (119,134,135,136,137). The major sources of impurities in this membrane preparation appear to be the golgi apparatus and the lysosomes.

Figure 32. Identification of cell surface ConA binding sites. SDS-polyacrylamide gels of isolated plasma membranes infiltrated with Fl-ConA. In A to C, regular membranes are on the left, membranes from cells which had internalized all their visible ConA binding sites are in the middle and the upper band material from the continuous sucrose gradient (i.e. fractions 15-19) is on the right. A, 45 μg membrane protein stained with Coomassie blue; B, 135 μg membrane protein stained with Fl-ConA; C, 135 μg membrane protein treated with Fl-ConA + 0.2 M α-methyl mannoside. The arrowheads in B indicate the bands absent from the gels of plasma membranes of cells which had internalized all their visible ConA binding sites.


C
B
A
120
107
95
83
75
52
43
30
27
DYE
MW x 10⁻³

However, both acid phosphatase, the lysosomal marker (81,122,138) and galactosyl transferase (139), the marker for the golgi apparatus, have been reported to be present in the plasma membranes of several different cell types as well as in their respective organelles. Thus, the recovery of these enzyme activities in the isolated plasma membrane may be a reflection of their presence in the plasma membrane as well as indication of a certain amount of contamination of the plasma membrane preparation by these two organelles.

The recoveries of both plasma membrane markers (acid-precipitable ^{125}I and Na^+, K^+ ATPase) are similar and in the same range as that reported for two other plasma membrane markers (5'-nucleotidase and ^{3}H-fucose) used with the same membrane isolation procedure and a similar neuroblastoma cell line (81). Similar recoveries of plasma membrane marker activities have been found with several other cell lines and the same type of membrane purification (119,131). The increases in specific activities for both the plasma membrane markers used in this study are in close agreement to those seen for purified plasma membranes from a variety of different cell lines using several different membrane isolation techniques (119,131). The major losses of plasma membrane appear to be due to the trapping of some plasma membrane material with the nuclei and to formation of small membrane vesicles during homogenization. The latter leads to the presence of plasma membrane in the upper portions of both the continuous and discontinuous sucrose gradients. These upper fractions probably also contain pinocytotic vesicles. Analysis of samples from all the fractionation steps by autoradiography revealed the same banding patterns, indicating that specialized portions of the plasma membrane were not being lost during its purification.

The recovery of protein in the plasma membrane fraction is also in agreement with that reported for other cell lines when a similar type of membrane preparation technique was used (119,134,141). It is also consistent with the recovery of Na^+, K^+ ATPase activity and acid-precipitable ^{125}I radioactivity if the plasma membrane makes up 8-11% (119) of the total cell protein.

The general molecular weight distribution of the Coomassie blue-staining membrane polypeptides is similar to that seen by the three other groups who have studied neuroblastoma cell membranes (80, 81, 132). In all cases, the majority of proteins have apparent molecular weights greater than 50,000, and less than 150,000, daltons. Since different SDS-polyacrylamide gel systems were used in each of these studies it is difficult to make an accurate comparison of the different gel protein patterns. However, several major Coomassie blue-staining bands have been identified previously and can be correlated with bands seen in this study. The major 49,000 molecular weight protein seen by Charalampous (132) probably corresponds to the 45,000 molecular weight band observed in the present study. The band comigrates with chicken gizzard actin. Hela cell plasma membranes have also been found to contain fairly large quantities of actin (119). The double bands at ~54,000 daltons have also been observed by Zisapel and Littauer (142) in their membrane preparation from neuroblastoma cells. They identified them as tubulin dimer.

The PAS reaction did not stain any of the proteins on the slab gels but it did stain a large amount of material of high mobility which is probably glycolipid. Poor labeling of gels using the PAS reaction has also been noted by other groups (43,97) and is probably due to the fairly

low sensitivity of the technique. This conclusion is reinforced by the fair staining seen here when gels treated with periodic acid are stained with dansyl hydrazine rather than the Schiff reagent. Garvican and Brown (81) noted an increase in the glycosylation of two membrane components of 60,000 and 70,000 daltons following the differentiation of neuroblastoma cells. These may correspond to the 60,000 and 70,000 dalton bands seen here.

The labelling of whole cells with ^{125}I revealed a number of polypeptides. All the same bands were present in isolated plasma membranes indicating that only cell surface polypeptides whose tyrosine residues were accessible to ^{125}I and lactoperoxidase were labeled by this procedure. A number of the polypeptides labeled with ^{125}I seem to correspond to bands which stained strongly with dansyl hydrazine, particularly those at 48,000; 74,000; and 90,000 daltons. This may indicate that large portions of these proteins protrude from the cell surface or that they have a lot of tyrosine residues. The band at 74,000 daltons may correspond to the 78,000 dalton polypeptide found in another study (80) to be preferentially labeled following neuroblastoma cell differention. The relative simplicity of the ^{125}I polypeptide banding pattern agrees with that of the earlier study (80) as well.

The labelling of the gels with the fluorescent lectins indicated that RCAI and WGA bound predominantly to the same polypeptide. This is in agreement with the results of the double labelling studies discussed in Chapter 3 where it was suggested that the major WGA and RCAI binding sites are located on the same or directly-associated cell surface proteins. The isoelectric focussing gel results further substantiate these findings. This 30,000 molecular weight polypeptide also stains heavily for carbohydrate using dansyl hydrazine. It does not label with ^{125}I, however, suggesting

that either heavy glycosylation makes its tyrosine residues inaccessible to lactoperoxidase or that it has no tyrosine residues in the portion that extends from the cell surface. A number of other bands which also stain with both WGA and RCAI are revealed at the higher membrane protein concentration. There are, however, a few additional bands for RCAI which may correlate with the low affinity RCAI binding sites discussed in Chapter 3.

When gels were pretreated with RCAI before labelling with Fl-WGA no change in the staining pattern was observed. Identical results were found in the reverse experiment. Thus, the RCAI-binding groups and the WGA-binding groups must be well separated when the polypeptide is dissociated in SDS.

The staining pattern for ConA was considerably different. ConA labeled a large number of polypeptides with molecular weights over 50,000 daltons. The major ConA-binding polypeptide at 105,000 daltons which also appeared to label heavily with ^{125}I may correspond to the 105,000 dalton glycoprotein observed by Truding *et al.* (80) to be preferentially synthesized in differentiated neuroblastoma cells. ConA also appeared to label the 30,000 molecular weight glycopeptide which bound both WGA and RCAI.

The co-ordinate redistribution of ConA receptors discussed in Chapter 3 does not suggest such a complicated array of cell surface ConA binding sites. Thus, the experiments on the internalization of cell surface ConA binding sites were carried out to determine whether or not all the ConA binding polypeptides revealed in the gel staining experiments are accessible to ConA at the cell surface. The method used brought about internalization of all the ConA receptors visible under the fluorescent microscope. The continuous sucrose gradient appeared to adequately separate

the plasma membranes from the internalized vesicles. However, the upper band, which contained these vesicles, also contained natural pinocytotic vesicles and probably some endoplasmic reticulum. Staining of gels of this plasma membrane preparation indicated that the majority of the ConA binding polypeptides revealed by SDS-gel electrophoresis were not accessible to ConA at the cell surface at the resolution of the fluorescent microscope. The four polypeptide bands whose staining was diminished or lost following internalization of the cell surface ConA binding sites are all of high molecular weight and all appear to label with ^{125}I in the presence of lactoperoxidase. However, only one of the four bands stains with dansyl hydrazine after periodate oxidation. The WGA and RCAI binding glycopeptide was not affected by the internalization of the cell surface ConA binding sites indicating that although this polypeptide apparently contains mannose residues these are not available for ConA binding at the cell surface. This is in agreement with the double labelling studies discussed in Chapter 3. It also suggests that cell surface ConA binding sites may be undergoing internalization only in certain places across the neuroblastoma cell surface where a concentration of these binding sites exists or develops. If the binding sites were simply internalized randomly over the entire cell surface, a diminution of all the ConA binding polypeptides seen on the gels would be expected.

The vesicle fraction appears to contain the ConA binding polypeptides absent from the plasma membranes of ConA-treated cells. However, this fraction also contains natural pinocytotic vesicles and so the presence of these ConA binding polypeptides in this fraction may reflect predominantly the presence of these natural vesicles rather than the ConA-induced vesicles.

A highly complex pattern of ConA binding sites as revealed by staining of SDS-polyacrylamide gels has also been observed with *D. discoideum* plasma membranes (110) and CHO cell plasma membranes (144). In both cases, the majority of the polypeptides had molecular weights greater than 50,000. *D. discoideum* plasma membranes (143) had a much lower number of WGA binding polypeptides and these tended to have lower molecular weights than the ConA binding polypeptides. Since a co-ordinate redistribution of ConA, but not WGA, binding sites has also been seen on *D. discoideum* cells (108) it may be that many of the ConA binding polypeptides, revealed by infiltration of SDS-polyacrylamide gels of *D. discoideum* plasma membranes with Fl-ConA, are not accessible to ConA at the cell surface.

The finding that *Ulex europeus* lectin, which is specific for L-fucose, did not label whole cells or gels of isolated plasma membrane proteins is interesting in view of the studies by several groups on the incorporation of L-fucose into neuroblastoma cell glycoproteins (80, 81). The present results suggest that fucose is only a minor component of neuroblastoma cell glycopeptides and/or that *Ulex europeus* lectin has a low affinity for fucose groups on neuroblastoma cells. The fucose should be accessible to the lectin, at least in the gels, since fucose is always found at the ends of oligosaccharide chains (45, 130).

None of the lectins showed any labelling of gels in the region below the dye marker. Since glycolipids appear to migrate in this region, it can be concluded that the lectins do not bind to neuroblastoma cell glycolipids in gels. This suggests that they also do not label glycolipids on the cell surface.

Each technique used in the present study to study cell surface glycoproteins revealed different information. It is apparent that a polypeptide which does not stain with dansyl hydrazine may still be a glycopeptide as indicated by staining with fluorescent lectins. In addition, polypeptides may be exposed at the cell surface but not be accessible to ^{125}I and lactoperoxidase. Together these different pieces of data can be put together to give a more detailed picture of the nature of glycoproteins on the neuroblastoma cell surface.

The results presented here confirm and extend those discussed in Chapter 3. WGA and RCAI do bind to the same membrane polypeptide which is why they can affect each other's binding. ConA appears to bind to four polypeptides at the neuroblastoma cell surface. These may be directly associated in a macromolecular complex or may exist separately. Nevertheless, they must associate in an indirect manner once some of them are labeled in order for the ConA receptors to undergo co-ordinate redistribution.

Chapter 5

MATERIALS AND METHODS

Reagents

The sources of the reagents were: complete Freund's adjuvant was purchased from Gibco; dimethyl-3-3'-dithiobispropionimidate hydrochloride (DTBP) from Sigma; goat anti-rabbit γ globulin from Antibodies Inc. All other chemicals were of reagent grade.

Methods

Preparation of Triton Cytoskeletons for SEM

Neuroblastoma cells were stripped of their membranes to allow examination of their cytoskeletons in the SEM by the method of Bell *et al.* (145). Cells grown on glass coverslips were cross-linked to the coverslips by treatment with DTBP (4 mg/ml in DPBS, pH 7.4) for 5 min at 37°C. The cells were rinsed in PBS and exposed to 0.5% Triton X-100 in PBS for 5 min at 37°C. The cells were then rinsed in PBS, fixed 1 hr in 1.25% glutaraldehyde in PBS at 23°C and processed for examination in the SEM as described in Chapter 3. In some experiments the cells were pretreated for 1 hr at 37°C with WGA or ConA (100 µg/ml in DPBS) or for 30 min at 37°C with cytochalasin B (5 µg/ml) or colchicine (10^{-6} M).

Identification of Cytoskeletal Components - a modification of the method of Brown *et al.* (146).

Neuroblastoma cells grown in 100 mm tissue culture dishes were rinsed several times with DPBS and cross-linked with 2 ml DTBP in DPBS for 5 min at 37°C. The cells were rinsed with PBS and treated with 1 ml of Triton X-100 (0.5%, 1.0%, 5.0% in PBS) for 5 min at 37°C. The supernatant was removed and saved and the residue was rinsed once with PBS. 1 ml of PBS, 0.1 ml of solubilizing solution (10% SDS, 50% glycerol in 0.05 M Tris-HCl, pH 6.8) and 25 µl of β-mercaptoethanol were added to the residue and it was collected by scraping with a rubber policeman.

To each ml of Triton supernatant was added 0.1 ml solubilizing solution, 0.1 ml 10% SDS, 25 µl β-mercaptoethanol and 25 µl 0.05% bromophenol blue. 0.1 ml 10% SDS and 25 µl 0.05% bromophenol blue were added to the residue as well and all the samples were heated at 100°C for 5 min. The samples were cooled and 30 µl aliquots were applied to 9% Laemmli slab gels. The gels were run and stained with Coomassie blue as described in Chapter 2.

Preparation of Actin

Actin was purified from chicken gizzards as described (147). The actin was further purified by preparative SDS-gel electrophoresis according to the method of Lazarides (10). The preparation gave a single peak when run on 9% Laemmli gels.

Preparation of Rabbit-Anti-Actin γ Globulin

500 μg of purified actin were injected subcutaneously in a rabbit in complete Freund's adjuvant. This injection was repeated 7 days later with an additional 500 μg actin in Freud's complete adjuvant. The rabbit was bled from the ear vein 21 days after the second injection.

The blood was allowed to clot overnight at 4°C and the serum was clarified by centrifugation at 12,000 x g for 10 min. 1-2 ml of the supernatant were applied to an actin-Sepharose column prepared from purified actin by the CNBr method (86,149). The column was washed with PBS until $A_{280} = 0.0$. The anti-actin antibodies were then eluted with 4.0 M $MgCl_2$ (149) and the peak fractions were pooled and dialyzed immediately against PBS.

Staining of Cells with Rabbit Anti-Actin and Globulin

Cells were prepared for staining with anti-actin by the method of Heggeness *et al.* (150). Cells on coverslips were fixed for 30 min at 23°C in 3% formaldehyde in PBS. The cells were rinsed in PBS, treated with 0.1 M glycine in PBS for 10 min at 23°C and rinsed again. They were then made permeable by a 2 min exposure to 0.1% Triton X-100 in PBS at 23°C. The cells were rinsed well in PBS and treated with rabbit anti-actin γ globulin (0.05-0.09 mg/ml in PBS) for 20 min at 23°C. After thorough rinsing the cells were stained with Fl-goat anti-rabbit γ globulin (0.05 mg/ml in PBS) for 20 min at 23°C. The coverslips were rinsed well, suspended over 50% glycerol and examined in the fluorescent microscope. Controls were run using the unadsorbed material from the actin-Sepharose column instead

of the anti-actin antibodies. In some experiments cells were pretreated for 5-60 min with WGA or ConA (100 μg/ml in PBS).

The Fl-goat anti-rabbit γ globulin was prepared by conjugation of goat anti-rabbit γ globulin with fluorescein isothiocyanate as described in Chapter 2 for lectins followed by purification of the fluorescent antibodies on a rabbit IgG-Sepharose column.

RESULTS

Analysis of Triton Cytoskeletons on SDS-Polyacrylamide Gels

SDS-gel electrophoresis of the residue left on the tissue culture plates after treatment with Triton X-100 revealed the presence of 5 major and a number of minor polypeptide bands (Fig. 33). Analysis of the Triton X-100 solubilized material by SDS-gel electrophoresis showed that some of these polypeptides were greatly enriched in the residue as compared to the supernatant whereas others were divided between the two fractions. A number of the polypeptides present in the supernatant were not found at all in the residue. The gel patterns were essentially the same at all three Triton X-100 concentrations tested. Omission of the cross-linking step resulted in complete solubilization of the cells.

Examination of the Triton Cytoskeletons in the SEM

The treatment of the cells with Triton X-100 brought about a decrease in the opacity of the extranuclear region of the cells when they were examined using phase contrast in the light microscope. When viewed in the SEM (Fig. 34) two different types of structures were apparent; a large, oval cell nucleus and a fibrous network. This network covered the substratum

Figure 33. SDS-polyacrylamide gels of fractions obtained by extraction of neuroblastoma cells with Triton X-100. A, protein remaining attached to the tissue culture dish after extraction of DTBP-treated cells with 0.5 % (left), 1.0 % (center), and 5.0 % (right) Triton X-100; B, protein released into the supernatant by extraction of DTBP-treated cells with 0.5 % Triton X-100 and; C, protein remaining attached to the tissue culture dish after extraction of untreated cells with Triton X-100. Residue and supernatant were prepared as described in Materials and Methods and a standard volume of each (30 µl) was applied to the gel.

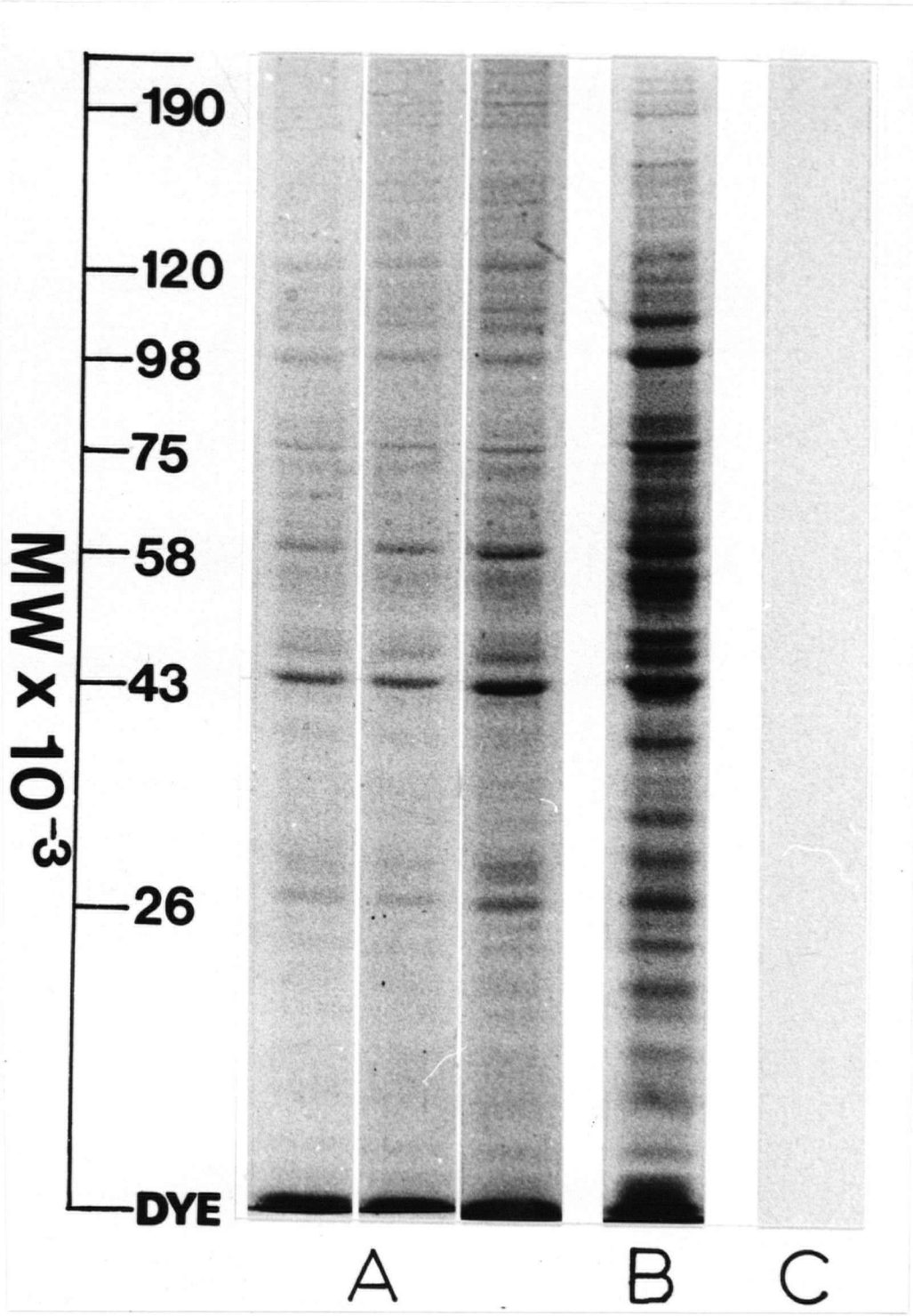
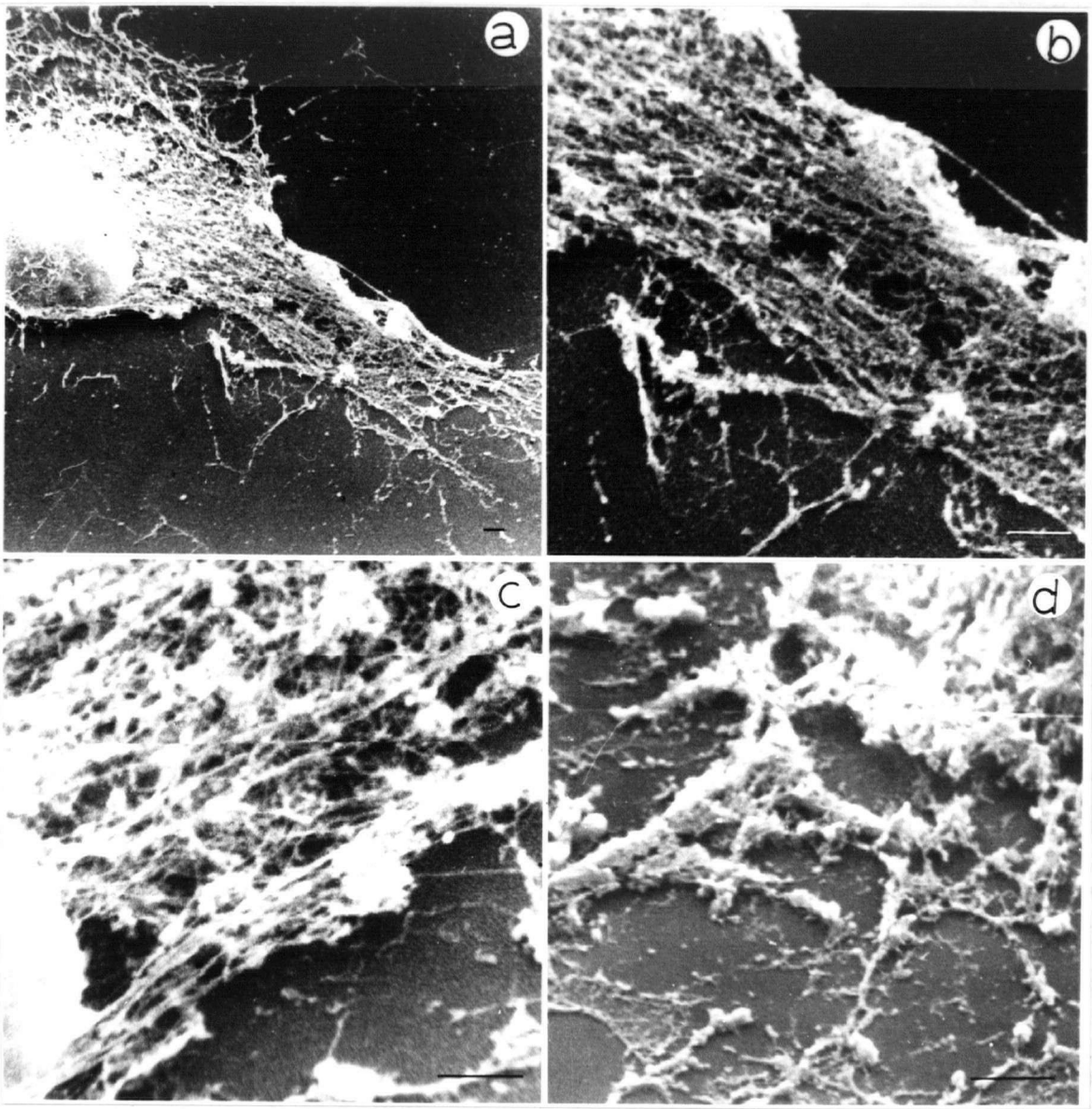

190
120
98
75
58
43
26
DYE
MW x 10-3
A
B
C

Figure 34. SEM micrographs of Triton X-100 cytoskeletons from neuroblastoma cells. (a) Cell crosslinked with DTBP and extracted with 0.5% Triton X-100; (b) higher magnification of same cell as in (a); (c) cell treated with 10^{-6} M colchicine for 30 min at 37°C, cross-linked with DTBP and extracted with 0.5% Triton X-100; (d) cell treated with 5 μg/ml cytochalasin B for 30 min at 37°C, cross-linked with DTBP and extracted with 0.5% Triton X-100. Bar = 1 μm in all SEM micrographs.


a
b
c
d

surrounding the nucleus and its outline appeared to closely approximate that of the original cell. The fibrous network was extremely dense in the areas of the cells closest to the nucleus and progressively thinned out toward the cell edges. The network appeared to consist of both individual fibers and fiber bundles. The fibers ranged in diameter from 0.05-0.2 μm. The thicker bundles appeared to form a framework which was connected by numerous smaller filaments. Often individual fibers were seen extending out from the cell periphery. Numerous globular structures were seen to be interspersed throughout the network.

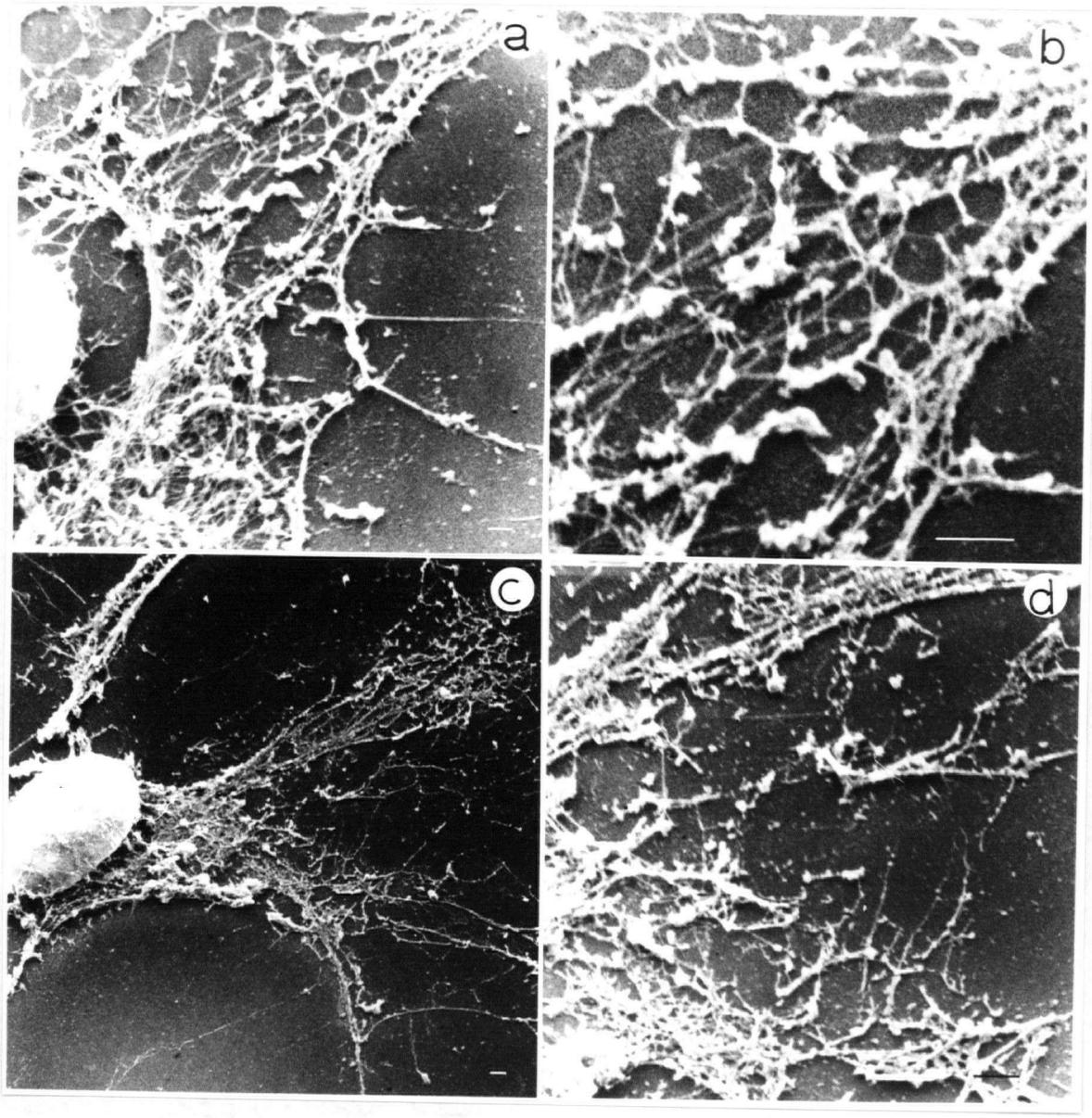
Cells treated with colchicine showed no changes in their cytoskeletons (Fig. 34c) whereas in cells treated with cytochalasin B (Fig. 34d) the cytoskeleton had formed a large, tight clump around the nucleus.

When cells were treated with WGA (Fig. 35a,b) there were no apparent changes in the organization of the Triton cytoskeleton. However, cells treated with ConA did show some alterations (Fig. 35c,d) in the structure of the fibrous netowrk. Near the nucleus the cytoskeleton remained the same but toward the cell periphery the highly organized lattice structure appeared to have broken down. The large fiber bundles were still present but the interconnecting fibers were reduced or, in some cases, completely absent.

Staining with Anti-Actin γ Globulin

The immunofluorescent staining of actin in differentiated neuroblastoma cells is shown in Fig. 36a. The majority of the fluorescence is localized to the cell membrane and the neurites. Microspikes on both the

Figure 35. SEM micrographs of Triton X-100 cytoskeletons from neuroblastoma cells treated with lectins. Cells were pretreated for 1 hr at 37°C with ConA or WGA (100 µg/ml), rinsed, cross-linked with DTBP and extracted with 0.5% Triton X-100. (a,b) Cell pretreated with WGA; (c,d) Cell pretreated with ConA.


a
b
c
d

cell body and along the neurites were heavily stained as were areas of apparent membrane ruffling. The staining around the cell membrane was nonuniform with areas of light fluorescence interspersed with bright patches. In some cells, numerous fluorescent dots were seen over the cell surface and these may be due to microvilli or other similar structures. There was little definite staining in the central portions of cells. Stress fibers were never seen.

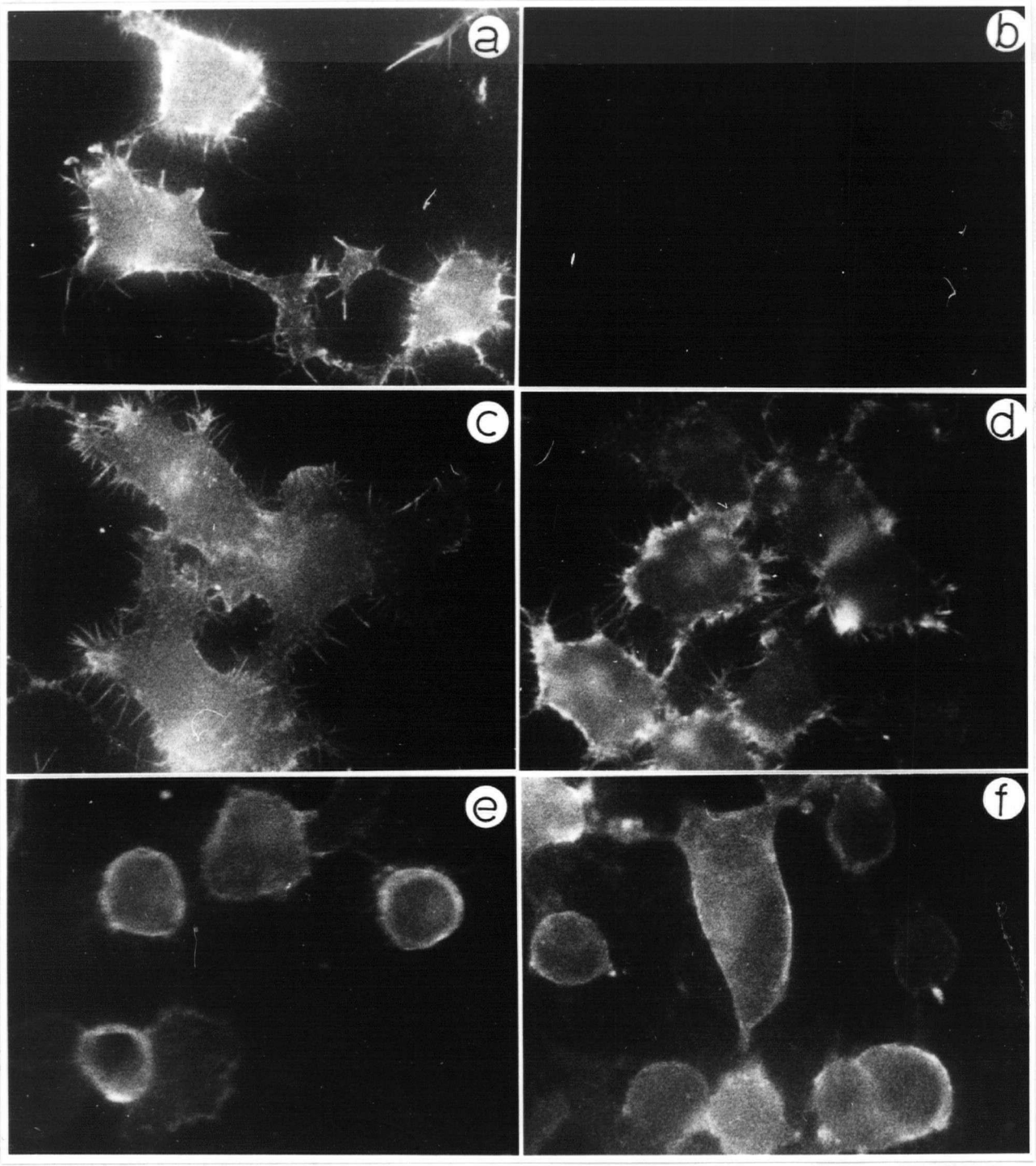
In control experiments, where the cells were treated with the globulin not bound to the actin-Sepharose column, only a faint, diffuse fluorescence present throughout the cells was observed (Fig. 36b).

Treatment of the cells for up to 1 hr with WGA (100 μg/ml) had no apparent effect on the fluorescent pattern of actin staining (Fig. 36c,d). When cells were treated for 15-60 min with ConA (50 or 100 μg/ml) a marked alteration in the actin staining pattern was observed (Fig. 36e,f). Neither microspikes nor areas of apparent ruffling stained with the anti-actin antibodies under these conditions. The fluorescence of the neurites was also sharply reduced. The fluorescence around the cell membrane appeared to be somewhat brighter than in control or WGA-treated cells.

DISCUSSION

The extraction of neuroblastoma cells with Triton X-100 brings about a loss of the soluble cellular components and leaves behind a cytoskeleton consisting predominantly of 5 polypeptides of molecular weights 26,000; 43,000; 58,000; 75,000; 98,000 and 120,000 and the cell nucleus. Based on the results of similar studies with other types of cells (145,151,152) several of these

Figure 36. Fluorescent micrographs of neuroblastoma cells treated with antibody against smooth muscle actin to visualize actin-containing structures in the cells. The cells were fixed and permeabilized as described in Materials and Methods, treated for 20 min at 23°C with rabbit anti-actin antibody (90 μg/ml), rinsed and stained with Fl-goat anti-rabbit antibody (50 μg/ml). (a) Untreated cells; (b) control cells treated with non-specific rabbit γ globulin; (c,d) cells pretreated for 60 min at 37°C with WGA (100 μg/ml) before staining for actin; (e,f) cells pretreated for 60 min at 37°C with ConA (100 μg/ml) before staining for actin.


a
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e
f

polypeptides can be identified. The 42,000 dalton polypeptide is most likely actin, the 57,000 dalton polypeptide is probably the monomer of the 10 nm filaments and the large amount of material near the dye marker is the histones from the nucleus. No polypeptides corresponding to tubulin are apparent. Both the 57,000 and 115,000 dalton polypeptides are almost exclusively found in the Triton residue whereas the actin appears to be divided equally between the residue and the supernantant. The three other polypeptides found in the residue are mostly extracted by the Triton X-100. A similar pattern of extraction has been observed with fibroblasts (146, 151). The reason for the extraction of a large percentage of the actin by the Triton X-100 is not known but it may be due to actin which is not in the form of microfilaments. It is interesting to note that there is very little residual material of molecular weight over 200,000. With fibroblasts a dark band at 230,000 daltons, which is thought to be the LETS protein, is always found (146,151). The LETS protein is apparently absent from the neuroblastoma cells. It has been suggested (146) that the absence of the LETS protein, which is thought to anchor cells to the substratum, may be the reason why certain types of cells must be cross-linked to the substratum before Triton extraction will work.

Examination of the cytoskeleton in the SEM revealed a pattern of filament organization similar to that seen when neuroblastoma cells were examined in the TEM (153). Based on the SDS-polyacrylamide gel studies, the fibrous network appears to be made up predominantly of actin and 10 nm filaments. This is in agreement with the results of TEM studies on neuroblastoma cytoskeletons (153). In addition, it is possible to specifically stain the Triton cytoskeletons with anti-actin antibodies confirming the presence of actin.

The effects of colchicine and cytochalasin B on the appearance of the cytoskeleton are in agreement with the known effects of these drugs on cytoskeletal elements (5). Since no microtubules are present in the Triton cytoskeleton as indicated by the lack of tubulin on the SDS-polyacrylamide gels, colchicine would not be expected to have any effect. The marked alteration in the appearance of the cytoskeleton brought about by cytochalasin B is consistent with the evidence that microfilaments are a major component of the Triton cytoskeleton. Bell *et al.* (146) observed a similar effect of cytochalasin B on CHO cell cytoskeletons.

The fluorescent staining pattern for differentiated neuroblastoma cell actin seen in this study is very similar to that observed previously with several other neuroblastoma cell lines (152,154,155). In all cases, the staining was concentrated to the cell membrane, neurites, microspikes and ruffles. Stress fibers, characteristic of fibroblast microfilaments, were never seen. The apparent large concentration of neuroblastoma cell microfilaments around the cell periphery and in microspikes and neurites has been confirmed by TEM (152). Thus, neuroblastoma cell microfilaments appear to play a more dynamic role in cellular functions than do fibroblast microfilaments. The localization of actin to the cell membrane agrees with the finding that actin is found on SDS-polyacrylamide gels of purified neuroblastoma cell plasma membranes.

ConA has no apparent effect on the appearance of differentiated neuroblastoma cells under phase contrast in the light microscope. However, the present studies indicate that it can bring about alterations in the cytoskeleton. The experiments on the staining of intracellular actin showed that certain structures were no longer fluorescent after treatment

with ConA. The results on the Triton cytoskeletons suggest that this lack of fluorescence is due to a loss of microfilaments from these structures rather than an inhibition of staining brought about by ConA. This effect of ConA was localized only to peripheral actin-containing structures suggesting it is due to a rearrangement of the microfilament network rather than a simple destruction of the microfilaments. This would also explain the earlier results with cytochalasin B since it appears to destroy the network.

The alterations in microfilament organization brought about by ConA may be related to the ability of the ConA receptors to redistribute in a co-ordinated manner. This concept is reinforced by the finding that WGA had no effect on microfilament organization and has receptors which redistribute independently of each other. It is tempting to speculate that the interaction ConA with its receptors on the cell surface somehow brings about a reorganization of the peripheral microfilaments into a new type of network which is then responsible for the co-ordinate redistribution of ConA receptors.

Chapter 6

SUMMARY

1. ConA, but not WGA, can bind to both RCAI and RCAII.

2. ConA, WGA and RCAI each have over 10^7 binding sites on neuroblastoma cells.

3. ConA, WGA and RCAI are all internalized by neuroblastoma cells in an energy dependent process.

4. There is a co-ordinate redistribution of labeled and unlabeled ConA receptors.

5. Cytochalasin B, but not colchicine, uncouples this co-ordinate redistribution.

6. Labeled WGA and RCAI receptors redistribute independently of their unlabeled counterparts.

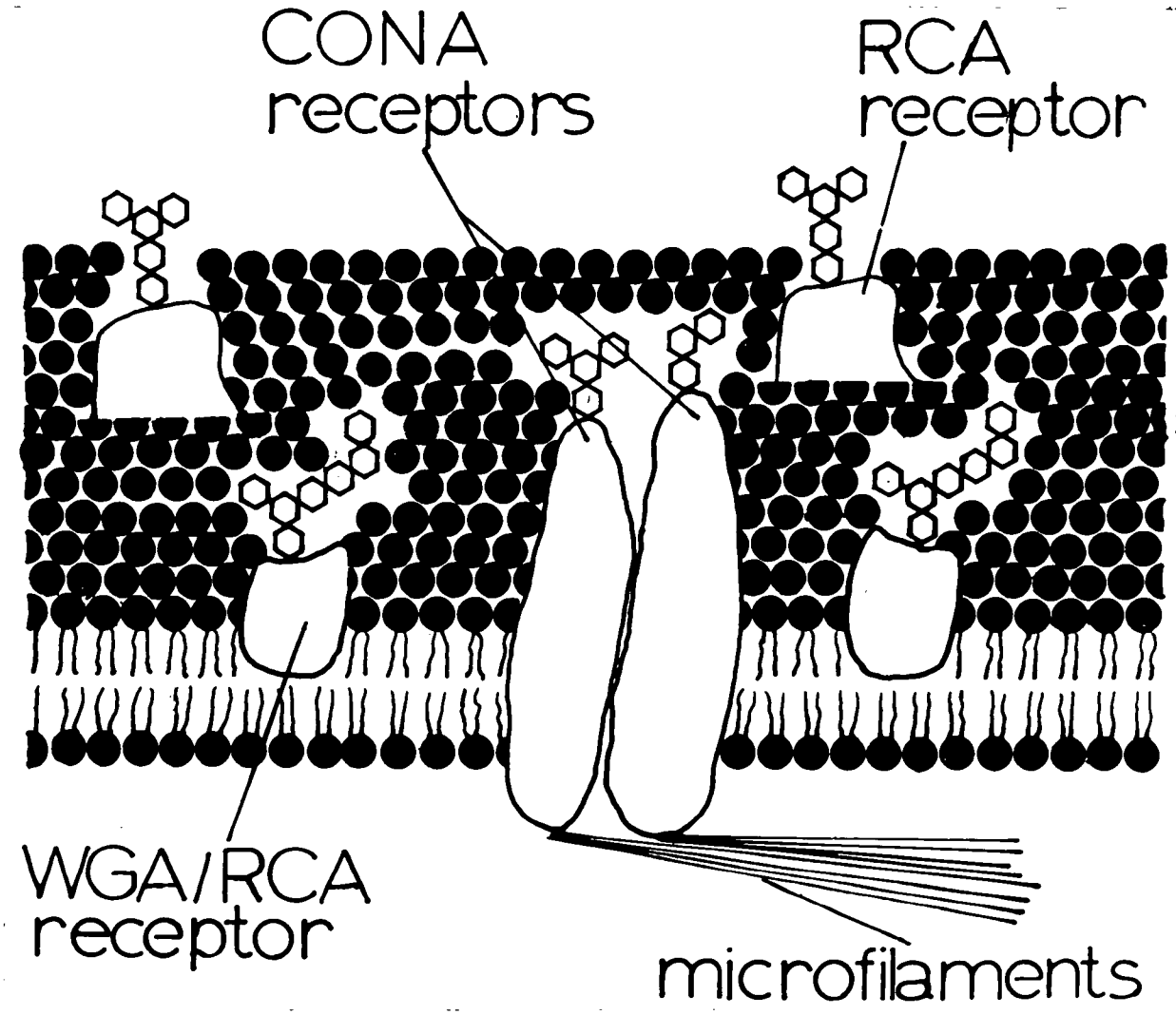
7. WGA and RCAI share the same receptor; a 30,000 dalton glycopeptide.

8. Although ConA can label many membrane polypeptides when they are dissociated in SDS only four polypeptides ranging in molecular weight from 90,000 to 120,000 daltons appear to be cell surface binding sites for ConA.

9. ConA, but not WGA, can alter the organization of microfilaments in the neuroblastoma cells.

A diagram, based on the results of the preceding four chapters, which depicts the organization of lectin receptors in the neuroblastoma cell membrane is shown in Fig. 37. The membrane polypeptide which has binding sites for both WGA and RCAI is pictured as being a heavily glycosylated, non-transmembrane protein. There is no evidence, however, that it doesn't span the plasma membrane. An additional RCAI binding site, separate from the WGA-RCAI receptor,is also shown. The ConA receptor is pictured as consisting of a complex of two or more transmembrane polypeptides. Although there is good evidence that ConA has several cell surface binding sites and that these sites undergo co-ordinate redistribution, there is no evidence that these sites exist as a complex in the unlabeled cell membrane. There is also no evidence that these binding sites span the plasma membrane. In addition, the ConA binding sites are shown to be interacting directly with microfilaments. Although there is evidence that these receptors do interact with microfilaments nothing is known about the nature of this interaction.

Figure 37. Hypothetical arrangement of lectin receptors in the neuroblastoma cell membrane based on the data presented in the previous three chapters. WGA and RCA were shown to share a receptor of 30,000 daltons. These receptors all redistributed independently of each other. Thus, this receptor is pictured as single, small integral membrane proteins. The results also suggested that RCA might have a second receptor independent from the WGA-RCA receptor. This is pictured as larger, single integral membrane proteins. ConA was shown to have several receptors, all of high molecular weight. They were shown to redistribute in a co-ordinated manner. This co-ordinate redistribution was dependent on an intact microfilament system. In addition, ConA was shown to alter the organization of actin-containing microfilaments associated with the cell membrane. Thus, the ConA receptors are pictured as existing as small complexes of two or more large transmembrane polypeptides. In this model, they are shown to interact directly with microfilaments on the cytoplasmic side of the cell membrane. However, the interaction could also be an indirect one mediated by another integral membrane protein which was directly associated with both the microfilaments and labeled ConA receptors.


CONA
receptors
RCA
receptor
WGA/RCA
receptor
microfilaments

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