(1) GLYCOPROTEINS AND DEVELOPMENT IN THE CELLULAR

SLIME MOLD DICTYOSTELIUM DISCOIDEUM. (2) SEPARATION

OF CELLS USING ISOPYCNIC CENTRIFUGATION IN LINEAR

DENSITY GRADIENTS OF COLLOIDAL SILICA

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ABSTRACT

I. Two methods were adapted for the study of glycoproteins in
Dictyostelium discoideum. Both techniques relied upon prior separation
of glycoproteins on one- or two-dimensional SDS-polyacrylamide gels.
The first method was a modified form of crossed immunoelectrophoresis
which substituted the carbohydrate-binding protein (lectin) concanavalin
A (Con A) as the precipitating agent. In the second method, polyacryl-
amide gels were simply fixed and incubated in the presence of fluorescein-
tagged lectins. After washing away free lectin, the gels were photo-
graphed and stained for protein. Identified glycoproteins were defined
by their apparent molecular weights, identity and, anomeric linkage of
some of their monosaccharides and in two-dimensional gels, their ap-
parent isoelectric points. The ability of these techniques to specifically
identify authentic glycoproteins was confirmed using a defined membrane
system, the erythrocyte ghost, other known proteins, and hapten inhibitors.
Of the two techniques, lectin diffusion was capable of higher resolution
but did not give information about receptor multivalency. Both methods
were more sensitive than the commonly-used periodic acid-schiff's base
stain.

More than fifty different glycoproteins were detected in vegetative
cells on the basis of their labeling with Con A or wheat germ agglutinin
(WGA) and their sensitivity to proteolysis. These glycoproteins were
distributed throughout the cell, giving each of several subfractions,
including the plasma membrane, its own profile of glycoproteins. WGA
receptors were apparently membrane bound and predominantly localized in the
plasma membrane. A small number of glycoproteins were also detected with a

galactose-binding protein, but these glycoproteins were not present in
the plasma membrane or on the cell surface as determined by an independent
technique. Receptors for L-fucose binding proteins were also absent
from the plasma membrane of these cells. In contrast, another eukaryotic
cell plasma membrane, the erythrocyte ghost, contained receptors to
all lectins tested.

During the formation of the pseudoplasmodium from vegetative cells,
many glycoproteins were lost, modified, or many new ones were synthesized
in all cell subfractions, including the plasma membrane. In addition,
there was an asymmetry of distribution of glycoproteins in the pseudo-
plasmodium. There were three prestalk-cell specific glycoconjugates,
which were all restricted to the plasma membrane, and two prespore-specific
glycoproteins, which were present in plasma membranes as well as in a
potential plasma membrane precursor. These plasma membrane differences
occurred prior to overt differentiation of any stalk or spore cells. The
region-specific glycoproteins were also present in the plasma membranes of
cells which had not yet formed pseudoplasmodia, but were absent from
vegetative cells.

In order to reinforce the temporal evidence that these region-specific
glycoproteins were involved in the creation of the pseudoplasmodium,
dissociated pseudoplasmodial cells were treated with the lectin which
originally identified the region-specific molecules (WGA). Although
dissociated cells typically reformed pseudoplasmodia, in the presence of
WGA, they did not. This effect of WGA was blocked by a hapten inhibitor.

II. In other work, separation of cells with different densities was
attempted. When cells of Dictyostelium discoideum were centrifuged to

density equilibrium in linear gradients of colloidal silica (Ludox),
approximately 40 discrete bands appeared. A similar result was found for
formalinized red blood cells and plastic beads. Isolated bands of cells
rebanded faithfully in new gradients and band spacing depended upon
gradient steepness. It was found that cell bands resulted from micro-
scopic discontinuities in the linear gradients caused by centrifugation.
When the gradients were analyzed in the analytical ultracentrifuge,
absorbance scans revealed that cell bands coincided with "bands" of
Ludox, which formed even without cells. Evidence ruling out other
possible causes for cell bands is presented and procedures which avoid
this condition are described.

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CHAPTER I

The Role of Carbohydrate in Glycoproteins

INTRODUCTION

Many if not most protein species contain covalently-linked carbo-
hydrate and are called glycoproteins. As an example of the prominence
of glycoproteins, approximately one-half as many glycoproteins as
proteins can be detected in plasma membranes from Dictyostelium
discoideum electrophoresed on one-or two-dimensional SDS-polyacrylamide
gels (Chapters 2-4; C. M. West and D. McMahon, unpublished). This holds as
well for whole cells of D. discoideum electrophoresed on one-
dimensional gels (Chapter 4). These facts have recently become
accessible as a result of a new technique which unites the specific
binding of carbohydrate-binding proteins and the high resolution
separation of proteins afforded by SDS-polyacrylamide gel electro-
phoresis (Robinson et al., 1975; Tanner and Anstee, 1976; Chapter 2).

From a structural point of veiw, protein-linked oligosaccharides
contain from one to twenty of any of approximately ten different
kinds of monosaccharides arranged in partially branched structures.
Carbohydrate-peptide linkages are restricted to certain amino acids
within characteristic short amino acid sequences in the protein, and
can occur multiple times in a given polypeptide change (Spiro, 1973).
Due to their hydrophilic nature, the oligosaccharides are thought to
always be located on the surface of proteins.

Why has evolution gone to the expense of attaching complex carbo-
hydrate to proteins? Are there unique functions which can only be
served by these carbohydrate structures?

ROLE OF GLYCOPROTEIN CARBOHYDRATE

In Dictyostelium, the identity of most glycoproteins is unknown although a few of them are enzymes (Every and Ashworth, 1973; Parish, 1976; Crean and Rossomando, 1977). The cell surface glycoproteins serve important functions, however, since treating the cells with multivalent carbohydrate-binding proteins stalls or blocks the time course of development (Gillette and Filosa, 1973; Darmon and Klein, 1976; Chapter 4). However, no function has yet been directly demonstrated for the carbohydrate linked to any of the Dictyostelium proteins. The strongest evidence for a role of glycoprotein carbohydrate to date is in cell adhesion and this is discussed below.

In other systems, several functions have been ascribed to protein-linked carbohydrate and examples of these will be evaluated in detail below. As a summary in advance, the following hypotheses about protein glycosylation are presented:

1. The result of protein glycosylation is to form a relatively passive protuberance on the surface of proteins. This protuberance may potentially serve several functions. It may be bound or "gripped" by other proteins or may associate with other carbohydrate chains, resulting in a linkage between two molecules. This protuberance may also, however, block the grip which a protein, e.g., a protease, may ordinarily be able to make on a protein. In playing other roles, this carbohydrate may improve the solubility of peptide regions in normal aqueous solvents or improve the stability of protein conformation in unfavorable conditions.

As a corollary to this, glycosylation usually subserves a second biochemically significant property of the glycoprotein. The peptide portion usually has an identifiable function of its own.

2. In some cases glycosylation confers a shared property upon a variety of otherwise unrelated or diverse proteins. On the other hand, differential glycosylation can instead result in very specific protein modifications which can distinguish each of a number of otherwise similar proteins.

3. Both economy of energy consumption, and the characteristic chemistry of sugars, justify the cell's use of glycosylation of proteins over other means of protein modification in some cases. It may be economical for the cell to evolve the process of protein glycosylation, since much of the enzymatic mechanism already exists for synthesizing polysaccharides.

These hypotheses address the biological significance of glycosylation, the mechanism by which glycoprotein carbohydrate seems to subserve its functions, and why glycosylation is used in the place of other possible processes of providing second functions to polypeptide chains. Now we turn to the examples on which these hypothese are based.

PROTEIN-PROTEIN INTERACTIONS WHICH DEPEND ON CARBOHYDRATE

The following three systems to be described illustrate that carbohydrate can be attached to a protein to give a new biochemical property. This property is a new capability for intermolecular interaction.

Serum Protein Turnover

Most mammalian serum protein species are glycosylated and contain sialic acid at their non-reducing oligosaccharide termini (Winterburn and Phelps, 1972). When sialic acid is removed, galactose usually becomes exposed, resulting in rapid clearance (with a few exceptions) from the circulation by being bound to multivalent galactose-binding proteins situated on the surface of liver cells (Ashwell and Morell, 1974). Sialylation and desialylation have been proposed as mechanisms for controlling the turnover of serum proteins. In general, these proteins have functions unrelated to their carbohydrate; many are carrier proteins for small molecules or are hormones and others have enzymatic activities or are zymogens.

Experimental modification of serum glycoprotein carbohydrate has uncovered the potential importance of some of the other sugars comprising the carbohydrate chains as well. Removal of the terminal three monosaccharides in the complex carbohydrate chain of Immunoglobulin G (IgG), which exposes α-linked mannose residues, causes a marked increase of uptake of IgG in mammalian kidney. In contrast, there is no preferential uptake by any organ tested if only two sugars are removed, which exposes β-linked N-acetyl-D-glucosamine residues (Winkelhake and Nicolson, 1976). Exposure of mannose in ribonuclease has also been found to signal serum depletion of this glycoprotein in a mammalian species (Baynes and Wold, 1976). Both of these glycoproteins are also taken up in the liver when their sialic acids are removed.

Immunoglobulin Activity

A subset of serum glycoproteins, IgG, contains an oligosaccharide chain in the Fc region of the molecule which participates in a different kind of molecular recognition (Winkelhake and Kasper, 1972; Koide, Nose and Muramatsu, 1977). Removal of portions of this oligosaccharide chain of anti-red blood cell IgG impairs its ability to fix complement on the surface of red blood cells, to mediate antibody dependent cell-mediated cytotoxicity, and to mediate rosetting of red blood cells by lymphocytes (Koide et al., 1977). The hemagglutination titer of this antibody remains unaffected. In addition, each of the processes blocked by the treatment with glycosidases could also be inhibited by certain sugars, while not affecting hemagglutination titer. This suggests that the Fc region oligosaccharide is directly involved in the binding of IgG to complement and Fc receptors.

Uptake of Enzymes by Cells

Several inherited disorders of lysosomal metabolism have been described which result in the accumulation of secretory product precursors due to the absence of specific enzymes. In some cases the metabolic defect can be corrected by incubating mutant cells in a medium containing a normal form of the enzyme. Surprisingly, the cells have a very selective, specific and saturable uptake mechanism for these enzymes. Furthermore, it was found that enzymes from mutant cells could not be taken up. A critical variable for whether the enzymes are taken up is their carbohydrate. Although the extent of glycosylation has no effect on enzyme activity, chemical or enzymatic

removal of carbohydrate inhibits its uptake (Hickman, Shapiro and

Neufeld, 1974). In recent work it has been shown that phosphate

monoester derivatives of glycoenzyme carbohydrate are the real high-

uptake forms of the enzymes (Kaplan, Achord and Sly, 1977). The

uptake of the enzyme could be blocked by simple and complex mannose

phosphate monoesters. Alkaline phosphatase treatment of these high-

uptake glycoenzymes also inhibit their uptake. Phosphomannosylation

may be a chemical passport for an enzyme to enter into lysosomes. One

could speculate that this mutant has uncovered what may be a general

mechanism for the population of lysosomes with enzymes.

Carbohydrate can give a second property to proteins. In these

examples, the acquired property seems to depend at least in part on the

identity of the terminal sugar. A second interesting observation is

that this carbohydrate gives the same property to each of a heterogeneous

group of proteins. This post-translational modification has the effect

of giving a hierarchy of various molecular functions to a set of proteins.

Cell Binding or Adhesion

The adhesion of cells to cells or non-living surfaces can often

be reduced to interactions between molecules which contain carbohydrate.

The significance of carbohydrate in intermolecular interactions spreads

beyond simply linking two molecules together to binding macroscopic

objects to one another. For example, a scavenging system for red blood

cells similar to that for desialylated serum proteins also occurs.

Kupfer cells isolated from the liver will rosette desialylated but not

intact erythrocytes (Aminoff et al., 1977). A cell surface lectin is thought to mediate this cell binding. Treatment of lymphocytes with neuraminidase (to remove cell surface sialic acid) similarly results in a selective uptake of lymphocytes in the liver. The appearance of lymphocytes in all other tissues studied was diminished (Woodruff and Gesner, 1969). It may not be necessary that glycoproteins are involved in this process although it is known that many glycoproteins on the surfaces of these cells are terminally sialylated.

Cell recognition of a different type has also been found to involve cell surface glyco-conjugates (again, not necessarily glyco-proteins). When thoracic duct lymphocytes are treated with glycosidases which are inhibitable with N-acetyl-D-glucosamine or L-fucose the lymphocytes fail to lodge in the spleen and lymph nodes but are preferentially found in the liver (Gesner and Ginsburg, 1964). The mechanism does not appear to be the same which binds erythrocytes and lymphocytes in the liver on account of exposed galactose because of the sugar specificity of glycosidation and because the ability of these lymphocytes to populate the lung is normal.

Several other interesting studies have implicated carbohydrate in cell adhesion or contact. Roseman and his colleagues have derivatized Sephadex beads with monosaccharides and found that the ability of fibro-blasts to adhere to these beads depended upon the kind of sugar which was linked to the Sephadex (Chipowsky, Lee and Roseman, 1973). Galactose was a key sugar in fibroblast adhesion. In a different system, much evidence has been garnered for the involvement of collagen galactose and platelet cell surface glucosyltransferase in platelet-collagen adhesion

during the first step of hemostasis (Jamieson, Urban and Barber, 1971).

Agents or treatments which modify the collagen glucose acceptor,

galactose, or the platelet glucosyltransferase, seem to modify the

collagen-platelet adhesion similarly (Jamieson et al., 1971; Chesney,

Harper and Colman, 1972; Brass and Bensusan, 1976). This is probably

the strongest evidence for the highly referenced but weakly supported

hypothesis by Roseman (1970) that trans-cell glycosyltransferase

carbohydrate acceptor systems play an important part in cell interactions.

Other evidence in favor of this theory includes a small increase of

cell surface glycosyltransferase activity following association of sperm

and egg (Durr, Shur and Roth, 1977) or during the mating reaction of

Chlamydomonas (McClean and Bossmann, 1975). It is not known whether

this increased activity results in a cis- or a trans-glycosylation, nor

what would be the direct consequences of glycosylation for cell adhesion.

Cell Adhesion in Solid Tissues

What is known about the involvement of glycoprotein carbohydrate in

adhesion between cells of a solid tissue? As discussed below, glyco-

protein carbohydrate seems to be involved in a primary way.

Sponge cell adhesion

Molecules involved in dissociated sponge cell reaggregation provide

a good example for study. Reaggregation in marine sponges is relatively

complex because phenomenologically there are three aggregation systems:

one which results in clumps containing approximately eighty cells, a

secondary system which aggregates these clumps and activates growth and

differentiation, and a third system which organizes the secondary aggregates

into water channels (Muller et al., 1976). The current hypothesis

for the second system of cell adhesion involves an aggregation factor,

an acidic proteoglycan with a molecular weight of 3×10^6 and composed

of 50% carbohydrate (Henkart, Humphreys and Humphreys, 1973; Cauldwell,

Henkart and Humphreys, 1973); an aggregation factor receptor or base-

plate which contains greater than 80% carbohydrate; and a yet uncharacter-

ized baseplate receptor. According to the hypothesis, cells which

contain baseplates anchored to baseplate receptors in their plasma

membranes are linked by a dimerized pair of aggregation factors which

bind to the baseplates. In some cases where aggregation is species-

specific the aggregation factor is too (Humphreys, 1963). This chain

of events has been reconstructed in vivo by recovering aggregation

ability of cells from which aggregation factors and baseplates have

been removed by adding them back in the correct sequence. The proposed

linkages have also been reconstructed in vitro by coupling the factors

to agarose beads and finding the correct interactions between aggre-

gation factor-beads and aggregation factor-beads, and between aggre-

gation factor-beads and baseplate-beads (Turner et al., 1974; Kuhns

et al., 1974). There is evidence that the aggregation factor serves as

the physical cross-link between cells from experiments that aggregation

factor can agglutinate glutaraldehyde-fixed cells (Gasic and Galanti,

1966). In addition, it has been suggested that a series of species-

specific lectins found in sponges may be involved in one of the three

levels of adhesion (Bretting and Kabat, 1976). The chemical composition

of the lectins make them unlikely candidates for the aggregation factor

or baseplate as described.

Evidence for the involvement of carbohydrate in adhesion was the finding that periodate treatment inactivated the ability of either base-plate or aggregation factor to promote adhesion of cells in in vivo reconstitution of adhesion (Turner et al., 1974; Kuhns et al., 1974). Later it was found that incubation of baseplate with glucuronic acid (but not galacturonic acid) inhibited its ability to associate with the aggregation factor. Furthermore, glucuronidase treatment of the aggregation factor inactivated its ability to associate with baseplate. Together these suggest that baseplate associates with aggregation factor by binding to its glucuronic acid, of which aggregation factor is partially composed. Whether or not carbohydrate is involved in aggregation factor dimerization is not known. However each of these factors, baseplate and aggregation factor, undergo one of their intermolecular associations by way of carbohydrate.

Intercellular adhesion in cellular slime molds

Several studies suggest that cell surface carbohydrate plays an integral role in at least one of the two known cell adhesion systems in cellular slime molds. Gerisch and his collaborators found that Fab fragments prepared from antisera developed against membranes isolated from aggregation-stage cells blocked adhesion but not chemotaxis in both vegetative and aggregation stage D. discoideum cells (Beug et al., 1970). If the serum was absorbed with vegetative cells the Fab fragments no longer blocked adhesion between vegetative cells, although aggregation-stage aggregation was still blocked. Adhesion between vegetative cells can also be selectively blocked with EDTA. 3×10^5 Fabs adsorbed per

cell was required to block the aggregation of aggregation-stage cells, although 2×10^6 Fabs against another plasma membrane component did not affect adhesion (Beug et al., 1973). The ability of Fab to block aggregation-stage cell adhesion (named contact system A) could be absorbed with aggregation stage crude membranes. The membrane's ability to absorb adhesion blocking activity was markedly reduced by treatment with periodate under conditions which specifically destroyed sugars (Beug et al., 1970). Furthermore, treatment of partially purified contact system A antigens after solubilization in detergents with periodate also destroyed its ability to absorb adhesion blocking activity (Gerisch, 1976). Pronase digestion also inactivated the ability of solubilized material to absorb adhesion blocking activity and thus the carbohydrate may be linked to protein.

Using another approach with a different cellular slime mold, Polysphondylium pallidum, and with Dicytostelium discoideum, Rosen and his collaborators have proposed that a lectin-lectin receptor coupling system mediates adhesion in aggregation-stage cells. They found that certain sugars could block cohesion between heat-killed aggregation-competent P. pallidum cells in the presence of EDTA (Rosen et al., 1974). The glycoprotein asialofetuin (but not fetuin itself) also could block cohesion, though this was only under abnormal metabolic conditions - either high osmotic pressure or the presence of metabolic poisons (Rosen, unpublished). Both the sugars which blocked adhesion and asialofetuin were hapten inhibitors for a lectin which is located on the surface of the cells (Chang, Rosen and Barondes, 1977), while the sugars which did not inhibit adhesion and fetuin were not. Fabs prepared from an antibody

against the cell surface lectin also inhibited cohesion of aggregation-
competent cells, although this was also only under conditions of high
osmotic pressure (Rosen, Haywood and Barondes, 1976). This is a serious
shortcoming which must be overcome by a more complete description of
the system, including the receptor conjugate. We must turn to work on
a related cellular slime mold, D. discoideum, to gain further evidence
that the putative lectin-lectin receptor pair constitutes the physical
bridge between adhering cells. Aggregation-competent D. discoideum cells
will themselves rosette sheep red blood cells and this is probably due
to the lectin on the cell surface (Reitherman et al., 1975). In addition,
the lectin can agglutinate glutaraldehyde-fixed cells from the aggre-
gation stage, but not vegetative stage, of development (Reitherman et al.,
1975), suggesting that the lectin plans a trans-cell rather than a cis-
cell role in adhesion. There are a restricted number of high-affinity
sites for discoidin on aggregation stage but not vegetative stage cells
(of strain NC-4) (Reitherman et al., 1975) and binding of the lectin to
cells is series-specific in pairs of species which also do not coaggregate.
Gerisch's anti-contact sites A serum does not recognize the cell surface
lectin but there is some question as to whether it could recognize the
lectin's putative receptor (Huesgen and Gerisch, 1975). Although the
evidence is compelling that Rosen's lectin system is involved in
aggregation-stage cell adhesion and consequently in Gerisch's contact sites
A system, acceptance of this theory awaits at least an agreement in the
results of these two experimental approaches.

Cell adhesion in chick central nervous system

The tissue specificity of cell adhesion in the embryonic chick central nervous system may rely on glycoprotein carbohydrate. Embryonic chick neural retina and cerebral lobe cells have an adhesion system which is formally analogous to the sponge system described above (Balsamo and Lilien, 1974). The distal component (analogous to aggregation factor in sponges) is released from embryonic chick cells and can promote the agglutination of chick cells which have been fixed with glutaraldehyde in the presence of an aggregation-promotion factor (analogous to baseplate in sponge). Under the same conditions the fixed cells can co-aggregate with living cells of the homologous type. Since the fixed cells are relatively inert this suggests that the distal component forms a physical bridge between cells. The intermediate component, or aggregation-promoting factor, is a glycoprotein. Its protein part is necessary for interaction with the external component and its carbohydrate region is sufficient alone for its binding to cells. The carbohydrate is responsible for the tissue specific binding to cells by this factor (Balsamo and Lilien, 1975). By treating the factor with specific glycosidases and by inhibiting the association of the factor with cells with simple sugars, mannosamine has been identified as a key sugar in cerebral lobe cell aggregation and N-acetyl-D-glucosamine as a key sugar in neural retina cell adhesion.

Other cell adhesion systems

In some cell-cell adhesion systems, the toxic effects of some carbohydrate binding proteins (lectins) have been used to select mutants which bind less of the lectin. This approach has been applied to baby hamster kidney cell lines and these cells bind less Ricinus communis

agglutinin-60. These cells display reduced intercellular adhesion

though they were not selected for this property (Edwards, Dysart and

Hughes, 1976). Glycopeptides isolated from wild-type baby hamster

kidney cells also block cell adhesion (Vicker, 1976).

Glycoproteins, oligosaccharides and carbohydrate-binding proteins

are used by bacteria, viruses and plants as well as by animals in

intercellular adhesions. This application of carbohydrate, potentially

linked to protein, is consequently very widespread, suggesting that

evolutionarily it is a rather primitive device for cells. For example,

bacteria (Escherichia coli) can infect human mucosal tissue by adhering

to the cells. This adhesion can be blocked by the simple sugar D-

mannose and a lectin with a corresponding specificity, concanavalin A

(Ofek, Mirelman and Sharon, 1977). Other sugars and lectins were in-

effective. An endogenous carbohydrate-binding protein with a specifi-

city for D-mannose was found in stationary phase bacteria and a crude

extract containing this lectin also inhibited adhesion of bacteria with

cells. It was suggested that the bacteria use this lectin as a tool for

infecting foreign tissue.

Carbohydrate-binding proteins involved in cell adhesion are also

found in the plant world. One example involves the symbiosis between clover

root hairs and the N_2-fixing Rhizobium trifolii (Dazzo and Hubbell, 1975).

The carbohydrate is located on the bacterium but does not seem to be

linked to protein.

Some viruses have been shown to absorb to cells by way of sialic

acids located on the surfaces of those cells. Influenza viruses bind

to glycoproteins as cell receptors and this can be blocked by prior treat-

ment of the glycoprotein with neuraminidase(Gottschalk, et al., 1972).

A conclusion from studies of cell adhesion is that as far as we know,
carbohydrate invariably plays a direct physical role in linking cells
with cells or other physical objects. Although the carbohydrate usually
seems to be part of a glycoprotein, there may be no fundamental require-
ment for the carbohydrate to be attached to protein; in fact it may
be simply a part of a large polysaccharide molecule (Dazzo and Hubbell,
1975; polysaccharides may promote nonspecific cell agglutination, see
B. Pessacto and V. Defendi, 1972). This is consistent with the carbohydrate
having a function independent of the protein to which it is attached
- that protein is not even needed at all.

HORMONE-RECEPTOR INTERACTIONS

Intermolecular linkages mediated through carbohydrate have other
biological ramifications as well. For instance, some hormones use
carbohydrate as their cell surface receptors. In one example, carbo-
hydrate seems to determine the unique structure of each of a family of
distinct hormone-like molecules.

Carbohydrate in hormone

In mice there are soluble antigen-specific and non-specific factors
coded by the H-2 genetic locus which are involved in T-B cell collaboration
and suppression. These factors have been defined serologically and
named Ia antigens 1 through 21. These factors also occur on the surfaces
of lymphocytes. Different serologically-defined classes modulate the
immune response to different foreign antigens or are not antigen specific
at all. The part of the Ia antigen which is recognized by the anti-
serum is presumably the structurally unique part of each Ia and recent

evidence suggests that it is carbohydrate (McKenzie, Clarke and Parish, 1977). The ability of Ia factors from four classes studied to date to bind with their respective antisera is blocked, respectively, by N-acetyl-D-mannosamine, N-acetyl-D-glucosamine, L-fucose and D-galactose.

Thus, in addition to conferring a shared property upon each of a group of different proteins, such as for the serum proteins discussed above, it seems that the carbohydrate may also deliver a biologically relevant uniqueness to each of a series of immunologically similar Ia peptides.

Carbohydrate in the hormone receptor

The hormones thyrotropin, human chorionic gonadotropin and cholera toxin (this must be considered as hormone-like) all bind, as a first step in their action, to carbohydrate linked to lipid in the plasma membrane. This may serve as an example for a potential role for glycoproteins.

Cholera toxin is a bacterial toxin which is a powerful stimulant of adenylate cyclase in most vertebrate cells. Monosialoganglioside (Gm_1) blocks the lipolytic response (mediated by cAMP) of adipocytes to cholera toxin, but not the response to epinephrine (Cuatrecasas, 1973a; Cuatrecasas, 1973b). Preincubation of cells with Gm_1 increases the binding of cholera toxin to these cells and the lipolytic response. The carbohydrate region seems to be involved since two glycoproteins, fetuin and thyroglobulin, also bind cholera toxin and bind with Gm_1 for binding. Some evidence that thyrotropin (Mullin et al., 1976a) and human chorionic gonadotropin (Lee et al., 1976) bind to gangliosides

with different carbohydrate structures has also been found. Because

of primary sequence homologies between these hormones and leutinizing

hormone and follicle-stimulating hormone it has been suggested that

these latter hormones may also bind to cells through similar receptors.

There is evidence that cholera toxin, upon binding to its

receptor, undergoes a conformational change which releases an active

subunit which travels elsewhere in the membrane to perform its function

(Sahyoun and Cuatrecasas, 1975). This would be analogous to the

activation of cAMP-dependent protein kinase by cAMP. Accordingly, the

receptor oligosaccharide could play a relatively passive role simply

involving association with the binding subunit of the hormone. A

similar mechanism has been proposed for the other possibly homologous

hormones mentioned above.

Insulin also binds to cell surface carbohydrate but the carbohydrate

seems to be linked to protein instead. Treatment of adipocytes with

neuraminidase and subsequently with β-galactosidase abolishes insulin

binding to the surfaces of these cells (Cuatrecasas, 1974). The carbo-

hydrate-binding proteins wheat germ agglutinin and concanavalin A can

compete with insulin for binding to its receptor (Cuatrecasas, 1974).

Trypsinization of the cells blocks insulin binding as well and suggests

that the insulin receptor is a glycoprotein.

Whether the insulin receptor plays a 'passive' role as has been

suggested for cholera toxin, thyrotropin and human chorionic gonado-

tropin is not yet known. There are suggestions that insulin can act

intracellularly, and binding to a 'passive' plasma membrane receptor

could be a first step in a mechanism of this sort. This mechanism is

used by diptheria toxin.

CARBOHYDRATE INVOLVED IN REGULATION OF ENZYME ACTIVITY

The activity of some cell surface glycoenzymes is regulatable
by artificial means and this represents potential mechanisms of control
in cells. The activity of the isolated glycoenzyme 5'-nucleotidase
is inhibited when a mannose-binding protein, concanavalin A, binds to
the enzyme through its carbohydrate moiety (Slavik, Kartner, and
Riordan, 1977). 5'-Nucleotidase activity is sensitive to concanavalin
A in isolated plasma membranes and intact cells as well (Pomier et al.,
1975; Riordan and Slavik, 1974). The activities of a number of other
enzymes in membranes are also modified by lectins (Pomier et al., 1975;
Swann et al., 1975). Inasmuch as lectins seem to be common constituents
of cells, cell surfaces and extracellular matrices (Simpson, Thorne
and Loh, 1977; de Waard, Hickman and Kornfeld, 1976; Sakakibara et al.,
1976; Reitherman et al., 1975; Kauss and Bowles,
1976), this may be an important but undemonstrated mechanism of control.
It seems likely that the effect of the carbohydrate-binding proteins
is due to a steric blockage of the catalytic site of the enzyme.

The sugar of glycoenzymes has not been found to be directly im-
portant for enzyme activity. For instance, porcine pancreatic ribo-
nuclease naturally occurs as at least ten different isozymes and these
are distinguished only by their varying carbohydrate content ranging
from zero to 40% by weight; they all have identical enzymatic properties
(Pazur and Aronson, 1972). The activity of egg white trypsin inhibitor
(Krysteva and Dobrev, 1977), glucose oxidase (Nakamura, Hayashi and
Koga, 1976), glucoamylase I (Pazur, Knull and Simpson, 1970), pineapple
stem bromelain (Yasuda, Takahashi and Murachi, 1971), glucohydrolase I

(Pazur, Simpson and Knull, 1969) and several other glycoenzymes is not altered by enzymatic removal or chemical (periodate) destruction of carbohydrate.

PRESERVATION OF PROTEIN STRUCTURE

In addition to promoting interactions between molecules, carbohydrate can protect proteins from proteolysis, increase the solubility of proteins, or protect them from denaturation. An example of protection is the resistance to proteolysis conferred upon a mucoprotein by its sugar chains (Gottschalk and de St. Groth, 1960). Carbohydrate has been shown to be important for the solubility of fetuin (Spiro, 1960), α-glycoprotein (Schmid, 1953), and thyroglobulin (Tarutani, Kondo and Shulman, 1977). There are several examples of carbohydrate stabilizing protein against denaturation and these experiments are usually done by comparing the stability of an enzyme before and after removal of some or all of its carbohydrate. The sugar of glucose oxidase stabilizes the enzyme against SDS or urea denaturation (Nakamura, Hayashi and Koga, 1976). Yeast β-fructofuranosidase becomes increasingly resistant to heat denaturation in naturally occurring forms containing more carbohydrate (Arnold, 1969). When the glycoenzyme glucoamylase I is subjected to modification of its carbohydrate with periodate, its stability to low temperature storage is reduced (Pazur, Knull and Simpson, 1970). Any of these carbohydrate-dependent properties could be important for proteins in normal environments. This might be especially true for cell surface proteins which are exposed to a less well-controlled environment, where stability to a greater variety of conditions might be required.

GLYCOPROTEINS AND A CONVECTION-FREE ZONE AT THE SURFACES OF CELLS

The aim of the following section is to present an argument that

the primary reason for most of the carbohydrate in the plasma membrane,

including that attached to protein, is to form a protective shell or

net around cells. This region has been termed the greater membrane

(Revel and Ito, 1967). Its physiological role might be related to

that of the perioplasmic space located on the surface of bacterial cells.

According to this hypothesis, many proteins in the plasma membrane

have carbohydrate attached to them simply to create this net or shell.

The normal function of the protein remains unaffected.

The evidence for a zone on the surface of cells is as follows.

Fuzzy coats on the order of 100-200 Å thick are found to some extent

on the surfaces of most cells and are where studied predominatly carbo-

hydrate in composition and often contain anions (Revel and Ito, 1967).

In marked contrast, rarely is there detectable carbohydrate on the

cytoplasmic face of the plasma membrane (Nicolson and Singer, 1974;

Boller, Durr and Wiemken, 1976; Walsh, Barber and Crumpton, 1976).

Consistent with this visible zone, there is considerable functional

evidence that an unstirred water layer exists on the surface of cells

and this may be due in part to a carbohydrate matrix. A series of studies

on the uptake of intestinal contents through intestinal epithelial

cells have identified a region near the surface of the cell, outside

the lipid bilayer, which is often rate-limiting for the uptake of

digestive products. The kinetic effect of this region can be rationalized

mathematically as an unstirred layer through which transport is governed

by diffusion (Thomson and Dietschy, 1977). In another experimental approach, Stoker and his colleagues have disturbed resting.(non-dividing) 3T3 cells by directing a jet of growth medium at the cells or shaking them (Stoker, 1973; Stoker and Piggot, 1974). Either of these methods stimulated the cells to increase their rate of division and this was rationalized as the result of disturbing an unstirred layer at the surfaces of the cells, thus permitting increased fluxes of growth promoters, nutrients and waste substances. Perturbation of the cell surface with an enzyme which released sialic acid also stimulated cell division (Vaheri, Ruoslahti and Nordling, 1972). This stimulation may also be due to a perturbation of a surface layer. Likewise treatment of fibroblasts with proteases in vitro released them from density-dependent inhibition of growth and rendered them more agglutinable with lectins (Burger, 1971). Lectins, which can patch and cap and cause loss of cell surface glycoconjugates and periodate, which can destroy cell surface sugars, stimulated cell division in lymphocytes. These results are consistent with the presence of a glycoprotein-carbohydrate matrix on the surface of cells acting as a barrier to diffusion of key molecules regulating cell division.

Other studies have used serological techniques to identify a privileged region on the surface of lymphoid and glial cells where antigens seem to be hidden. Neuraminidase treatment of these cells significantly increased complement mediated killing or complement fixation on the cell surface in the presence of antibodies against specific cell surface molecules or against purified plasma membranes (Schlesinger and Amos, 1971; Herschman, Breeding and Nedrud, 1972). Since it is unlikely that neuraminidase is creating new antigens recognized by these antibodies,

it seems that formerly cryptic sites have become exposed to the macro-molecular reagents. This is consistent with disturbing a convection free layer.

I speculate that most carbohydrate on the external face of the plasma membrane is linked to protein and that its purpose is to stabilize the plasma membrane against osmotic and mechanical insults, and to provide a microenvironment on the cell surface whose composition of macromolecules and small molecules can be very different from that of the external medium. To do this glycoprotein-carbohydrate must self-associate in some manner. Can carbohydrate, especially glycoprotein-carbohydrate, self-associate to create a three dimensional zone with some mechanical stability?

Polysaccharides such as mannan, agarose, l-carageenan, mucin, glycosaminoglycan, etc. are well-known for their structural and space-filling roles in yeast and algal cell walls and animal extracellular materials. In some cases this structural stability is achieved through double and triple helix interactions between polysaccharide chains; these require no coulombic interactions (Rees, 1975; Morris et al., 1977). In some cases where the polysaccharides are charged, divalent cations can participate resulting in cooperative interactions between chains which can be very stable. In addition, some of these poly-saccharides can bind considerable amounts of water and form a highly porous gel which in marine red algae maintains a special osmotic environment close to the cell and regulates transport of metabolites to and from the cells (Rees, 1962). A gel of hyaluronic acid, a common extracellular matrix element in vertebrates, excludes macromolecules (Laurent, 1964).

The properties of these polysaccharides serve as an introduction
to potential capabilities of glycoprotein oligosaccharides. The basic
differences between these two sugar structures are that the glycoprotein
sugars are more heterogeneous in the same polymer and occur in short
branched chains. Structure stabilizing associations of some kind do
apparently occur, however. An example is gastric mucous gel. The gel
protects the lining of the stomach from the harmful effects of the
stomach fluid. Each molecule results from the tetrameric association
of subunits 5×10^5 in molecular weight. Physical studies suggest that
these glycoproteins exist as fully hydrated extended spheres which
are packed so closely together that the sphere borders overlap. The
close contact of the spheres is apparently related to the high viscosity
of this material. The glycoprotein oligosaccharides contain a complement
of sugars and have a length typical of glycoproteins (Allen, Pain and
Robson, 1977). In a related glycoprotein, ovine submaxillary gland
mucoprotein, terminal sialic acid is important in the intermolecular
interactions which result in the high viscosity of solutions of this
substance. The viscosity can be reversibly decreased by neutralizing
the negative charge of the sialic acid (Gottschalk and de St. Groth,
1960). Can cell surface glycoproteins form structures of this type?

The answer is unknown but it is known that in Halobacterium salinarium
the carbohydrate on the 200,000 dalton plasma membrane glycoprotein is
essential for rod-shaped morphology. This glycoprotein forms an
electron-microscopically visible fuzzy coat on the cell surface. If the cell
surface glycoprotein is digested with proteases (in the intact cell) or
if carbohydrate addition to the protein is prevented by the inhibitor

bacitracin (the protein part still appears normally on the cell surface),

the cells lose their rod-shaped morphology and become spheres (Mescher

and Strominger, 1976). Divalent cations are required for rod morphology

in untreated cells and this may be associated with the glucuronic

acid known to be present in the glycoprotein. The density of the

glycoprotein on the bacterial cell surface is similar to or less than that

on the surface of eukaryotic cells. Although eukaryotic cell surface

glycoproteins may not be involved in preserving cell morphology, the

Halobacterium example suggests that cell surface glycoproteins can

interact through their carbohydrate to carry out an important function.

In one example, large amounts of a single glycoprotein,

epiglycanin, is known to be the basis of a relatively prominent cell

surface zone, or "fuzz," on the surface of certain carcinoma cells

(Miller, Hay and Codington, 1977).

One can readily imagine the importance for cell physiology of a

convection-free zone like that occurring as the periplasmic space in

bacteria. Many cell surface enzymes need cofactors and it would seem

easier for the cell to concentrate these and substrates in a convection

free zone. Extrinsic membrane proteins may be localized in part by

such a zone as this. Furthermore, extrinsic proteins which are glyco-

sylated may be bound through the same carbohydrate-carbohydrate inter-

actions which are postulated to create this zone. Oligosaccharide may

be well suited as the skeleton for a zone due to its ability to take up

water very well (Rees, 1975).

In summary, the argument that general glycoprotein carbohydrate

serves as a structural layer around cells is as follows. 1) There is

ultrastructural evidence for the existence of a layer superficial

to the lipid bilayer and that it is composed of carbohydrate. 2) There

is functional evidence for a convection-free, diffusion-limited, zone

around cells. 3) A large proportion of the cell surface proteins are

glycosylated in one detailed study (Chapters 2 and 3). 4) Glycoproteins

seem to be capable of interacting closely in order to form ordered

structures in solution or on cell surfaces. 5) It is known that carbo-

hydrate linked to protein can carry out functions unrelated to that of

the protein, and need not interfere with the function of the protein.

EVALUATION

Carbohydrate attachment is a post-translational modification of

proteins which often allows for new intermoelcular associations between

proteins so modified and other molecules, such as carbohydrates, proteins,

lipids (Rees, 1975), and possibly even nucleoside hormones (Shida and

Shida, 1976). These new intermolecular associations appear to be vital

for cellular functions such as intercellular adhesion, response to

hormones, serum protein turnover, immunoglobulin functions, hormone

identity, and integrity of cell surface functions. Many other inter-

molecular interactions which are carbohydrate-obligate may be discovered

as they are studied. Many roles for this prosthetic group seem possible

due to the diversity of possible carbohydrate structures.

What relevance does this have for the glycoproteins in D. discoideum?

First, many of the glycoproteins are apparently not membrane

bound (Chapter 4), and thus not all glycoproteins are involved in

membrane structure. The carbohydrate in these non-membrane bound

molecules may be for providing intermolecular interactions, protecting

polypeptides from protelysis (e.g., regulating protein turnover),

or enhancing protein solubility. Possibly intracellular lectins are

involved in the turnover of cell proteins just as hepatic lectins seem

to be involved in serum protein turnover.

In the plasma membrane and elsewhere in the cell, carbohydrate

of glycoproteins may serve as "landing sites" for soluble

molecules coming from other cells or "fastening points" for molecules

situated on adjacent cells. On the basis of the postulated "passivity"

of glycoprotein carbohydrate, it is hypothesized that glycoproteins

which act as receptors would not be responsible for transmitting the

reception event to other molecules of the cell. That would be the

responsibility of the molecule which actually binds to the glycoprotein,

as occurs for cholera toxin when it binds to its receptor. Alternatively,

the carbohydrate could be the "fastening point" for some agent which

relocates the glycoprotein receptor in the plasma membrane, thus

permitting a new function for the protein moiety due to a new environment.

Protein-linked carbohydrate with structures distinct from those

subserving other functions could constitute the framework for a con-

vection-free, diffusion limited zone at the surface of D. discoideum

cells. This could serve as a site for macromolecular synthesis,

metabolism of intercellular signals, or serve as protection against

osmotic shock or mechanical insult. Carbohydrate of some form does

constitute a coat around the lipid bilayer. For example, using classical

carbohydrate stains a layer of carbohydrate is seen to extend 125 $\overset{\circ}{A}$

beyond the lipid bilayer; the stain is denser in the proximal 30 Å

(H. Aldrich, University of Florida, personal communication). Gerisch

and his collaborators have measured the distance from the lipid bilayer

at which antibodies against contact sites A, which are thought to

be glycoproteins, are located, and this is about 150 Å.

The glycoprotein composition of Dictyostelium plasma membranes

is altered considerably as a consequence of development (Chapters 2,

3). The affinity for the majority of Con A receptors to Con A is

reduced 4- to 5-fold compared to vegetative cell surface receptors,

although the number of receptors is similar (Weeks, 1975). If Con

A receptor carbohydrate is generally involved in creating a cell

surface convection-free zone, this suggests a major reorganization

during development. One might expect that requirements of a cell for

a surface zone might be quite different for free-living amoebae and

cells of a multicellular organism.

Many WGA receptors are lost or created during development.

Changes in the cell surface glyco-conjugates are consistent with these

receptors being involved in the different kinds of cell-cell inter-

actions, e.g., hormonal or adhesive, which are involved in development.

There are also several molecular differences between the plasma membranes

of prestalk and prespore cells. These differences are in glyco-

conjugates and occur relatively early in development and may mediate

some cell interactions responsible for appearance or position of pre-

stalk and prespore cells.

The capability of carbohydrate to perform some of these functions

does not seem to be unique, however. Many kinds of intermolecular

associations occur in the absence of carbohydrate, such as polymerization

of sugar-free collagen (Prockop, 1972), tetramerization of hemoglobin

subunits or binding of metabolites to enzymes. The cell certainly

has other tricks for conferring a shared property upon a set of proteins.

For instance, it could be genetically programmed in the amino acid

sequence, such as seems to occur for a hydrophobic tail in the precur-

sors for several secreted proteins (Blobel and Dobberstein, 1975) or in

the lymphocyte system for immunoglobulin constant regions (Gally and Edelman,

1972). Alternatively, two polypeptides could be non-covalently or

covalently joined (Apte and Zipser, 1973), only one of which would

display variability. Different post-translational modifications are

also conceivable, such as aminoacylation (Raybin and Flavin, 1977),

phosphorylation, acetylation, etc. Finally, many proteins exist which

are quite soluble and are stable to proteolysis and other denaturing

forces in biological fluids without containing carbohydrates.

Glycosylation may have an advantage over these other mechanisms

since it can create a sizeable domain (Kirschner and Bisswanger, 1976),

of easily varied structure, requiring a minimum amount of metabolic

energy and genetical modification. Energetic economy is a result of

the absence of an intermediate template for carbohydrate synthesis

like that in protein synthesis. Economy of genetic modification is most

pronounced when a shared oligosaccharide or sugar is attached to each

of a set of distinct proteins. However, some genetic signal is necessary

for sugar attachment since glycosylation is coded at least in part by a

primary amino acid sequence of approximately five amino acids in length,

in the receptor protein. Other advantages of carbohydrate may be that

its distinct chemistry permits independent processing of glycoprotein

carbohydrate without danger of interfering with other chemical systems

such as protein. Carbohydrate is also rather special in the ability

of its polymers to absorb much water in its loose networks. This

may be important in a cell-surface convection-free zone or in situations

where the solubility of a protein or peptide region needs to be enhanced.

The possible roles of carbohydrate linked to so many different

proteins does not seem to be so mysterious now. However, much work

needs to be done to turn these spectulations into facts.

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CHAPTER II

IDENTIFICATION OF CONCANAVALIN A RECEPTORS AND

GALACTOSE-BINDING PROTEINS IN PURIFIED PLASMA

MEMBRANES OF DICTYOSTELIUM DISCOIDEUM

IDENTIFICATION OF CONCANAVALIN A RECEPTORS AND

GALACTOSE-BINDING PROTEINS IN PURIFIED PLASMA

MEMBRANES OF <u>DICTYOSTELIUM DISCOIDEUM</u>

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ABSTRACT

Two techniques have been modified to provide simple means for the
identification of molecules which bind concanavalin A (Con A). Crossed
immunoelectrophoresis was altered by replacing antibody with Con A,
and receptors were identified by the precipitin arcs which they pro-
duced. Con A, tagged with fluorescein isothiocyanate, was also
diffused into prefixed sodium dodecyl sulfate (SDS)-polyacrylamide
gels, and additional receptors identified by fluorescence. More than
35 molecules in the plasma membrane of the cellular slime mold
<u>Dictyostelium discoideum</u> which bind Con A were identified with these
techniques. At least 12 of these diminish and 12 increase in importance
as receptors during differentiation of the cells from the vegetative
to the preculmination stage of development. In the course of these
experiments, it was possible to confirm the presence of the galactose-
binding protein discoidin, in the plasma membrane, by electrophoresing
membrane proteins into an agarose gel. This lectin regains its sugar-
binding activity after denaturation and electrophoresis in SDS.

The molecules of the plasma membrane of the cellular slime mold, Dictyostelium discoideum, are potentially involved in many aspects of development. Considerable evidence indicates that the membrane is involved in cellular adhesion, induction of developmentally coupled enzymes, the production and response to chemotactic signals, and several other areas of development (see reference 16 for review). Therefore, we have examined two particular classes of molecules of the plasma membrane, concanavalin A (Con A) receptors and agarose-binding proteins.

Con A induces a plethora of responses at the cell surface of D. discoideum. Living cells can be agglutinated by Con A and this agglutinability decreases during development so that 15 times more Con A is required to maximally agglutinate preculmination cells as compared to vegetative cells (32,33). Con A postpones the onset of aggregation, but this effect saturates at a much higher concentration of Con A than does agglutination (8,9,32). All of the Con A receptors on the surface of D. discoideum cells patch in the presence of Con A and are translocated to a cap which is distinguished by an accumulation of all the microvilli on the cell surface (9,17). Treatment with Con A results in a fourfold increase in intracellular cAMP phosphodiesterase activity within 30 min (8). This result is especially interesting because a model for cellular position in D. discoideum posited such a regulation of cAMP phosphodiesterase activity by receptors on the plasma membrane (14). Other effects of Con A on D. discoideum are a delay in the appearance of large intramembranous particles (37), and inhibition of the appearance of extracellular cAMP phosphodiesterase inhibitor, and extracellular N-acetyl glucosaminidase activities during development (6,8).

A number of additional effects of Con A have been characterized in other cell types. In addition to its mitogenic properties and its ability to return normal growth inhibition to transformed cells (23), Con A has been found to affect membrane enzyme activities such as 5'-nucleotidase (22,26), Na^+, K^+-stimulated, Mg^{+2}-dependent ATPase (28,21) and glycosyltransferases (20).

Most studies have suggested and even assumed that the number of Con A receptor species is small. For instance, binding studies of radiolabeled Con A suggest that there is only one class of binding sites in vegetative cells from D. discoideum (34). We have determined, however, that there are over 35 different kinds of receptors for Con A in the plasma membrane of D. discoideum.

Another lectin, discoidin, occurs naturally in D. discoideum. This protein, which binds to galactose residues, has been suggested to be involved in adhesion of aggregating D. discoideum (4,24,25). One of the methods used to identify receptors for Con A proved also to be a sensitive assay for agarose-binding proteins and was used to verify the presence of discoidin in the plasma membrane.

Preliminary accounts of a portion of this work have appeared elsewhere (35,36).

MATERIALS AND METHODS

General Methods

D. discoideum, strain A3, was grown axenically (15). Development was induced by plating on filter paper for 12 h (aggregation stage) or 18 h (preculmination stage) as described (15). There are some bio-chemical idfferences between strain A3 cells grown axenically or on

bacteria (13), but cells grown under both regimes have a similar course

of morphogenesis. Plasma membranes were purified by isopycnic centrif-

ugation of cell-free extracts in sucrose and renografin gradients as

described by McMahon et al. (15,18), unless otherwise indicated. This

preparation is normally contaminated to the extent of 5% with mitochon-

drial inner membrane (18). A mixture of discoidins 1 and 2 (prepared

according to reference 7) was the generous gift of D. Lesikar and S.

Barondes (University of California, San Diego, La Jolla, Calif.).

In some experiments, plasma membranes were purified by a modified

method which reduced contamination by inner mitochondrial membrane

although this method gives a lower yield of material. Cells were

broken in a French press with a pressure of 56.4 kg/cm^2 (170 kg/cm^2

for cells in aggregation or preculmination) and a mitochondrial pellet

was obtained according to the methods of Stuchell et al. (26). The

post-mitochondrial supernate was then pelleted at 40,000 g for 30 min

at 2°C, and resuspended in homogenization buffer (18). Plasma membranes

were purified from this fraction in the normal way. At the final

step of purification, when the membranes were collected from the

renografin gradient, care was taken to collect only the upper portion

of the turbid region. This region is white in color. The ratio of

succinate dehydrogenase (12) to 5'-nucleotidase (18) activities was 0.05

of that found in the standard method of purification, suggesting that

the content of mitochondrial inner membrane was reduced to 0.25%.

Following solubilization in boiling sodium dodecyl sulfate (SDS)

and β-mercaptoethanol, membranes (60-260 µg of protein per gel lane) were

electrophoresed in discontinuous polyacrylamide slab gels (8-15% expo-

nential gradient of polyacrylamide) containing 0.1% SDS, modified slightly

(11) from reference 5. Polypeptides were stained with Coomassie blue
(31) and glycolipids and glycoproteins by the periodic acid-Schiff's
base technique (PAS) according to Hoffman and McMahon (11) as modified
from reference 10.

Lectin-Electrophoresis

Lanes, sliced from the slab gel and frozen in SDS electrophoresis
buffer (30), were thawed and lectin-electrophoresed by a procedure
adapted from reference 5. Two glass plates cleaned in Chemsolve
(Mallinkrodt Chemical, St. Lous, Missouri, 63147) were clamped around
three 1.4 mm thick plexiglass spacers, and several molten agarose
solutions were successively poured. For contact with the cathode, a
solution (90°C) of 1.33% (wt/vol) agarose (electrophoresis grade,
Nutritional Biochemicals, Cleveland, Ohio, 44128) dissolved in electro-
phoresis buffer ([EB]:80 mM Tris Base, 40 mM sodium acetate, 100 mM
NaCl, 2 mM $CaCl_2$, to pH 7.4 with glacial acetic acid) was poured to a
depth of 4 cm in the prewarmed (60°C) mold and cooled. In a similar
way, a 0.5 cm layer of 0.01% trypan blue in 1.33% agarose solution was
cast. The third layer was 0.8 cm of a solution (55°C) consisting of
0.15 part of 10% (wt/vol) Lubrol PX (Sigma Chemical, St. Louis, Missouri,
63178) in EB and 0.85 part of 1.33% agarose in EB. For the next layer
(4 cm), the mold was not preheated. The Con A-agarose solution (1.2 ml
of 0.004% [wt/vol] Con A and 0.012% [wt/vol] NaCl in EB at 25°C mixed
quickly with 4.8 ml of 1.33% agarose in EB at 50°C) was poured with a
warm pipette immediately after it was mixed. Finally, the mold was
filled (5 cm) with a 1.33% agarose solution in EB to contact the anode.

After cooling, the plates were separated, exposing one face of the gel.
A strip of agarose equal in width to a lane of the SDS gel was carved
from the gel leaving only a narrow strip of the layer containing the
trypan blue abutting the Lubrol layer. The SDS gel lane was inserted,
moved into contact, and the agarose gel extending beyond the ends of
the polyacrylamide gel was trimmed away. The agarose gel was covered
with a square of cellophane except at the points of contact with the
wick. The buffer reservoirs contained EB. Electrophoresis was performed
in the cold ($4°C$) at a potential of 7-10 v/cm for 2-4 h. The gels were
washed in two changes of 0.075 M NaCl for 15 h to remove nonprecipitated
proteins, dried underneath a sheet of Whatman No. 1 paper in a $55°C$
incubator, and then stained with Coomassie blue for 5 min and destained
(31). When hapten sugars were present, they were dissolved in the
Lubrol and Con A solutions before dilution with agarose. The concentra-
tions given are those after dilution. Conclusions were based on at
least three independent experiments.

Lectin-Diffusion

SDS polyacrylamide gels were prepared as above, sliced into lanes,
then fixed with methanol, glutaraldehyde, and sodium borohydride (29).
For labeling, gel lanes were rocked for three days in a small plastic
tray containing 16 ml of a solution composed of 0.1 M NaCl, 0.033 M
sodium phosphate, pH 8.0, 0.2% bovine hemoglobin, 0.05% sodium azide,
0.4-0.6 mg Con A conjugated with fluorescein isothiocyanate per gel
(Miles Laboratories, Elkhart, Indiana or Vector Laboratories, Ignacio,
California; $OD_{495}:OD_{280} = 0.95$) in the presence or absence of 0.25 M
α-methyl-D-mannopyranoside (αMM). The gel was washed for 2 days in two

changes of 200 ml per lane of a solution containing 0.1 M NaCl, 0.033 M

sodium phosphate, pH 8.0, 0.05% sodium azide, and 0.25 M αMM if appro-

priate. Gels illuminated from below with a short wavelength ultraviolet

light box were photographed for 5-10 min with a Wratten filter No. 65.

Conclusions were based on two or three independent experiments.

Some gels were postfixed overnight with 6.5% (vol/vol) 2-amino-

ethanol in place of the NaBH$_4$, or, alternatively, not fixed and postfixed

at all but labeled with Con A immediately following a 1/2 h incubation

in 25% isopropanol. The pattern of Con A bands was essentially the

same, but in the absence of fixation there was some loss of resolution.

Molecular weight positions were calibrated in the lectin diffusion

gels by poststaining with Coomassie blue and, in the case of lectin-

electrophoresis, by staining nearby lanes of the same SDS polyacrylamide

gel with Coomassie blue.

RESULTS

Identification of Con A Receptors by Crossed Lectin-Electrophoresis

Con A receptors were identified in two ways after prior separation

in SDS polyacrylamide gels. The first technique was a modified form

of crossed immunoelectrophoresis (5) substituting a lectin for anti-

body. The feasibility of the system was explored with a glycoprotein

which contains mannose, avidin, and a nonglycosylated protein, cyto-

chrome c. In Fig. 1, avidin is located to the right of cytochrome c

in the SDS polyacrylamide gel. After electrophoresis in the second

dimension gel, which contained Con A, a precipitate (stained with

Coomassie blue) was produced by avidin but not by cytochrome c. The

Figure 1. Crossed lectin-electrophoresis of avidin and ctyochrome

c.

2 μg each of avidin (on right), a glycoprotein containing

mannose, and cytochrome c (on left), a non-glycosylated protein,

were electrophoresed on SDS polyacrylamide gels and then electro-

phoresed into an agarose gel containing, successively, Lubrol

and Con A. A composite picture containing a duplicate of the

original polyacrylamide gel (bottom) and the agarose gel (top),

each stained with Coomassie Blue, is presented.
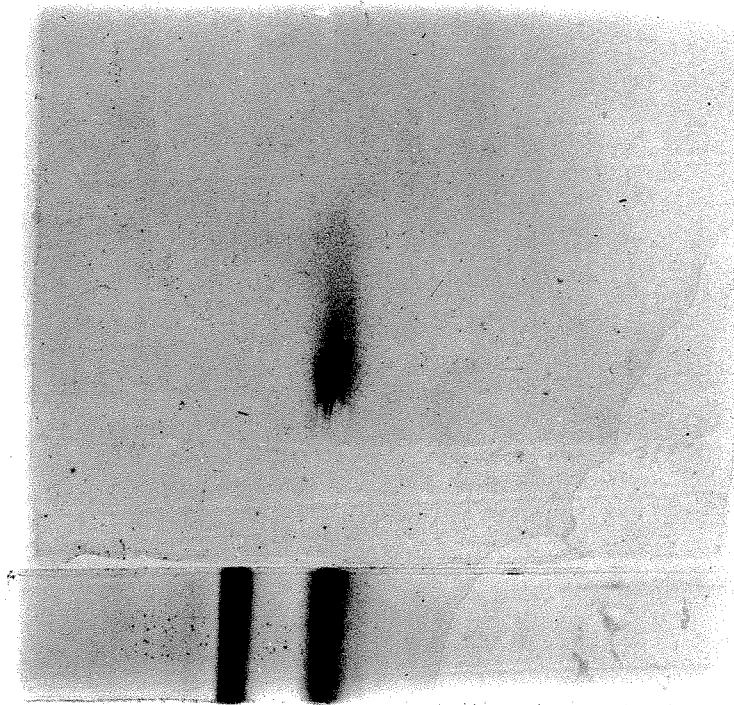
precipitation was reduced in the presence of 0.1 αMM and not present

in the absence of Con A (not shown). It is probable that only multi-

valent Con A receptors would form precipitates with this method. Such

receptors might contain a branched polysaccharide, multiple polysac-

charides attached to a single polypeptide, or aggregates of polypeptides

each linked to a minimum of a single polysaccharide.

This method was used to analyze plasma membranes isolated from

vegetative cells and resolved by SDS polyacrylamide gel electrophoresis.

The gels in Figs. 2a and 2b illustrate the molecular weight profile of

proteins and glycoproteins, respectively, as they were stained with

Coomassie blue and PAS. Fig. 2c shows the stained profile of precipi-

tation arcs which developed when 60 μg of plasma membrane protein,

resolved on an SDS gel, was lectin-electrophoresed. Only the part of

the gel which contained Con A is shown. At least six receptors with

apparent molecular weights of 124,000, 87,000, 76,000, 68,000, 58,000,

and 42,000 daltons are visible. Several other relatively minor arcs

indicate that there are potentially more Con A receptors.

We examined the specificity of the reaction in several ways. The

precipitin arc reactions depended on Con A since they were absent when

Con A was replaced by the same concentration of bovine serum albumin

(Fig. 2g). Furthermore, the reactions were the result of Con A's sugar-

specific binding properties since, when the hapten sugar αMM (0.15 M) was

present in Lubrol and Con A layers of the gel, the arcs did not form

(Fig. 2e). On the other hand, the same or higher concentration of

L-fucose (not shown) or D-fucose (Fig. 2f) did not affect the pattern

of arcs. However, a spot of adsorbed protein, of molecular weight

Figure 2. Components of the plasma membrane (60 μg protein) of

D. discoideum.

(a) Polypeptides from vegetative stage plasma membranes

resolved on an SDS polyacrylamide gel and stained with Coomassie

blue. (b) Glycoproteins and glycolipids on an equivalent gel

stained with PAS. (c-g) Crossed lectin-electrophoresis of the

components of the SDS polyacrylamide gel as given in the legend

to Fig. 1. The Con A-containing portion of the gel is shown.

(c) Plasma membranes from vegetative amoebae were in the polyacryl-

amide gel and Con A (40 μg) was in the agarose gel. (d) Plasma

membranes from cells in the preculmination stage of development

in the polyacrylamide gel were electrophoresed into agarose

containing Con A (40 μg protein). In this case, part of the

Lubrol gel is shown. The bar marks the bottom of the Con A part

of the gel. (e) Plasma membranes from vegetative cells were

electrophoresed into an agarose gel containing Con A (40 μg) and

0.15 M αMM. (f) As in (e) except that αMM was replaced with

0.5 M D-fucose. (g) As in (c), but Con A was replaced by bovine

serum albumin (40 μg). The arrows mark components with apparent

molecular weights of 124,000, 58,000 and 27,000 daltons, from

left to right, respectively.

a

b

c

d

e

313 215 160 124 87 68 58 48 42 35 27 21 15

MOLECULAR WEIGHT × 10⁻³
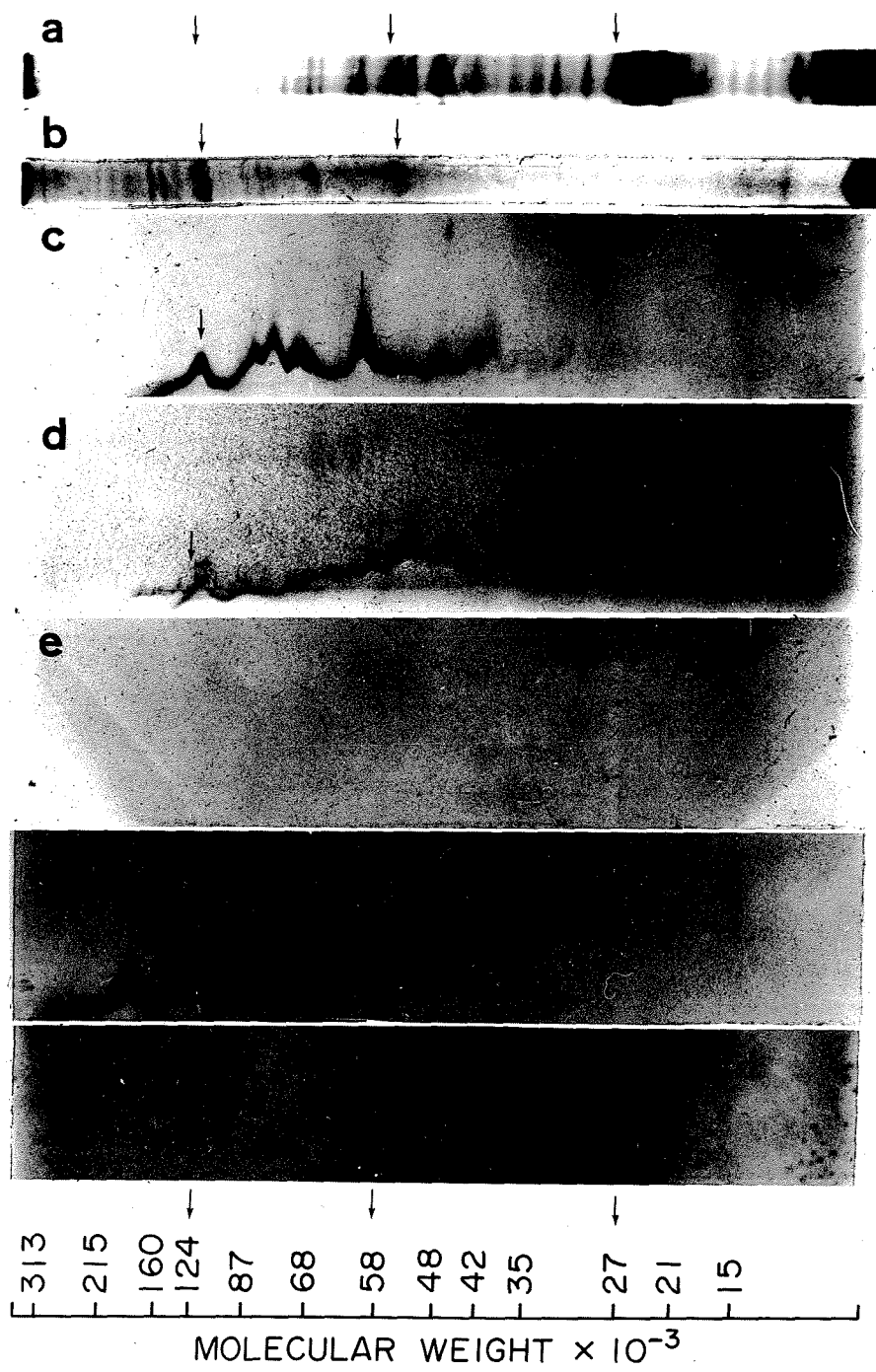
27,000 daltons, was not removed by αMM or L-fucose, and was present
when the Con A was replaced by bovine serum albumin. This will be
discussed later.

At this relatively low concentration of membrane protein, the
major Con A receptors comigrated with several major glycoproteins in
the SDS gel (compare Figs. 2b and 2c). It appears that the majority
of glycoproteins revealed by the PAS method are Con A receptors.

It was of interest to determine whether there were any changes
in the Con A receptors when cells had differentiated. When similar
concentrations of preculmination stage plasma membranes were studied,
the precipitin pattern was consistently very weak and difficult to
interpret (Fig. 2d). Higher concentrations of protein gave clear
patterns.

Crossed lectin-electrophoretic analysis of higher concentrations
(190 μg protein) of plasma membranes from vegetative cells showed
several additional Con A receptors (Fig. 3a). Examination of precul-
mination stage plasma membranes with this higher concentration also
showed a complex set of Con A receptors (Fig. 3c). The majority of the
receptors of the two developmental stages have similar molecular weights,
but some are clearly different. It appears that the receptor at 58,000
daltons decreased or disappeared from the plasma membrane as develop-
ment progressed from the vegetative to the preculmination stage, while
a new one with an apparent molecular weight of 115,000 daltons replaced
with an apparent molecular weight of 124,000 daltons. In pseudo-
plasmodial plasma membranes, new precipitin arcs were produced by
receptors whose apparent molecular weights were 63,000 and 48,000 daltons.

Figure 3. Alteration of Con A receptors during development.

The components of the SDS polyacrylamide gel were electro-
phoresed into agarose gels containing Con A (40 μg). (a) Vege-
tative stage plasma membranes (190 μg protein). (b) Vegetative
stage plasma membranes (190 μg protein) plus preculmination
stage plasma membrane (190 μg protein). (c) Preculmination
stage plasma membranes (190 μg protein). In the four panels on
the bottom, fluorescent Con A was allowed to diffuse into
prefixed SDS polyacrylamide gels. (d) Vegetative plasma
membranes (260 μg protein). (e) Preculmination stage plasma
membranes (260 μg protein). (f) As in (d) plus 0.25 M αMM.
(g) As in (e) plus 0.25 M αMM. Arrows are described in legend
to Fig. 2.

MOLECULAR WEIGHT × 10⁻³
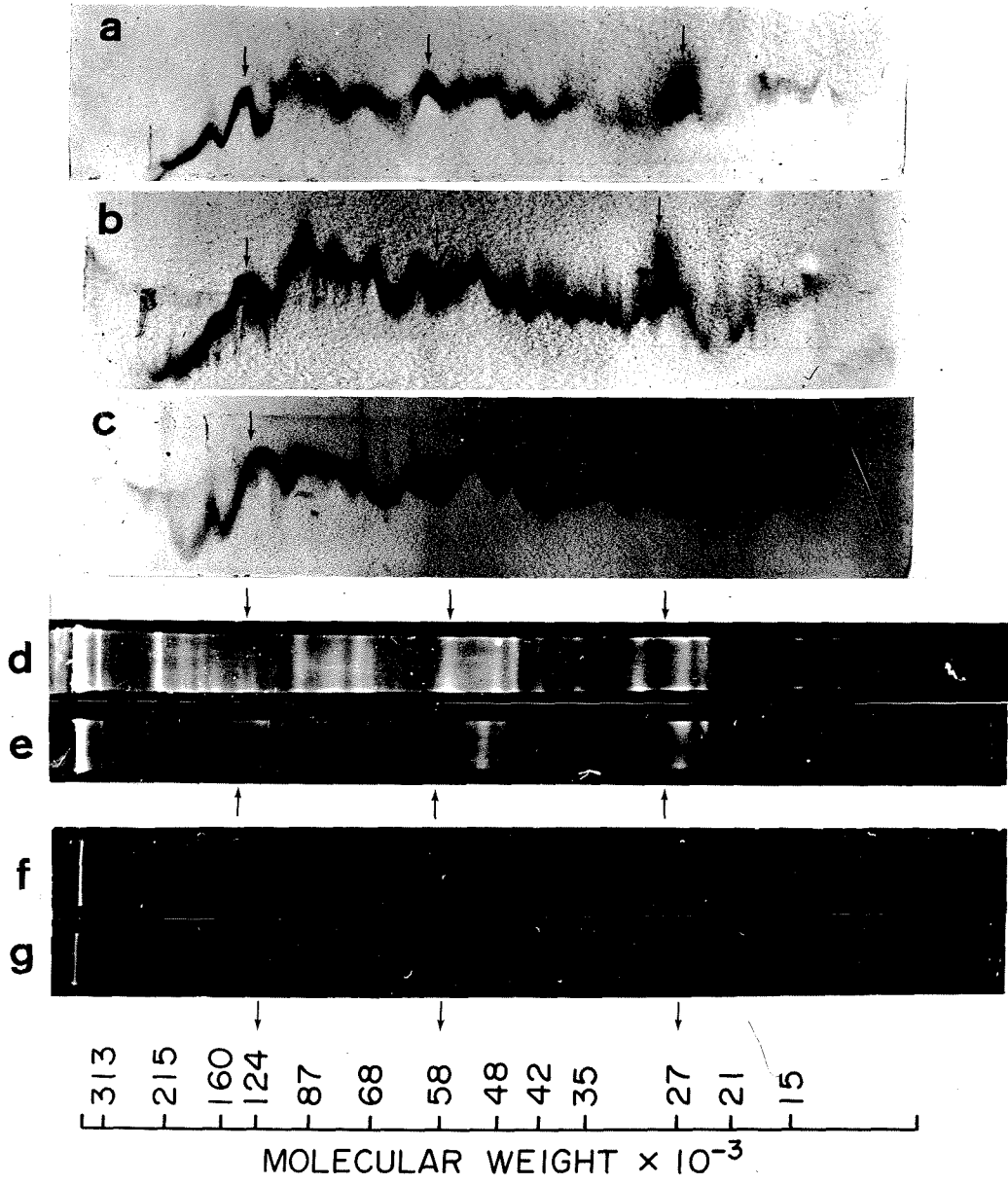
A comparison of membranes from the two developmental stages was least
ambiguous when vegetative and preculmination plasma membranes were
co-electrophoresed together in the SDS gel and then lectin-electropho-
resed (Fig. 3b). The exchange of the 124,000-dalton receptor for
one of 115,000 daltons is supported by the presence of two superimposed
arcs in the mixture. Furthermore, a complex set of changes occurred
in the molecular weight region between 58,000 and 124,000 daltons.

In summary, crossed lectin-electrophoresis with Con A showed a
complicated pattern of receptors between 30,000 and 160,000 daltons.
Below 30,000, the pattern of arcs was ill-defined and detection of
potential receptors above 160,000 daltons was hindered because they
were trapped in the polyacrylamide gel, although this could be overcome
by electrophoresis for longer times (data not presented).

Identification of Con A Receptors by Lectin-Diffusion

Another technique for detecting Con A receptors was adapted in
order to complement the findings of crossed lectin-electrophoresis.
This procedure, modified from that used by Tanner and Anstee (29), used
glycoproteins resolved on SDS polyacrylamide gels and then fixed. After
washing, fluorescein isothiocyanate-conjugated Con A (FITC-Con A) was
diffused into the gel. Fig. 3d illustrates the bands which stain with
FITC-Con A. The presence of 0.25 M αMM prevents labeling (Fig. 3f).
The residual apparent labeling with αMM present is visible even when
the Con a is not added and is presumably due to autofluorescence.
Preculmination stage plasma membranes examined in a similar way are
presented in Figs. 3e and 3g.

These Con A receptors were detected in a plasma membrane preparation
which contained about a 5% contamination with inner mitochondrial
membrane.   In order to determine whether the Con A receptors came
from the plasma membrane, we studied plasma membranes purified by a
modified procedure which contained mitochondrial contamination reduced
by twentyfold.

The overall qualitative fluorescence intensity produced by labeling
these plasma membrane glycoproteins with FITC-Con A (Fig. 4b) was
similar to that seen before with less purified plasma membranes (Fig.
3d).   The greater amount of fluorescence intensity associated with
vegetative stage plasma membrane glycoproteins compared with that from
pseduoplasmodial plasma membrane glycoproteins was also preserved in
the more purified plasma membranes.   Most of the receptors were found
in both preparations, but there were distinctive differences as well.
It was concluded that those bands which were reduced in amount in Fig.
4 either do not reside in the plasma membrane or reside in a portion
of the plasma membrane which does not co-purify with the marker enzyme
5'-nucleotidase.   In vegetative stage plasma membranes, there were five
differences, including the pair of Con A receptors migrating at 48,000
daltons and another band at about 215,000 (Fig. 3d).   The pair of major
bands migrating near 24,000 daltons were relatively diminished in inten-
sity of fluorescence and staining by Coomassie blue (compare Fig. 4a and
Fig. 2a).

The major conclusions from crossed lectin-electrophoresis were
corroborated by the results from lectin-diffusion shown in Fig. 4.   In
both cases, the pattern of Con A receptors was very complex.   The changes

Figure 4. Con A receptors in highly purified plasma membranes.

100 µg of plasma membrane protein per gel lane were electro-
phoresed into polyacrylamide gels in the presence of SDS and 2-
mercaptoethanol. After fixation in methanol, glutaraldehyde and
$NaBH_4$, the gels were incubated in FITC-Con A followed by washing
in buffer. After photography of fluorescent staining, the gel
was stained with Coomassie blue and destained. (a) Gel shown
in part b stained with Coomassie blue. (b) Gel stained with
FITC-Con A. (c) Gel stained with FITC-Con A and washed, both
in the presence of 0.25 M αMM. V, A, and P refer to plasma
membranes isolated from cells in the vegetative, aggregation and
preculmination stages of development, respectively. The irregu-
lar band with a molecular weight of approximately 30,000 daltons
is an artifact.

MOLECULAR WEIGHT x 10⁻³

313
215
160
124
87
68
58
48
42
35
27
21
15

A   B   C
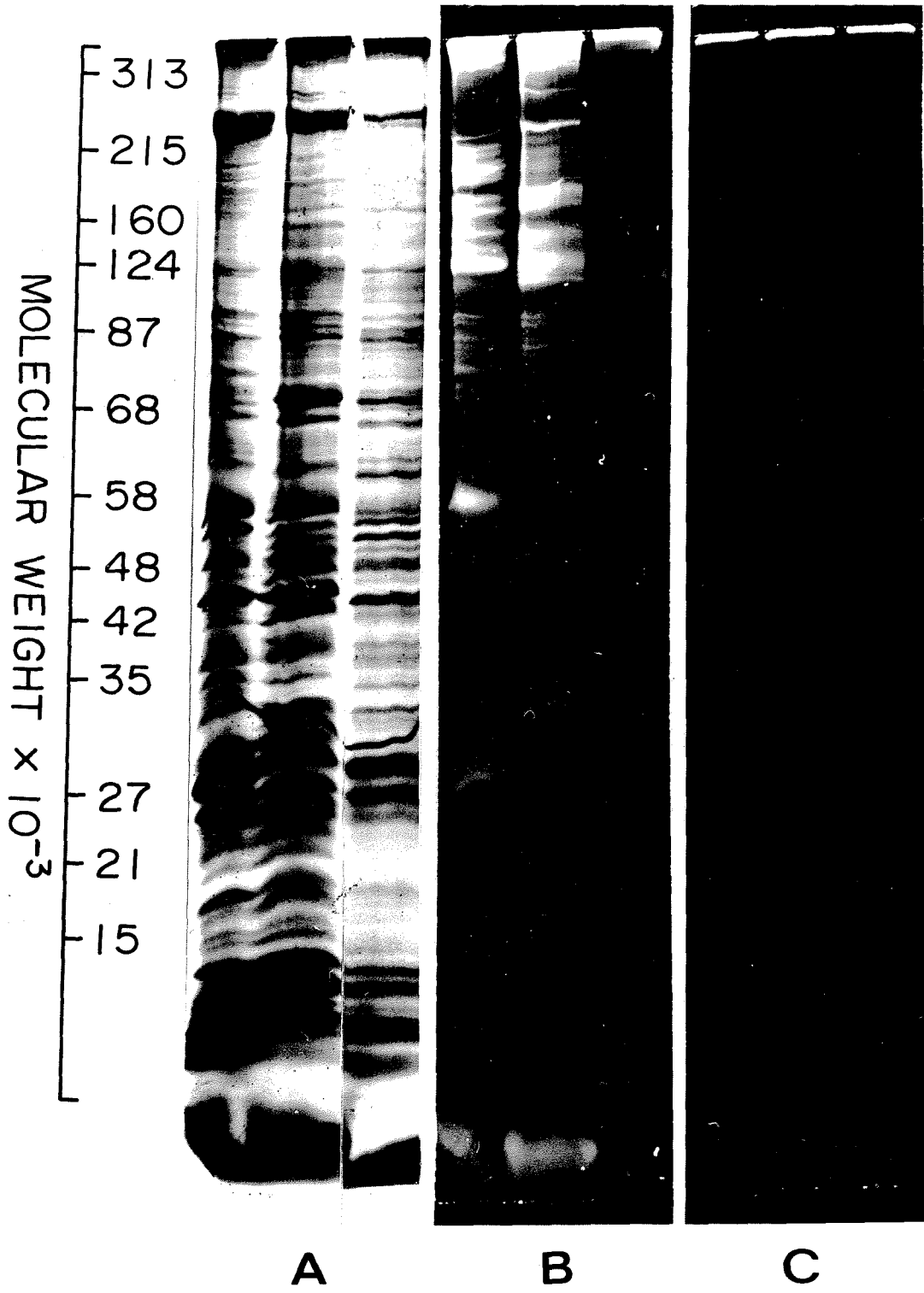
described to take place during the course of development were also

observed in lectin-diffusion. However, the lectin-diffusion technique

was more sensitive and had greater resolution, and this permitted a

number of additional receptors to be identified (Fig. 4b). A list of

Con A receptors in the vegetative stage plasma membrane is given in

Table I. There are at least 8 additional unlisted minor bands which do

not photograph well. Many of the Con A receptors from aggregation and

preculmination stage plasma membranes were similar on the basis of their

comigration with vegetative stage receptors (Fig. 4b). There were

differences as well, and these are summarized in Table I.

Identification of Discoidin

As described above, a protein with a molecular weight of 27,000

was detected by staining in the agarose gels after crossed lectin-

electrophoresis (Figs. 2e and 2g). This is discoidin, a galactose-

binding protein, known to exist on the surface of D. discoideum (4,25).

The identification of this spot was established by the following

observations: The protein remained bound to the agarose gel when Con

A was replaced by bovine serum albumin (Fig. 2g); discoidin binds to

galactose and would be expected to bind to agarose; the presence of

0.5 M D-fucose (a hapten of discoidin) in the gel substantially reduced

the amount of protein which was bound to the gel (Fig. 2f); L-fucose

(not shown) and αMM (Fig. 2e), sugars which are not haptens for discoidin,

did not have this effect; the molecular weight of the bound protein was

identical, within experimental error, to the molecular weight of

discoidin (26,000) (7,18); this protein was destroyed when

TABLE I

*Apparent Molecular Weights of Receptors for Concanavalin A in the Plasma Membrane of D. discoideum*

Apparent molecular weight ($\times 10^{-3}$)[*]	Stage of development		
	Vegetative	Aggregation	Preculmination
313	+ +‡	+ +	0
260	0	+ +	0
250	0	+	0
213	0	+ +	+
210	+ +	+ +	+
205	+ +	+ +	+
200	+ +	+	+
175	+ +	+ +	+
165	+ +	+ +	+
145	+ +	+ +	+
128	+	+	+
124	+ + +	+ +	+
115	+	+ +	+ + +
93	0	+	+
91	+	+	+
87	+	+	+
84	+	+	0
82	+	+	+
80	+	+	+
76	+ +	+	+
73	+	+ +	+
71	+	+	+
70	0	+	0
68	+	+	+
65	+	+ +	+
63	0	+	+
61	0	+	0
58	+ + +	+	+
55	0	+ +	+
52	0	0	+
48	+	+ +	+ +
42	+ +	+	+
39	+ +	+ +	+
37	+ +	+	0
32	+ +	+	0
30	+ +	+ + +	+ +
19	+	+	+ +
15	+ +	+ +	+ +
12	+ +	0	0
11	+ +	0	0
8	+ +	+ +	+ +

* Molecular weights calibrated with thyroglobulin (335,000 daltons), myosin (220,000 daltons), β-galactosidase (130,000 daltons), phosphorylase A (92,000 daltons), human serum albumin (68,000 daltons), immunoglobulin G (50,000 and 23,000 daltons), avidin (16,000 daltons) and cytochrome c (11,800 daltons).

‡ Relative fluorescence intensity of labeling, on a scale ranging from 0, not detectable, to + + +, a relatively large amount of fluorescence.

Figure 5. Detection of discoidin by electrophoresis into an
agarose gel.

Seventeen micrograms of a mixture of discoidins I and II
were subjected to SDS gel electrophoresis and then electro-
phoresed into an agarose gel, as described in Materials and
Methods, in the absence of Con A.
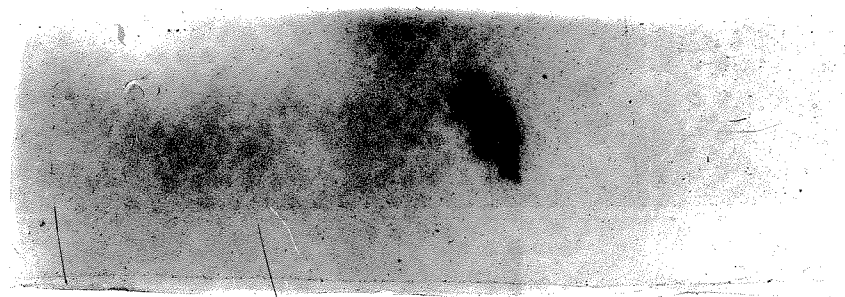
intact cells were treated with pronase (11), agreeing with the presence
of discoidin on the surface of the cell; and, finally, an authentic
sample of discoidin (7) behaved in a similar manner (Fig. 5). Recently,
a second galactose-binding protein (called discoidin II) which co-
purifies with discoidin and has a slightly lower molecular weight has
been resolved (7; see also Fig. 5). A second agarose-binding protein
at a position corresponding to the molecular weight of discoidin II
also appeared intermittently in preculmination stage plasma membranes
(Fig. 2d), and this may be discoidin II. Therefore, discoidin can
recover its native specificity after being denatured in boiling SDS
and β-mercaptoethanol. This procedure is a sensitive assay for the
presence of galactose-binding proteins.

DISCUSSION

As discussed in the introduction, Con A has a variety of bio-
chemical effects on plasma membrane-associated activities in many kinds
of cells. Because several effects of Con A on D. discoideum disrupt the
normal program of development, considerable effort has been devoted to
analyzing the cellular and biochemical properties of the molecules which
bind Con A. These studies produced a picture which suggested that a
relatively homogeneous class of Con A receptors, perhaps as few as one
to two molecular species, was present on the surface of D. discoideum
and also that some property(ies) of the receptor(s) changed during
development which resulted in lowered affinity for Con A (6,34).

The techniques used in this paper indicate that a minimum of 35
glycoproteins are Con A receptors in purified plasma membranes of D.
discoideum. This number is much greater than that determined for two

other types of cells, neurons and lymphocytes, which have seven to nine

Con A receptors as determined by affinity chromatography (1,38). This

may be due to a higher sensitivity to techniques presented here. Our

techniques would detect receptors regardless of whether they were

found on the exterior or interior face of the plasma membrane. Some

of the Con A receptors are located on the external face of the plasma

membrane (9,17), but it is not known whether all receptors have this

orientation. While all Con A receptors seem to be localized on the

external rather than the internal face of mammalian (19) and yeast (2)

plasma membranes, in an amoeba one is found on the cytoplasmic face (3).

Nevertheless, there is no reason to assume that the diverse effects of

Con A on D. discoideum are mediated by a single receptor.

The Con A-induced agglutinability of preculmination stage cells has

been shown to be reduced when compared to vegetative cells (32,33).

Changes in receptor mobility (17) or number of receptors (34) do not

appear to be responsible for this altered agglutinability. Weeks' findings

(1973) that the affinity of the majority of Con A Receptors in de-

creased in preculmination stage cells is, however, a potential explanation

for the altered agglutinability. The changes observed by Weeks could be

due to modification of preexisting receptors, alterations of their

environment, or replacement of receptors. Our results indicate that

many vegetative receptors are removed, and that new ones appear during

the course of development (Fig. 4b). In addition, many of the receptors

which seem to remain, during development, bind less fluorescent Con A

(Fig. 4b). This is consistent with a lowered affinity of receptors on

the surface of preculmination cells.

Comparison of the results of lectin-electrophoresis and lectin-diffusion allows inferences to be made regarding structural features of the Con A receptors. Lectin-electrophoresis would probably detect only those receptors that have multiple binding sites which are sterically independent or those which aggregate when they migrate from the SDS gel. Many of the receptors fit into this category (Figs. 3a and 3b). In contrast, lectin-diffusion should detect receptors of any valency. Comparison of the results of lectin-electrophoresis with those of lectin-diffusion suggests that the receptors migrating below 20,000 daltons are univalent.

The techniques discussed here will greatly facilitate the detection and identification of lectin receptors separated on SDS polyacrylamide gels. The lectin-diffusion technique possesses three important advantages over other techniques for identifying receptors. It requires only small amounts of material and is extremely convenient. Second, its resolution is equal to that of the electrophoresis system which is used. Finally, the method results in a profile of receptors which are stained in proportion to their lectin-binding, unlike other methods which may depend on the exposure of a receptor on the membrane, its mass, or its amino acid composition. Electrophoresis in a second dimension into agarose gels was also a sensitive assay for galactose-binding proteins, as shown with discoidin. This technique could potentially be expanded to identify proteins of many types by adding or coupling different ligands to the agarose. Likewise, other kinds of receptors could potentially be identified in prefixed SDS polyacrylamide gels by infiltration with fluorescein-tagged or radiolabeled ligands.

ADDENDUM

After the submission of this work for publication, Geltosky

et al. (Geltosky, J. E., C.-H. Siu, and R. A. Lerner, 1976. Cell.

8:391-396) published a paper describing Con A receptors on the D.

discoideum cell surface, using an alternative method. In the part

of the paper relevant to our work, whole cells were iodinated with

^{125}I and lactoperoxidase, solubilized with NP-40, and applied to a

Con A affinity column; material was eluted with α-methyl mannoside

and electrophoresed in an SDS-polyacrylamide gel which was examined

with autoradiography. They observed about 15 cell surface Con A

receptor species and suggested that there were two changes during

development: one with an apparent molecular weight of 180,000 which

decreased in amount or affinity to Con A, and another with an apparent

molecular weight of 150,000 which increased. This pair may correspond

to the two glycoproteins, with apparent molecular weights of 124,000

and 115,000, found in our work.

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APPENDIX TO CHAPTER 2

Several studies have indicated that concanavalin A (Con A) can self-associate to form turbid solutions or can associate with other proteins in the absence of specific binding to carbohydrate. Furthermore, there was evidence the self-association could be blocked with specific sugars and that the interactions were hydrophobic in nature. Conditions were found by these authors which prevented these non-carbohydrate specific interactions and thus it was possible to perform experiments to rule out these interactions using the lectin diffusion technique.

Background

Sawyer and his colleagues found that Con A, in buffers of pH 7.0 and ionic strength <0.1, or in buffers at pH 8.0 or higher and any ionic strength, formed turbid solutions (McKenzie, Sawyer and Nichol, 1972; Sawyer, 1975). There was indirect evidence that formation of turbidity was due to hydrophobic interactions between self-associating molecules. This raised the possibility that association could occur between Con A and a hydrophobic peptide region from any protein. The formation of turbidity was inhibited or blocked by specific hapten inhibitors to Con A, but not by sugars which did not bind to Con A. McKenzie et al. (1972) found that this self-association did not occur at pH 5.6 or at pH 7.0 and an ionic strength of 0.3 or greater.

Davey et al. (1976) found that human fibroblast interferon bound to Con A linked to agarose, but that it could not be eluted with a hapten-inhibitor to Con A. Release of Con A could only be achieved in

in the presence of chaotropic agents, such as 10% ethylene glycol,

dioxane, 1,2-propanediol or tetraethylammonium chloride, in addition

to the hapten sugar. The requirement for chaotropic agents could

be avoided at lower pH's.

Crystallographic (Becker et al., 1975) and other studies (Porter

and Goldstein, 1971) have provided the evidence for the existence of

a hydrophobic binding site on Con A which could be blocked or modified

by binding of a hapten inhibitor.

Results and Discussion

The Con A binding studies described in Chapter 2 were performed

at pH 8.0 at an ionic strength (I) of 0.167, where binding as described

above could conceivably have occurred. The experiments were repeated

on vegetative stage plasma membranes in $NaCl/NaPO_4$ buffers at pH 5.6,

I = 0.18; pH 7.0, I = 0.44; and pH 7.0, I = 0.44 in the presence of

10% (v/v) ethylene glycol. For 12 hours prior to photography, the gels

were incubated in buffer pH 8.0, I = 0.167, in order to maximize

fluorescein fluorescence.

Binding of FITC-Con A to gels was qualitatively similar under all

of these conditions (Fig. 1). The fluoresent intensity was somewhat

reduced for some Con A receptors at pH 5.6 (Fig. 1c) or in the presence

of ethylene glycol (Fig. 1d), but in each case the binding was still

blocked by the hapten inhibitor α-methyl mannoside. Reduced binding

was expected due to lower binding affinity of Con A to saccharides under

these conditions.

In conclusion, it is unlikely that the hydrophobic interactions

or other mechanisms which cause the artifactual self-precipitation of

Con A described above is responsible for Con A labeling in gels, because conditions found by several authors to abolish these artifactual interactions did not prevent hapten inhibitable binding to the membrane receptors. Con A binds to its receptors because of the carbohydrate they contain.

<u>Figure 1.</u> Analysis of Con A receptors in plasma membranes from vegetative cells of <u>Dictyostelium discoideum</u>.

Plasma membranes were isolated according to a modified procedure (Chapter 2) and 73 µg of protein per gel lane (1.2 x 5.5 mm) were electrophoresed in discontinuous exponential gradient polyacrylamide slab gels in the presence of 0.1% SDS according to Chapter 2. Following fixation, the gels were labeled with fluorescein conjugated-Con A in the absence (left-hand gel in each panel) or the presence (right-hand gel in each panel) of the hapten inhibitor α-methyl-mannoside (0.25 M). The first stage of washing was also done in the presence of the hapten inhibitor where appropriate. a) Incubation and wash were performed in the standard $NaCl/NaPO_4$ buffer, pH 8.0, ionic strength (I) = 0.167, described in Chapter 2. b) Incubation and wash in a modified $NaCl/NaPO_4$ buffer, pH 7.0, I = 0.44. c) Incubation and wash in a modified $NaCl/NaPO_4$ buffer, pH 5.6, I = 0.18. d) Incubation and wash in a modified $NaCl/NaPO_4$ buffer, pH 7.0, I = 0.44, supplemented with ethylene glycol, 10% (v/v). After 2.0 days of wash, gels were washed for a final 0.5 days in buffer used in (a). The modified appearance of the lower molecular weight region in the left-hand gel of (c) is different due to an artifact of electrophoresis, as determined by post-staining with Coomassie blue.

A   B   C   D

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CHAPTER III

Identification of Glycoproteins and Analysis of Their

Structure Using Lectins as Probes, in Plasma Membranes from

Dictyostelium discoideum and Human Erythrocytes[1]

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SUMMARY

The glycoproteins of plasma membranes from axenically grown Dictyostelium discoideum and human red blood cells (O^+) were characterized according to their apparent molecular weights in SDS-polyacrylamide gels and the identity of monosaccharides at their non-reducing oligosaccharide termini. This was achieved by diffusing each of several fluorescein-conjugated lectins into SDS-polyacrylamide gels which contained the purified plasma membranes. Subsequent to identifying fluorescent bands the gels were stained with Coomassie blue to relate the lectin receptors to known proteins. In D. discoideum plasma membranes over 25 macromolecules, ranging in apparent molecular weight from 8000 to 95,000, were identified with fluorescent wheat germ agglutinin. The binding of wheat germ agglutinin was specific and reflected the presence of receptors containing N-acetyl-D-glucosamine. Some of these receptors are distinct from those which bind concanavalin A [West, C. M. & McMahon, D. (1977) J. Cell Biol. in press]. The concanavalin A receptors and all but one of the wheat germ agglutinin receptors were shown to be glycoproteins since they were hydrolyzed by proteolytic treatment. In contrast, Ricinus communis agglutinins 60 and 120, soybean agglutinin and Ulex europeus agglutinin I, lectins with specificities directed toward the other common simple sugars of the plasma membrane, failed to label any glycoproteins in the SDS-polyacrylamide gel. The absence of glycoproteins recognized by the R. communis agglutinins on the plasma membrane was confirmed by conjugating a mixture of the two lectins labeled with fluorescein-isothiocyanate and microspheres, and assaying for binding to cells in a fluorescent or scanning electron microscope. Consequently, D. discoideum plasma membrane glycoproteins seem to be restricted to a class which binds concanavalin A and another which binds wheat germ agglutinin. This pattern persisted during the course of development, although within each class of glycoproteins there were many developmental changes.

In comparative experiments, the glycoproteins of the human erythrocyte ghost were also identified, using lectins with specificities against each of the preponderant simple sugars of the ghost. In contrast to D. discoideum, there were glycoproteins capable of binding, specifically, all of the lectins tested. In most cases glycoproteins had been known from previous experiments, but a few new glycoproteins were identified as well.

The cell surface of the cellular slime mold <u>Dictyostelium discoideum</u> is becoming described in increasing detail with respect to its structure, function and role in development (for review, see ref. 1). There are over sixty polypeptides which have been identified in the isolated plasma membrane (2-5); at least fourteen of these are located on its external surface (2,3). More than forty of the plasma membrane molecules are glycoproteins which bind Con A[2] (6-8); some of these had previously escaped detection as proteins or glycoproteins (2) using conventional staining procedures. Con A binding proteins total more than 1×10^7 per cell surface (8-10,14,15). A significant fraction of the proteins and glycoproteins are modified or replaced during the course of development (2,3,5-7) and have been shown to be under developmental control[3].

Glycoproteins have been suggested to play an important role in plasma membrane function in <u>D</u>. <u>discoideum</u>. For example, an antigen(s) (contact sites A) in the plasma membrane is known which if blocked with the Fab fragment of an appropriate antibody will result in a loss of adhesion between aggregation stage cells. This antigen is carbohydrate in nature (11, 55). Isolated plasma membranes induce a change in activity of enzymes in whole cells (4,12) and this effect can be prevented by pre-treatment of the membranes with periodate, under conditions which are relatively specific for destruction of carbohydrate (4; D. McMahon, unpublished results). An enzyme on the cell surface, acid phosphatase, is known to be a glycoprotein which binds Con A (13). Con A (16,17) and WGA (C. West, unpublished results), lectins which bind to specific classes of glycoproteins (18), interfere with the normal course of <u>D</u>. <u>discoideum</u> development. Finally, a D-galactose (or N-acetyl-D-glactosamine) specific lectin, discoidin, has been identified on the cell surface (19,20) and in purified plasma membranes (7) and has been postulated to play a role

in cell adhesion (21,40). The validity of this hypothesis is contigent upon
the presence of receptor-glycoconjugates, possibly glycoproteins, in the plasma
membrane.

In this paper we have continued our study of the glycoprotein structure of
the plasma membrane using lectins as probes. We and others have developed a
procedure where glycoprotein receptors can be conveniently identified _in situ_
after electrophoresis in an SDS-polyacrylamide gel (7,22), and we have ex-
tended previous observations to identify a number of other glycoproteins. For
comparative purposes we also studied glycoproteins in erythrocyte ghosts.

Materials and Methods

Lectins and lectin derivatives. FITC-Con A, FITC-WGA, FITC-RCA-60, FITC-RCA-120,
and FITC-SBA were obtained from Miles Biochemicals (Elkhart, Ind). Hapten sugars
for these lectins are, respectively, D-glucose or D-mannose, N-acetyl-D-glucosamine,
D-galactose or N-acetyl-D-galactosamine, D-galactose, and D-galactose or N-acetyl-
D-galactosamine. FITC-WGA and FITC-FBP were obtained from Vector Laboratories
(Ignacio, CA) and the FITC-WGA from Vector Labs gave results identical with that
from Miles Labs.   A hapten inhibitor for FBP is L-fucose. The A_{495}/A_{280}
ranged from 0.7 to 1.3 for these lectins and the FITC-RCA described below.
Unlabeled Con A (3X recrystallized), fucose binding protein (from Lotus Tetrago-
nolobus) and WGA were from Miles Biochemicals. RCA-60 and RCA-120, generous
gifts from Mr. H. Huang, were purified according to ref. 24. For cell surface
labeling experiments the two RCA lectins were purified as in ref. 24 but not
separated. The mixture, called RCA, contained about 90% RCA-120 and 10% RCA-60
as determined by SDS-polyacrylamide gel electrophoresis. FITC-RCA was prepared
by reacting 10 mg of RCA with 0.5 mg of FITC in 0.1 M sodium carbonate buffer
pH 9.0, containing 0.05 M

D-galactose and 0.02 M NaN_3. The total volume was 10 ml. The reaction was

carried out at $4°$ for 12 hr with constant stirring and unbound fluorescein

was removed by exhaustive dialysis against $NaCl/P_i$ (8.0 g NaCl, 0.2 g KCl, 1.15 g

Na_2HPO_4, and 0.2 g KH_2PO_4, to 1 liter with H_2O, pH 7.4). Precipitated protein

was removed by centrifugation at 12,000 g for 10 min. The FITC-RCA had an

agglutination titer of 2^9 against human red blood cells.

Fluorescein labeled copolymer methacrylate microspheres approximately 40

nm in diameter were prepared as previously described (23). The fluorescein

microspheres were derivatized with diaminoheptane using 1-ethyl-3-(3 dimethyl-

aminopropyl) carbodiimide, and subsequently coupled to RCA by the two-step

glutaraldehyde procedure as previously described (23). A protein concentra-

tion of 0.6 mg/ml of microsphere suspension (6 mg/ml) was used in the coupling

reaction. Lectin-microsphere conjugates were separated from unbound lectin

by centrifugation on a discontinuous sucrose gradient (23). FITC-RCA-micro-

sphere conjugates showed an agglutination titer with human red blood cells of

2^{12}.

Growth of cells and isolation of plasma membranes. Dictyostelium discoideum,

strain A3 (obtained from Dr. F. Rothman, Brown Univ.) was grown axenically (4,7)

and developed on filter paper as described (4). Ax-2 cells were also examined

for the presence of RCA-60 and RCA-120 receptors. Total cell protein was

measured as described in legend to Fig. 2 in ref. 25. Plasma membranes were

isolated from these cells by either of two methods. Plasma membranes purified

by the first method (26) were characterized by biochemical, ultrastructural

and histochemical methods and were found to contain a 5% inner mitochondrial

membrane contamination but to be free of other identifiable membranes (26).

In order to rule out a mitochondrial source for the lectin receptors under study,

plasma membranes were purified according to a modified procedure (7) which

reduced the inner mitochondrial membrane contamination to about 0.25% (7).

Plasma membranes prepared according to either procedure contained the same pattern

of receptors for wheat germ agglutinin and did not contain receptors for the

other lectins tested. Red blood cell ghosts were isolated from freshly drawn

blood (type O^+) according to the method in ref. 27.

Proteolysis. In some experiments plasma membranes were digested with pronase in

the presence of SDS prior to electrophoresis. Plasma membranes (10-15 mg protein/

ml in 20 mM Tris·HCl (pH 8.0) and 5 mM $MgCl_2$) were diluted with one volume of a

buffer containing 20 mM Tris·HCl (pH 6.7), 2% SDS (w/v), and 10% (v/v) β-mercapto-

ethanol, and boiled for 3 min. After cooling Pronase-CB (B grade, CalBiochem)

was added to 1 mg/ml and $CaCl_2$ was added to 2 mM. After 6 hr at 23°, an equivalent

amount of Pronase-CB was added again, and digestion was continued for 10 hr at

23°. Before use pronase was predigested for 20 min at 24 mg/ml in the presence

of $CaCl_2$ at 23°.

Electrophoresis. Plasma membrane polypeptides and glycoproteins were resolved by

electrophoresis on discontinuous polyacrylamide slab gels, which ranged exponen-

tially in polyacrylamide content from 10 to 15%, in the presence of 0.1% SDS and

β-mercaptoethanol (2). Molecular weight calibrations of these gels and staining

procedures were reported in refs. 2 and 7. Gel slices, measuring 14 cm long and

0.14 cm thick contained 0.35 mg/cm width (Fig. 1,3 & 4) or 0.18 mg/cm width

(Fig. 2) of membrane protein as measured by Lowry et al. (28) technique using

bovine serum albumin as a standard.

Fluorescent lectin labeling of glycoproteins. Polyacrylamide gels were fixed

according to ref. 7. Three to four gel slices were incubated in 8-16 ml of a

solution (7) containing 1.2 to 2.4 mg of FITC-lectin for 3.0 days. Identical

concentrations were used for samples which were compared. Gels were washed in
a buffer (7) which was changed after 1.0 days, and photographed after 2.0 or
3.0 days, over a short wavelength (predominantly 254 nm) transilluminator (UV
Products, San Gabriel, CA) through a Wratten type 65 filter (7) or type 61
filter. Type 61 is superior for these purposes. Photographic negatives were
scanned with a Syntex AD-1 autodensitometer and analyzed with a program which
automatically integrated the scan. In parallel experiments a hapten sugar
was present during both the incubation and the first wash. Data shown is rep-
resentative of at least three independent experiments.

Agglutination of cells by lectins. Agglutination of cells by lectins was inves-
tigated using both heat-killed and living cells. Strain A3 vegetative cells
were washed once in 0.4% NaCl and resuspended at 4×10^7 per ml in 0.15 M NaCl
and heat-killed cells were prepared as described by Gerisch et al. (29). Aliquots
from a 2-fold serial dilution of a stock lectin solution were combined with an
equal volume of cells. The agglutination titer was the minimum lectin concentra-
tion at which detectable agglutination occurred in a depression plate.

Labeling of cells with fluorescent lectins conjugated to microspheres. D.
discoideum cells (Ax-2) in vegetative or aggregation stage were washed with PBS.
Labeling was carried out on unfixed cells as well as on cells prefixed with
0.25% (v/v) glutaraldehyde-NaCl/P_i for 30 min at 25° or stabilized with 1.5%
(v/v) formaldehyde-NaCl/P_i for 1 hr at 25°.

Approximately 5×10^5 cells in 50 µl were incubated with 50 µl of FITC-RCA
(0.47 mg/ml) or FITC-RCA-microsphere conjugates in the presence or absence of
0.1 M D-galactose. In some experiments, human red blood cells (5×10^6 cells)
were added as an internal control. After 30 min at 25°, the cells were washed
with NaCl/P_i and observed directly under a Leitz Dialux flourescent microscope.

For visualization by scanning electron microscopy, glutaraldehyde-fixed cells in suspension or on filters were treated with lectin-microsphere reagents as described above and then refixed in 1.25% (v/v) glutaraldehyde-NaCl/P_1, dehydrated in acetone and critical-point dried from CO_2. Cells were examined with a Cambridge S-4 Stereoscan microscope.

Results

Lectin receptors in erythrocyte ghosts. Erythrocyte ghost proteins and the glycoproteins which stain by the periodic acid-Schiff's base technique are depicted in Fig. 1f after electrophoretic separation on an SDS-polyacrylamide gel. The components in the membrane which bind FITC-RCA-60 are visible in Fig. 1a. Those bands in which the intensity of fluorescence was markedly reduced in the presence of the hapten inhibitor 0.3 M N-acetyl-D-galactosamine (Fig. 1a) were considered to represent sepcific receptors for RCA-60. A similar pattern of receptors was found after labeling with FITC-RCA-120, although the fluorescence intensity was somewhat less (Fig. 1b). A hapten for RCA-120, galactose, also inhibited the binding of this lectin. FITC-RCA-60 and 120 receptors are present in the high molecular weight region of the gel greater than 260,000, the band 3 region, band 75,000 (4.1), the band 4.5 region, PAS 1 and PAS 2 (for nomenclature see legend to Fig. 1). The PAS bands bind FITC-RCA-60 relatively weakly if at all. Our results are similar to those of Steck and Dawson (30) and Gahmberg (31) where galactose-terminated glycoproteins have been identified by treatment of erythrocyte ghosts with galactose oxidase, followed by reduction with sodium borotritiide and autoradiography after electrophoresis on SDS-polyacrylamide gels. In contrast, however, band 4.1 was not labeled by that technique (30). Possibly we are identifying a glycoprotein which comigrates with this species. A similar pattern of receptors was also found when radio-iodinated RCA was diffused into SDS-polyacrylamide gels, but the resolution was much poorer (22).

In order to determine the threshold number of RCA-60 receptors required
for producing a detectable response in the polyacrylamide gel, a series of
decreasing amounts of red blood cell ghost protein was electrophoresed and
labeled. In Table 1, an analysis of denistometric scans of the fluorescent
labeling of bands migrating in the band 3 region and at molecular weight posi-
tions of approximately 75,000 (band 4.1 position) and 61,000 is presented.
The percent of stained protein in the polyacrylamide gel which these glycopro-
teins comprise is given in Table 1. Assuming 0.57 pg of protein per ghost
(32), we estimate there to be 8.5×10^5, 1.1×10^5
and 0.45×10^5 protein chains per ghost, respectively, which migrate at these
molecular weight positions in the SDS polyacrylamide gels (see ref. 32).
Because band 3 is known to be heterogeneous, and because the others may be
heterogeneous, these are maximum numbers of chains which bind RCA-60. Specific
binding of RCA-60 to each of these bands is readily detectable when 20 µg of
ghost protein is electrophoresed per gel lane. Consequently, in 200 µg of
ghost protein per gel lane, as in Fig. 1, 3 and 4, receptors present at a fre-
quency of 0.5 to 8.4×10^4 chains per ghost should also be detectable. Band 3
receptors could be detected at this threshold, even though they migrated as a
broad band 0.8 cm long.

The pattern of labeling with FITC-WGA is shown in Fig. 1c. In the center panel,
staining of receptors migrating with PAS 1, PAS 2, PAS 4, PAS 3, the region of
spectrin, and the band 3 and 75,000 (position of band 4.1) regions was evident
In the left panel of Fig 1c, at least fifteen additional receptors were identi-
fied when the photographic negative was printed under conditions which blanched
the higher molecular weight region of the gel. Binding of FITC-WGA by these
minor bands was also inhibited with FITC-WGA. Binding of WGA to PAS 1 has
previously been documented using column affinity chromatography (33), but
binding to the other glycoproteins has not been reported. The possibility that

WGA can bind to sialic acid-bearing glycoproteins (such as the PAS bands) as well as to glycoproteins containing the hapten inhibitor used here, N-acetyl-D-glucosamine (35), should be noted (33,34,51).

FBP had a binding specificity restricted to the band 3 region, PAS 1, band 75,000 (position of band 4.1) and the region of the spectrin bands (Fig. 1d). Although there was binding to band 4.2, this was not inhibited by L-fucose. FBP specifically agglutinates type O erythrocytes (37) and PAS I has previously been identified as a type O blood group determinant (49). The other glycoproteins have not previously been suggested to be type O determinants.

Con A bound only to the band 3 region and this was inhibitable with α-methyl-D-mannoside (Fig. 1e). This is in agreement with previous results (36,52).

Each FITC-lectin exhibited specific binding to at least one membrane glycoprotein. This is consistent with the hemagglutinating ability of each of these lectins (18,37). All of the lectins bound to receptors in the band 3 region and this is consistent with the sugar composition of this family of glycoproteins (38). The method used in this paper did not fail to identify known receptors and identified some new receptors as well. These new receptors may not have been identified in previous experiments using affinity (lectin) column chromatography, or other techniques, because of (1) failure to be solubilized from the ghost, (2) failure to elute from the column, (3) failure to be resolved in the SDS-polyacrylamide gel, or (4) lack of sensitivity. Since lectins were utilized which had specificities against the typical anomeric configurations of each of the sugars believed to exist in ghost glycoproteins (39), Fig. 1 probably identifies the apparent molecular weight of all of the glycoproteins which reside in the ghost, except for any which might be present at extremely low levels.

Lectin receptors in the plasma membrane of D. discoideum. Glycoproteins were assayed under similar conditions in plasma membranes isolated from vegetative or developing D. discoideum. The spectrum of proteins in these membranes, as revealed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue, is shown in Fig. 2a. These gels were previously labeled with WGA which contributes to the staining, so ref. 2 should be consulted for a more accurate pattern of staining.

FITC-WGA labeled many receptors at each of three stages of development (Fig. 2b), and the binding was specific for the sugar binding site of WGA (Fig. 2c). The majority of WGA receptors on vegetative cells disappeared or decreased greatly in amount (or affinity for WGA) when the cells differentiated to the pseudoplasmodial stage of the life cycle, and a new set of receptors appeared (Fig. 2b). The plasma membrane of aggregating cells appears to be mainly transitional in its composition, but there were some receptors peculiar to this stage as well (e.g., with apparent molecular weights of 19,000, 40,000 and 90,000). One vegetative stage receptor (or group of receptors) had a very broad and continuous (or closely spaced) distribution in the gel between the molecular weight positions 48,000 and 62,000. This species seemed to have less affinity for FITC-WGA than normal receptors, since an additional 1.0 day of washing diminished the relative fluorescence intensity of this band considerably when compared to other receptors (compare Figs. 2 and 3).

In order to increase the sensitivity of the technique, twice as much membrane protein was applied to the gel and the photographic negative was exposed twice as long as normal. A densitometric scan of a gel labeled and photographed in this way exhibited the major receptors seen in Fig. 2 described above and demonstrated at least twenty-five receptors at each stage of development (Fig. 3). The additional receptors identified in this manner might be lower affinity receptors or receptors which are present in small amounts.

When gels containing plasma membranes from either the vegetative, aggregation, or preculmination stages of development were labeled with either FITC-RCA-60, FITC-RCA-120, or SBA, there was no detectable binding of fluorescent lectin. Because of the negative result only examples of membranes from the aggregation stage are shown for these lectins (Fig. 4a, 4b, and 4c). This suggests that receptors for these lectins are relatively rare on the cell surface. Consequently, it was of interest to estimate the maximum number of RCA receptors in a single band which could have escaped detection. The data from red blood cell ghosts suggested that an RCA-60 receptor can be detected at a frequency of 0.5×10^4 to 8.4×10^4 per cell ghost (see above), depending on the receptor and the nature of its migration in the SDS-polyacrylamide gel. In $\underline{D}$. $\underline{discoideum}$, the cell protein content was measured as 89 pg of protein per vegetative cell and 2% of this, or 1.8 pg, is located in the plasma membrane (26). There is 0.57 pg (32), or one-third of this amount of protein, in the red blood cell ghost. Thus we can estimate that any plasma membrane RCA-60 receptors similar to those in red cell ghosts must be present in less than 1.5×10^4 to 25×10^4 copies per cell. The number of RCA receptors on the cell surface was examined more directly as described below.

FITC-FBP also failed to specifically label any glycoproteins in plasma membranes from either the vegetative (Fig. 4d) or pseudoplasmodial (Fig. 4e) stage of development. Although FITC-FBP strongly labeled several peptides this was not inhibited by L-fucose. While these might be very high affinity sites for FITC-FBP which are not competed effectively by the concentration of L-fucose employed, this is unlikely in view of the agglutination assays which are presented below.

The chemical composition of the plasma membrane lectin receptors was examined by exhaustively digesting dissolved membranes with pronase, prior to assay for lectin receptors. Proteolysis effectively hydrolyzed all of the receptors for Con A in vegetative stage plasma membranes to low molecular weight material (Fig. 5B) and had a similar effect on WGA receptors in red blood cell ghosts (Fig. 5A). This indicated that the receptors are predominantly protein in composition. Most of the WGA receptors in D. discoideum plasma membranes were also glycoproteins (Fig. 5C). However, one vegetative stage receptor, with a broad apparent molecular weight distribution ranging from 48,000 to 62,000, was not a glycoportein.

Lectin receptors on the cell surface. Analysis of plasma membrane glycoproteins indicates that there are relatively large numbers of receptors and receptor-species for WGA and Con A and relatively few receptors, if any, for RCA-60, RCA-120, SBA and FBP, on the surfaces of axenically-grown D. discoideum cells at any stage of development (Fig. 2,3,4). Because of previous reports that RCA-120 and lectins of similar specificities could agglutinate and/or bind to another strain of D. discoideum cells (19,20,40), this inference was investigated more directly by testing the ability of lectins to agglutinate the cells and by visualizing the binding of RCA, coupled to fluorescein and microspheres, to whole cells.

RCA-60, RCA-120, SBA and fucose-binding protein (from L. Tetragonolobus) were unable to agglutinate heat-killed D. discoideum cells (all three stages of development) at the highest concentrations tested (0.5 to 2.5 mg/ml). Similar results were found with living cells as well at the vegetative and aggregation stages of development. On the other hand, WGA and Con A agglutinated the cells at 0.08 and 0.06 mg/ml, respectively and this was inhibited by the appropriate hapten sugars. The minimal level of lectin which could agglutinate cells did not change more than two-fold during the course of development. This pattern of lectin-agglutinability corroborates the pattern of lectin-binding

to receptors in SDS-polyacrylamide gels as described above.

The presence of RCA-60 and RCA-120 receptors on D. discoideum cells was also examined by fluorescent light or scanning electron microscopy using FITC-RCA and FITC-RCA-microspheres. When living cells in either the vegetative or aggregation stage of development were treated with these fluorescent reagents, no labeling of cells was observed (Fig. 6). Likewise, cells stabilized with formaldehyde prior to labeling exhibited no fluorescence. When red blood cells were added as an internal control, only the red blood cells were fluorescent (Fig. 6). Labeling of red blood cells was inhibited if 0.1 M D-galactose was present (not shown).

Scanning electron microscopy was used to detect cell surface RCA receptors after glutaraldehyde fixation, as shown in Fig. 7 (upper panel), in order to increase the sensitivity of detection of ricin receptors. The D. discoideum cell surface, including the microvilli, bound less than fifty RCA-microspheres per cell. This binding was largely non-specific since the same degree of labeling was observed in the presence of 0.1 M D-galactose (Fig. 7, upper right panel). In contrast, red blood cells labeled with RCA-microspheres (not shown) and vegetative or aggregation stage D. discoideum cells labeled with Con A or WGA coupled microspheres (Fig. 7, lower panel) displayed a uniform tight packing of these markers when viewed under the scanning electron microscope (10).

Discussion

Glycoproteins can be conveniently identified in SDS-polyacrylamide gels by employing fluorescent lectins as probes. In contrast to chromatographic methods for assaying receptors, there is little danger of identifying a molecule as a lectin receptor on the basis of non-covalent association with an authentic receptor, because of denaturation of membrane proteins in SDS and β-mercaptoethanol. In addition, a lectin receptor is unlikely to be undetected because it is not

solubilized in preparation for chromatography or is protected from reaction
with a chemical probe such as lactoperoxidase (see ref. 41). The lectins WGA,
FBP, Con A (6,7), RCA-120, RCA-60 and SBA were employed to recognize, in
plasma membrane glycoproteins, the pyranose forms of the following sugars,
in the given anomeric configurations, respectively: N-acetyl-D-glucosamine,
α or β (35,53); L-fucose, α (37); D-mannose or D-glucose, α (42), D-galactose,
α or β (43,24); and D-galactose or N-acetyl-D-galactosamine, α or β (43,44)
for the last two. Glycoproteins which specifically bind these lectins pro-
bably contain the hapten sugar at the non-reducing terminus of one of their
oligosaccharide chains, or at an internal position where its glycosidic
linkages do not interfere with lectin binding.

D. discoideum (axenic) plasma membranes and cell surfaces are relatively
rich in glycoproteins which bind WGA and Con A, but have few, if any, receptors
for RCA-60, RCA-120, SBA and FBP. This was most clearly demonstrated by the
lack of binding of FITC-RCA-microspheres to whole cells, which indicated that
there were less than 50 binding sites per cell, under conditions where Con A
and WGA-microspheres cover the entire cell surface (10). These results support
the finding that RCA-60, RCA-120, SBA and FBP failed to bind specifically to
glycoproteins in plasma membranes subjected to SDS-polyacrylamide gel electro-
phoresis, under conditions which revealed numerous species of Con A and WGA
receptors. If any RCA-60 receptors were undetected by this method, they would
have to be located on the interior face of the plasma membrane and number less
than 1.5×10^4 to 25×10^4 copies per plasma membrane. This calculation was
based on the known frequency of receptors detected in erythrocyte ghosts and
consequently presumes an avidity of D. discoideum receptors for RCA-60 similar
to that of erythrocyte receptors.

There may be a conflict between our results and previous work. RCA-120
was reported to agglutinate aggregation stage NC-4 cells grown on bacteria

at a concentration much lower than concentrations at which we could find no

effect on axenic cells (40). Another lectin which, as far as has been examined,

also has a sugar binding specificity similar to RCA-60 (43,48), has been iso-

lated from D. discoideum and its binding to cells also studied (40). This lec-

tin, discoidin, was found to bind to NC-4 cells through 5×10^5 high affinity

sites per cell, a level of binding which should have been easily detected by

RCA-60 in our cells. This might be due to a very specific difference in

recognition properties between RCA-60 and discoidin or to a difference in strains

and culture conditions.

In contrast to D. discoideum the spectrum of lectin receptors was very

different in the erythrocyte plasma membrane. There were no similarities in

the molecular weight profiles of identified glycoproteins from the two species.

Receptors for RCA-60 and RCA-120 were richest in variety in red blood cell

plasma membranes, whereas Con A and WGA receptors were richest in variety in D.

discoideum. The red cell ghost contained receptors to lectins of all six of

the lectin specificity classes studied, while the D. discoideum plasma membrane

was devoid of receptors to four of these classes. There apparently were glyco-

proteins in the red cell ghost which could bind more than one lectin probe, and this

may be true of some glycoproteins of D. discoideum.

WGA bound to more than 25 different receptor species in plasma membranes from

each stage of development and is probably binding to N-acetyl-D-glucosaminyl-

residues since sialic acids are not present in this organism (45). α or β linked

N-acetyl-D-glucosamine could be located in either terminal (53,54) or internal (53)

positions for binding of WGA. The actual number of macromolecular species in the

plasma membrane which bind WGA may be less since subunits of different molecular

weights could combine to form multimeric proteins. The more important species

(those with the heaviest lectin-binding) present at the vegetative stage were

absent and replaced by new species in the pseudoplasmodial stage of development,

and the converse was also true. Most of the WGA receptors were glycoproteins
as evidenced by hydrolysis by treatment with proteases in the presence of SDS.
One receptor was not a glycoprotein.   This may correspond to glycos-
phingolipids which are located on the cell surface, contain large amounts of
N-acetyl-D-glucosamine, and migrate as a diffuse band in SDS-polyacrylamide gels(50).

   Most WGA binding proteins and Con A binding proteins are probably different
glycoproteins in the D. discoideum plasma membrane. There is limited correspondence
between the apparent molecular weight and developmental changes of glycoproteins
from these two classes of lectin receptors. At least some WGA receptors do not
co-cap with Con A receptors when Con A is added to cells (10). The change in
the molecular weight profile of WGA receptors during development is much more
profound than that of Con A receptors (6-8). Finally, except for material
which does not enter the polyacrylamide gel, all of the WGA receptors are
restricted to the apparent molecular weight region below 95,000, while Con A
receptors as large as 313,000 have been detected (7).

   Chemical analyses of the sugars of D. discoideum plasma membranes are rele-
vant to the interpretation of glycoprotein structure. The sugar composition of
vegetative stage plasma membranes isolated from an axenically grown strain was
recently determined by Gilkes and Weeks (45). There were very small amounts, if
any, of galactose (<1 nmole/mg membrane protein), corresponding to less than 10^6
galactose molecules per plasma membrane. This is consistent with the absence of
detectable receptors for RCA-60, RCA-120 and SBA (N-acetyl-D-galactosamine is
also not detectable in the plasma membrane[4]). Likewise, there was no detectable
sialic acid[4] (45), although sialic acid commonly terminates glycoprotein oligo-
saccharides in other systems (47). The absence of both of these sugars may not
be surprising, since sialic acid, when present, is glycosidically linked to
galactose, N-acetyl-D-galactosamine or another sialic acid in systems where this
has been studied (46). N-acetyl-D-glucosamine, D-mannose, and D-glucose,

possibly a contaminant (45), were the commonest sugars analyzed. This conforms

with our observation that the only lectins which bind to these sugars (WGA and

Con A) have receptors in the plasma membrane. L-fucose was also found in the

plasma membrane of D. discoideum[4] (45). Since we did not find any specific FBP

receptors and FBP did not agglutinate cells (see also ref. 40), L-fucose, if

it is an element of glycoproteins, probably lies at an internal position of

carbohydrates or in a structure inaccessible to FBP binding. These were the

only detectable sugars found in the vegetative stage plasma membrane, when the

plasma membrane was hydrolyzed by methanolysis, derivatized with trifluoroacetic

acid, and analyzed by gas-liquid chromatography[4], or studied by an alternate

gas-liquid chromatography procedure (45).

In summary, some general conclusions regarding D. discoideum plasma

membrane glycoprotein structure can be drawn. The non-reducing termini of the

glycoprotein oligosaccharide chains seem to contain predominantly, as far as

we can determine, α-linked D-mannose, α-linked D-glucose or α-or β-linked

N-acetyl-D-glucosamine. Furthermore, we conclude that some glycoproteins contain

either N-acetyl-D-glucosamine or D-mannose (or D-glucose) but not both as non-

reducing termini on separate oligosaccharide chains or branches. On the other

hand, some glycoproteins may bind both lectins, and

we can conclude for these that they contain both N-acetyl-D-glucosamine and

mannose or glucose in their oligosaccharide chains.

ACKNOWLEDGEMENTS

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FOOTNOTES

[1] This is paper number four in a series entitled "The Role of the Plasma
Membrane in the Development of <u>Dictyostelium discoideum</u>".
Paper number three is reference 7.

[2] Abbreviations are as follows: Con A, concanavalin A; WGA, wheat germ
agglutinin; RCA-60, <u>Ricinus communis</u> agglutinin 60 (II); RCA-120,
<u>R. communis</u> agglutinin 120 (I); SBA, soybean agglutinin; FBP, L-fucose
binding protein (<u>Ulex europeus</u> agglutinin I); SDS, sodium dodecyl sulfate;
FITC-, a prefix designating conjugation with fluorescein isothiocyanate;
$NaCl/P_i$, phosphate buffered saline, as defined in the Methods section.

[3] Hoffman, S. and McMahon, D., submitted for publication.

[4] Hoffman, S., Shively, J., Wrann, M., and McMahon, D., unpublished results.

TABLE 1

Sensitivity of Detection of Receptors for FITC-RCA-60 in Red Blood Cell Ghosts

Red blood cell ghosts were electrophoresed in SDS-polyacrylamide gels, labeled
with FITC-RCA-60, photographed, and the photograph scanned and integrated, as
described in the Methods section. Non-specific, background fluorescence in
the presence of 0.3 M lactose has been subtracted from the integration values
presented. The percent of stained protein, derived as in ref. 32., is the
fraction of coomassie blue absorption in the whole gel which is contained in
the band, as determined from an integral of a densitometric scan of coomassie
blue staining.

Amount of fluorescent lectin binding (arbitrary units)

Glycoprotein	% of stained protein in gel	Amount of total erythrocyte ghost protein in gel			
		100 µg	40 µg	20 µg	8 µg
Band 3 region	21.5	27.7	17.7	9.3	0.2
Band 75,000 (4.1)	2.5	3.9	2.9	1.5	0.1
Band 61,000	0.7	1.5	0.8	0.5	0.0

FIGURE LEGENDS

Figure 1: Identification of glycoproteins, using fluorescent lectins, in human erythrocyte ghosts. Purified ghosts (200 µg protein) were electrophoresed in SDS-polyacrylamide gels and the gels were fixed. Gel lanes were incubated with an FITC-lectin and a hapten sugar, if appropriate, and washed two days (as described, in the Methods section). In parts A-E, staining by the FITC-lectin alone is shown on the left; staining by the FITC-lectin in the presence of a hapten inhibitor, included during incubation and the first washing, is on the right. A) FITC-RCA-60 ± 0.3 M N-acetyl-D-galactosamine. B) FITC-RCA-120 ± 0.3 M D-galactose (in the control gel the glycolipid region is not stained at the bottom of the gel because it was broken off). C) FITC-WGA ± 0.3 M N-acetyl-D-glucosamine; two prints of the same gel are shown here in order to show detail in both the heavily and lightly staining portions of the gel. D) FITC-FBP ± 0.5 M L-fucose; E) FITC-Con A ± 0.25 M α-methyl-D-mannoside. F) Lanes stained with Coomassie blue for protein (left), and periodic acid Schiff's reagent for glycoproteins (right). Bands with the following apparent molecular weights were assigned names in accordance with reference 32: 220,000 to 260,000, bands 1 and 2 (spectrins); 90,000 to 135,000, band 3 region; 75,000, band 4.1 (doublet); 71,000, band 4.2; 50,000 to 65,000, band 4.5 region; 45,000, band 5; 34,000, band 6; 30,000, band 7; 80,000, PAS 1; 65,000, PAS 4; 38,000, PAS 2; 21,000, PAS 3.

Figure 2: Identification of receptors for wheat germ agglutinin in
plasma membranes isolated from D. discoideum. The procedure was as in the
legend to Fig. 1 except that there was 65 μg of protein per gal lane. Plasma
membranes isolated from cells of three stages of development, vegetative (v),
aggregation (a), and preculmination (p), are shown. A) Gel lanes illustrated
in (B) were stained with Coomassie blue to show protein after photographing the
fluorescence. B) Gel lanes incubated with FITC-WGA. C) Gel lanes incubated
with FITC-WGA in the presence of a hapten inhibitor, 0.3 M N-acetyl-D-glucosamine.

Figure 3: Densitometric scans of FITC-wheat germ agglutinin binding in D.
discoideum plasma membranes. Receptors were identified on a different set
of gels than those in Fig. 2, according to the method of Fig. 1 legend, except
that the gels were washed for three days following lectin labeling. Electro-
phoresis was from right to left. The ordinate is arbitrary units of optical
density on the photographic negative. The upper three panels, from top to
bottom, show receptors from the preculmination, aggregation, and vegetative
stages of development. In the bottom panel the individual scans are super-
imposed.

Figure 4: Analysis for receptors for several lectins in D. discoideum, as described in the legend to Fig. 1. In each section, labeling with the FITC-lectin is shown on the left and labeling, in the presence of a hapten inhibitor, is shown on the right. (A), (B), and (c) show plasma membranes from the aggregation stage of development. A) FITC-RCA-60 ± 0.3 M N-acetyl-D-galactosamine; B) FITC-RCA-120 ± 0.3 M D-galactose; C) SBA ± 0.3 M N-acetyl-D-galactosamine; D) FITC-FBP ± 0.5 M L-fucose, vegetative plasma membranes; E) same as (D), except with preculmination stage plasma membranes.

Figure 5: Identification of lectin receptors as glycoproteins. Plasma membranes were denatured and dissolved in SDS and β-mercaptoethanol, and treated with pronase, a group of proteases, as described in the Methods section. Lectin receptors were assayed as in the legend to Fig. 2. In each section (A-C) the panel on the left shows an untreated control, and the panel on the right shows the result after proteolysis. A) Red blood cell ghosts labeled with FITC-WGA. 3) Vegetative stage D. discoideum plasma membranes labeled with FITC-Con A. C) Same as (B) except labeled with FITC-WGA. Electrophoresis is from top to bottom.

Figure 6: A representative mixture of aggregation-stage D. discoideum cells and human red blood cells treated with fluorescein tagged microspheres conjugated to RCA. Left: phase contrast micrograph showing both cell types (red blood cells are smaller and discoid); right: fluorescent micrograph of the same field. Only the red blood cells are labeled and visible. Out of 62 D. discoideum cells which were studied, none bound detectable levels of FITC-RCA-60 microspheres. In contrast, each of the 53 red blood cells studied were fluorescent.
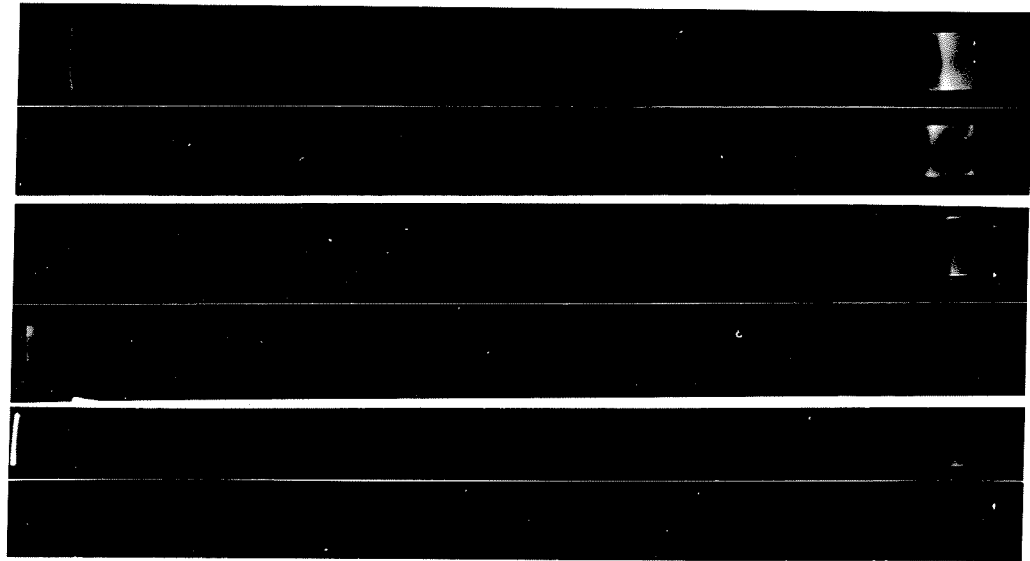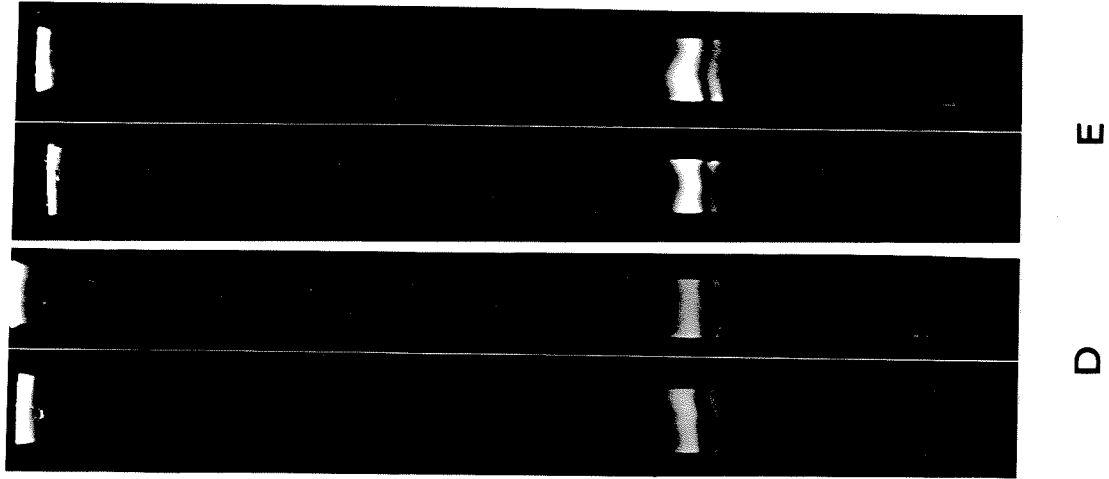
Figure 7: Scanning electron micrographs of $\underline{D}$. $\underline{discoideum}$ cells in the aggre-

gation stage of development. Cells were allowed to develop on filters for

12 hr, dissociated from filters, washed in $NaCl/P_i$, fixed in 0.25% glutaraldehyde

$NaCl/_i$ for 30 min and incubated in $NaCl/P_i$ containing 0.01 M glycine for 1 hr. Cells

in the upper panels were then treated with RCA-microspheres in the absence

(left) or presence (right) of 0.1 M D-galactose. Arrows show microspheres on

cell surface. (X 27,000). Cells in the lower panel were treated with FITC-WGA

coupled to microspheres in the absence (left) or presence (right) of 0.01 M

N-acetyl-chitobiose. (X 38,000).

MOLECULAR WEIGHT × 10^{-3}

313 215 160 124 87 68 58 48 42 35 27 21 15

A B C D E F

1

MOLECULAR WEIGHT × 10⁻³

313
215
160
124
87
68
58
48
42
35
27
21
15

v a p   v a p   v a p

A   B   C

2

MOLECULAR WEIGHT × 10⁻³

3
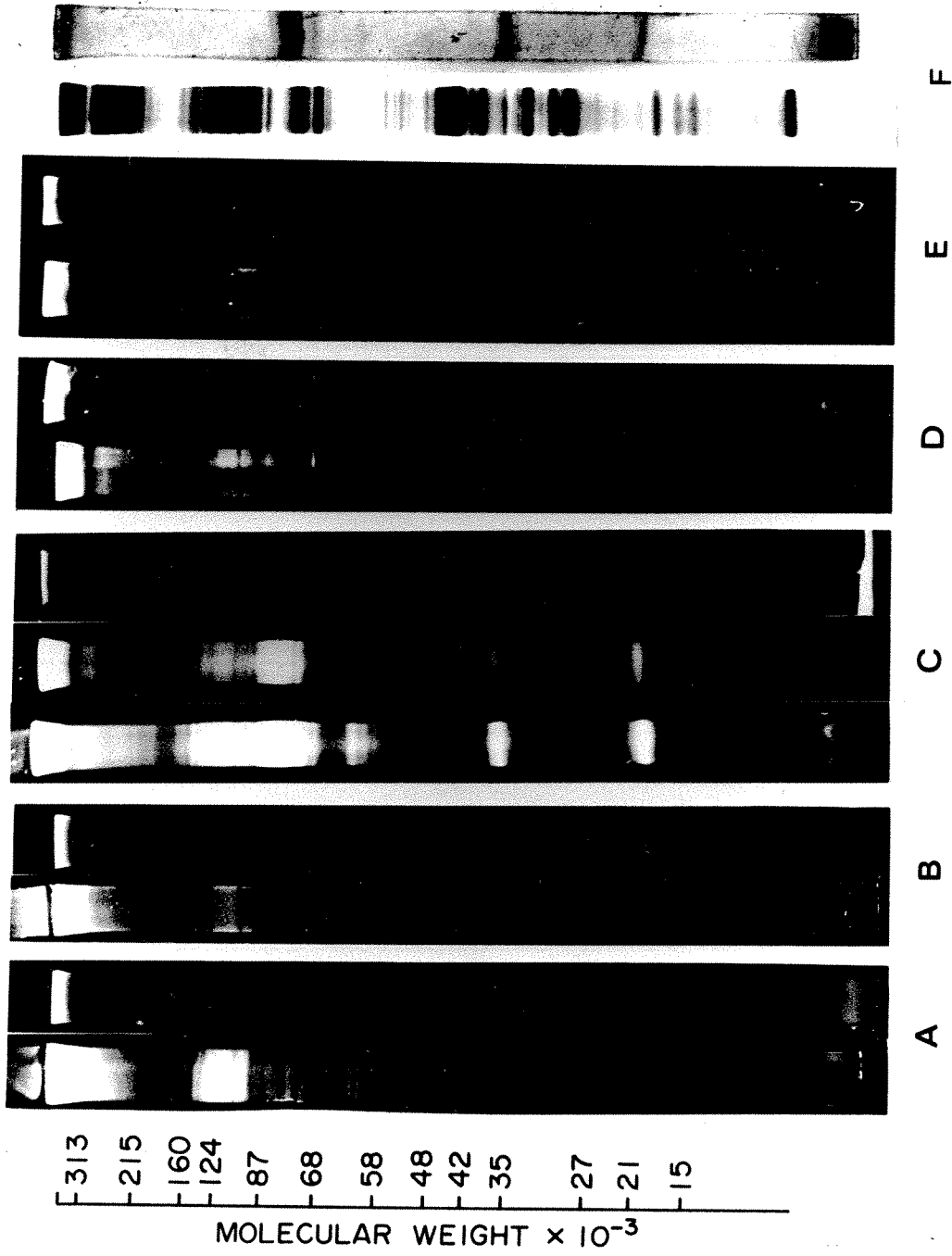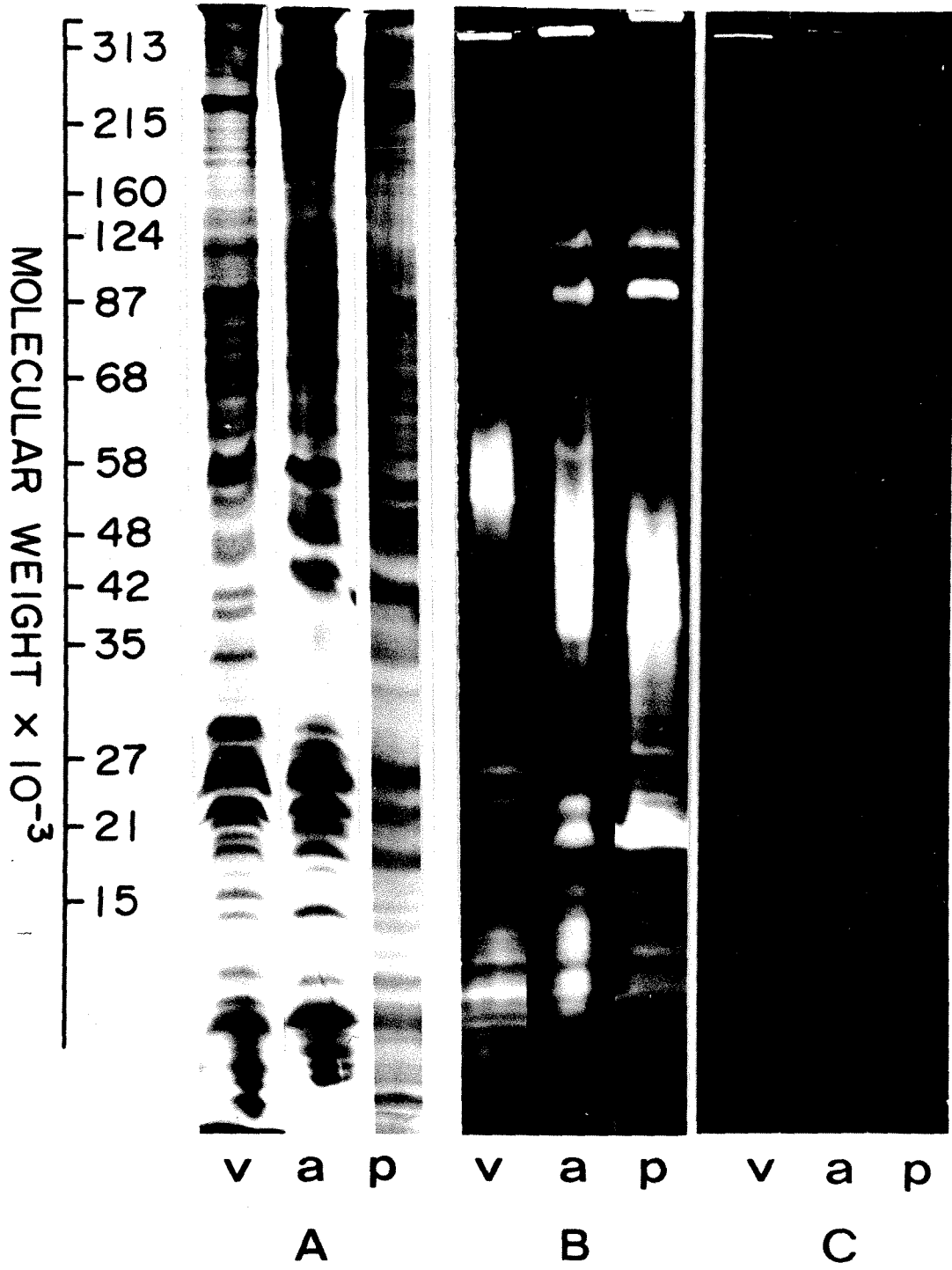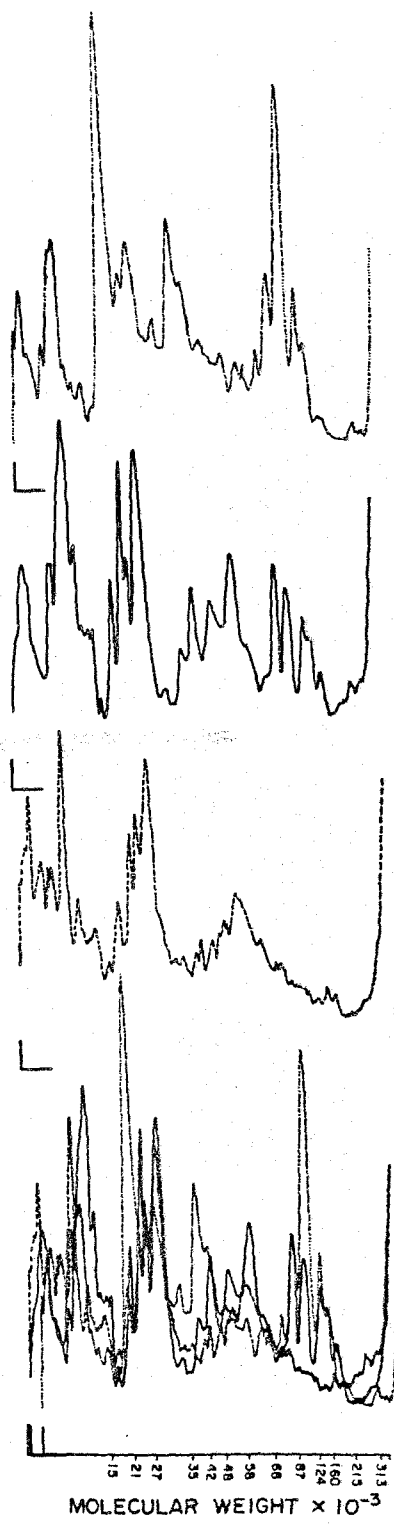
MOLECULAR WEIGHT × 10⁻³

A   B   C

5

6
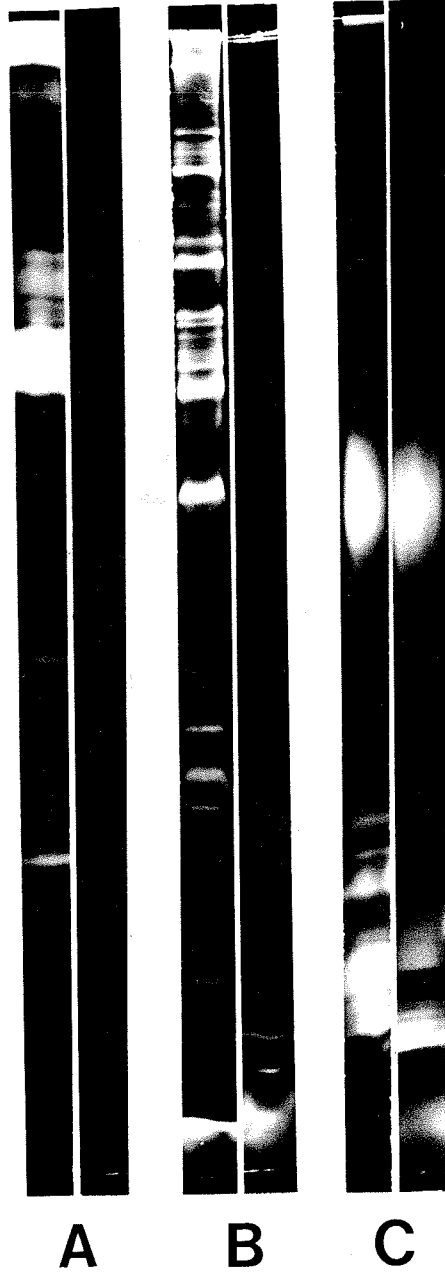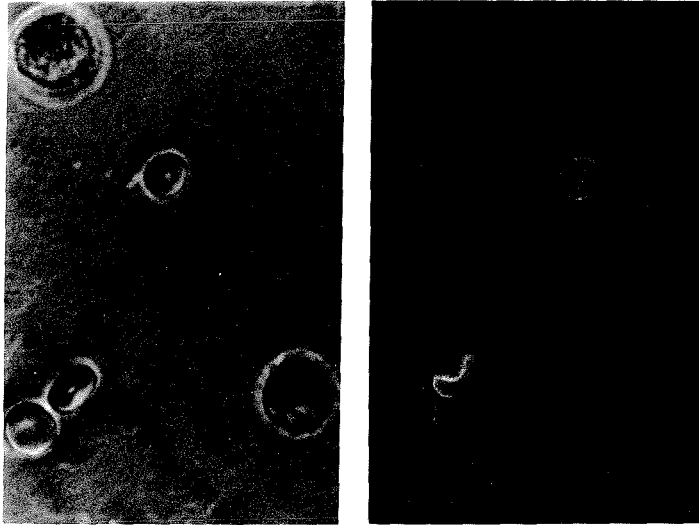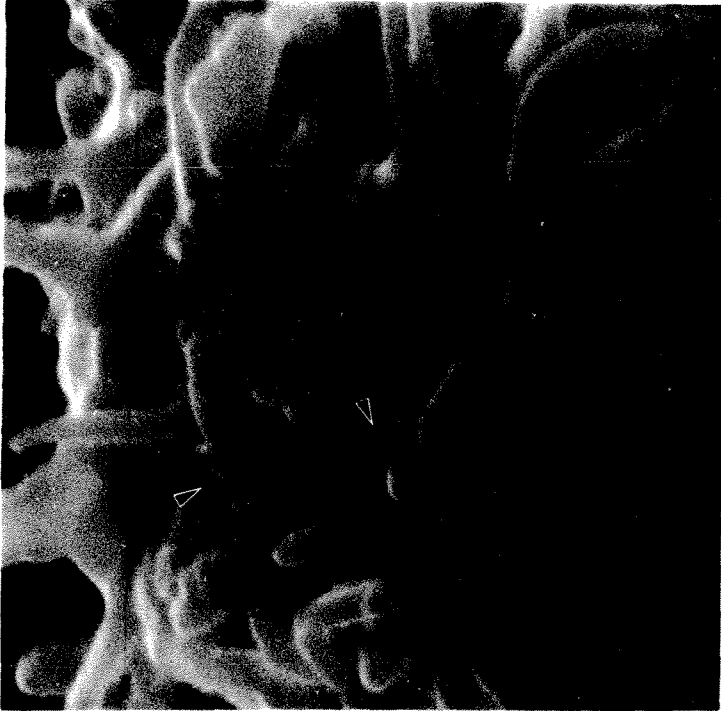
APPENDIX TO CHAPTER 3

The complexity of glycoproteins in cell organelles, as revealed

by lectin diffusion into one-dimensional SDS-polyacrylamide gels

(Chapters 2-4), makes it very desirable to find a method which is

capable of separating a greater number of glycoproteins. Such separation

is possible using the recently developed technique of two-dimensional

polyacrylamide gel electrophoresis (O'Farrell, 1975; Ames and Nikaido,

1976), where first-dimensional separation is on the basis of apparent

isoelectric point and second-dimensional separation depends upon

apparent molecular weight. We have extended our analysis of receptors

for wheat germ agglutinin in plasma membranes isolated from pseudo-

plasmodia of Dictyostelium discoideum using this technique.

Methods

Two-dimensional electrophoresis was adapted from the methods of

O'Farrell (1975) and Ames and Nikaido (1976). 2 mg of membranes,

10-20 mg/ml in 20 mM Tris (pH 8.0) and 5 mM $MgCl_2$, were digested 2 min

at room temperature with 15 μg of RNase A (Worthington N3J405) and 10

min on ice with 5 μg of DNase (Worthington DPRR3CA). This was diluted

with the same buffer and centrifuged at 48,000 g for 15 min in the cold.

0.1 ml of dissociation buffer (0.05 M Tris, pH 6.8, 2.0% (w/v) SDS, 0.5 mM

$MgCl_2$ and 10% (v/v) β-mercaptoethanol) was added to the pellet, and

the preparation was incubated in a boiling water bath for 3 min. Then

0.2 ml of a solution comprised of 9% (v/v) Triton X-100 and 2% in

ampholine (pH ranges 4-6:5-7:9-11:3.5-10.0; 1:1:2:14.2) from LKB was

added at room temperature followed by 100 mg urea (Schwarz Ultra Pure).

After the urea was dissolved, 100 µl of this, containing approximately 450 µg of protein, was used per isoelectric focusing gel.

Isoelectric focusing was performed in a cylindrical glass tube, 13 cm long x 4 mm inner diameter, capped at the bottom end with parafilm. For making a gel, the following components were combined: 22 g urea, 5.3 ml acrylamide stock solution (28.4% (w/v) acrylamide, 1.6% (w/v) bis-acrylamide), 8.0 ml 10% (v/v) Triton X-100 and 7.9 ml H_2O. After these were dissolved, the following amounts of ampholines (LKB) were added: 2.03 ml, pH 3.5-10.0; 0.286 ml, pH 9-11; 0.143 ml, pH 4-6; and 0.143 ml, pH 5.7. 0.06 ml of 10% (w/v) freshly made ammonium persulfate was added and the solution was degassed for 2-3 min in a side-arm flask connected to an aspirator. 0.04 ml of TEMED were added and 11 cm deep gels were immediately poured. Overlay was 0.05 ml of 8.0 M urea.

Following 2 hrs of polymerization, the 8.0 M urea overlay was removed and 0.1 ml of the dissociation mix described above, without protein, was placed on the gel. This was overalaid with 8.0 M urea containing 2% (w/v) ampholines proportioned as in the gel. This was pre-focused 350 V for 0.5 hrs. The upper buffer, 0.02 M NaOH (thoroughly degassed) was connected to the cathode, and the lower buffer, 0.01 M phosphoric acid, was the anode. Following prerunning, the gel overlay and electrode buffers were discarded, the sample was applied, overlaid as for prerunning, and focused for 12 hrs at 400 V, followed by 2 hrs at 800 V. The focusing gel was then incubated in the following solution for 0.5 hrs: 0.0625 M Tris (pH 6.8), 10% (v/v) glycerol, 2.3% (w/v) SDS, 0.002% (w/v) pyronin Y, 5% (v/v) β-mercaptoethanol. Then the gel

was laid upon a discontinuous, exponential gradient polyacrylamide slab gel made with 0.1% (w/v) SDS (as in Chapter 2) and sealed in with 1% (w/v) agarose containing 0.002% (w/v) pyronin Y. Electrophoresis was as described in Chapter 2.

Fixation and lectin labeling were as described in Chapter 4. pH was calibrated by incubating consecutive 5 mm slices of a gel that had been run in the absence of proteins in 2 ml of degassed 9.1 M urea. The pH of the solution was measured after 5 hrs.

Results and Discussion

Both the protein profile (Coomassie Blue) and wheat germ agglutinin (WGA) receptor profile of plasma membranes isolated (McMahon et al., 1977) from pseudoplasmodial cells and electrophoresed on two-dimensional gels are shown in Fig. 1. Over 150 proteins were resolved and they were well separated from one another. In many cases, proteins with similar molecular weights and thus not distinguished in one-dimensional gels were separated according to their isoelectric points.

Forty-nine different receptors for WGA were also detected. Many of the WGA receptors were relatively acidic compared to the bulk of the membrane protein. The two-dimensional pattern of WGA labeling was in general correspondence, in the second-dimension, with the WGA-labeling pattern in one-dimensional gels. Many of the carbohydrate-containing molecules identified with WGA also stained with Coomassie blue and could be located in gels using this property alone once the correspondence was made. This may be useful for elution and for further characterization of these molecules.

On the basis of their migration in the second-dimension, spots A, B, and C have been identified as the prestalk cell-specific plasma membrane molecules (Chapter 4), and spots 1 and 2 as the prespore cell-specific macromolecules (Fig. 1). The identification of spots 1 and 2 as being cell-type specific in the plasma membrane is subject to the assumptions discussed in Chapter 4.

<u>Figure 1</u>. Identification of wheat germ agglutinin receptors in
plasma membranes isolated from pseudoplasmodial cells and electro-
phoresed on two-dimensional polyacrylamide gels.

Plasma membranes were analyzed for WGA receptors as in Chapter
2 except that two-dimensional gels were used. The first dimensional
electrophoresis was from left to right, and second dimensional
electrophoresis was from top to bottom. The pH gradient is indicated
on top of the gels. The gel on the left was stained for protein
with Coomassie Blue and the gel on the right for carbohydrate
components with fluorescein-tagged WGA. Several spots have been
marked with letters or numbers. Those marked A, B, or C are prestalk
cell-specific WGA receptors and those marked 1 or 2 are probably
prespore cell-specific (see Chapter 4). Spot A did not appear in
the gel on the right but did appear in two other gels.

MOLECULAR WEIGHT × 10⁻³
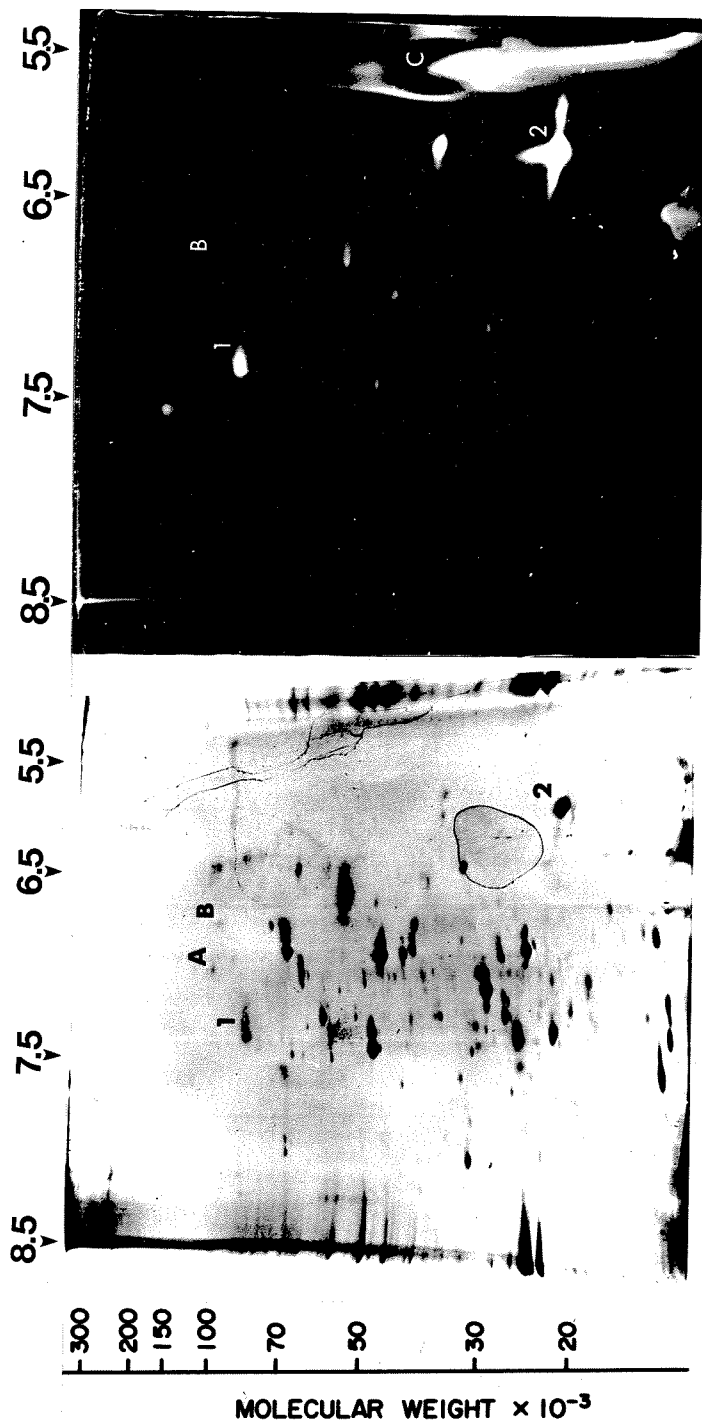
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CHAPTER IV

POSITION-SPECIFIC PLASMA MEMBRANE MOLECULES IN PSEUDOPLASMODIA

OF THE CELLULAR SLIME MOLD DICTYOSTELIUM DISCOIDEUM

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ABBREVIATIONS

Con A, concanavalin A; WGA, wheat germ agglutinin; FITC-, a prefix

designating conjugation with fluorescein isothiocyanate; SDS, sodium

dodecyl sulfate; 1/2PDF, 0.5 concentration of PAD dilution fluid

(ref. 24); NAG_5, N,N,N,N,N-pentaacetyl-chitepentose; NAG, N-acetyl-

D-glucosamine; BSA, bovine serum albumin; IgG, immunoglobulin G.

ABSTRACT

Pseudoplasmodia of the cellular slime <u>Dictyostelium</u> <u>discoideum</u>
were dissected into prestalk and prespore regions and assayed for their
lectin receptors in SDS-polyacrylamide gels which contained extracts
of the cells. Using the lectin wheat germ agglutinin (WGA), three
molecules, two of them glycoproteins and one not, were unique to the
prestalk region and two others, both glycoproteins, were predominantly
localized in the prespore region. In contrast, proteins identified
by Coomassie blue, receptors for the lectin concanavalin A, and other
WGA receptors were similar between the two regions. By analyzing
the subcellular distribution of these WGA receptors, all were found
in the plasma membrane, and the prestalk-region specific WGA recepotrs
were unique to the plasma membrane. The position-specific distri-
bution of these WGA receptors occurred prior to the formation of the
stalk. The four position-specific glycoproteins were also present
in twelve hour aggregates, and consequently, they appear before most
other markers of cell-type differentiation, and before the pseudo-
plasmodium is formed. At least one of the several cell surface WGA
receptors seems to be essential for development, since blocking these
receptors with WGA inhibited reformation of pseudoplasmodia from
dissociated pseudoplasmodial cells. This effect was blocked using a
competitive inhibitor for WGA.

Carbohydrate containing compounds are a prominent group of

molecules on the surfaces of cells (Hughes, 1976; Smith and Walborg,

1977). Key roles in cell adhesion (Balsamo and Lilien, 1975; Kuhns

et al., 1974) and hormone reception (Cuatrecasas, 1974) have been

ascribed to their carbohydrate moieties in many cell systems. Carbo-

hydrate-containing compounds seem to be equally important in Dictyostelium

discoideum. Their plasma membranes contain a large variety of glyco-

protein species, many of them being under developmental regulation

(Hoffman and McMahon, 1977b; West and McMahon, 1977a,1977b). When

carbohydrate-containing structures are blocked with carbohydrate-

binding proteins, the cells fail to undergo normal development (vida

infra; Gillette and Filosa, 1973; Darmon and Klein, 1976). Carbo-

hydrate moieties have been implicated in D. discoideum cell adhesion

in Gerisch's serologically defined contact system A (Beug et al., 1973;

Gerisch, 1976) whose antigens seem to be glycoproteins, and in Rosen

and Barondes lectin-lectin receptor hypothesis (Reitherman et al., 1975).

We have undertaken a study of position-specific, plasma membrane-

dwelling, carbohydrate-containing molecules in the pseudoplasmodium

of D. discoideum, in order to find a potential basis for the origin

of prestalk and prespore cells and/or the formation of a constant

pattern of these cell types in the pseudoplasmodium. Using a previously

described technique of labeling SDS-polyacrylamide gels with fluores-

cent lectins, several position-specific plasma membrane molecules were

found and characterized with respect to their apparent molecular weights,

presence of protein, and the identity of one of their sugars. These

position-specific molecules seem to occur early in the process of

pattern formation. Blocking carbohydrate containing molecules which contained the identified sugar common to these molecules and others in the plasma membrane with a specific carbohydrate binding protein inhibited normal development of the pseudoplasmodium.

These studies have indirectly given a picture of glycoprotein changes as a result of development, in whole cells and three sub-cellular fractions in addition to the plasma membrane. The glyco-protein composition of the different subcellular fractions was as complex as that of the plasma membrane; some of the glycoproteins were unique to one fraction or another. In general, the developmental changes in glycoproteins were more extensive than those in proteins which were identifiable with Coomassie Blue.

RESULTS

Cell Fractionation

The intracellular distribution of lectin receptors was studied in order 1) to find out if the glycoproteins of the plasma membranes could be studied by analyzing the whole cell and 2) to obtain an appreciation of the variety of cellular proteins which are glycosylated and how they change during development.

Four subcellular regions were analyzed for glycoproteins. A mitochondrial pellet, containing approximately 12% of the total cellular protein, was collected by differential cenfiguration and probably con-tained crystal bodies (Takeuchi, 1972), 90% of the mitochondria, 40% of the lysosomes, 20% of the peroxisomes (Stuchell et al., 1975) and 40% of the plasma membrane (West, unpublished) of vegetative cells. A

post-mitochondrial microsomal fraction, containing about 18% the

total cellular protein, was also obtained by differential centrifugation

and this was expected to contain the remaining cellular organelles

and membranes. The nuclei were probably broken during cell fractionation

due to the presence of EDTA (B. Seed, personal communication) and

the nuclear parts contents and nuclear membrane would be expected to

pellet in the microsomal fraction (T. Bakke, personal communication).

The cellular 100,000 g supernatant contained approximately 69% of the

cellular protein, and plasma membranes, which were purified directly from

the microsomal fraction, comprised of about 2% of the total cellular

protein (89 pg) (West and McMahon, 1977b).

For the analysis of proteins and glycoproteins, 100 μg of protein

from each of the four cell fractions was electrophoresed in adjacent

lanes of a polyacrylamide gel in the presence of 0.1% SDS. In the

surrounding lanes, 145 μg of whole cell homogenate protein, which was

calculated to contain 100 μg of cell supernatant protein, and 225 μg

of the same supernatant protein (to maximize sensitivity) were electro-

phoresed. As described previously these gels were fixed in glutar-

aldehyde in preparation for labeling with fluorescent lectins.

The pattern of proteins as revealed by staining with Coomassie

Blue is illustrated by Fig. 1a. Since an identity for bands in different

lanes rests at least in part on identical migration, it is worth

remarking that protein migration depends on the composition and amount

of the sample as well as on apparent molecular weight. For instance,

a molecule which is detectable with WGA labeling (Figs. 1c and 2c)

and often migrates on one side of the lane, retards the protein bands

which migrate in its vicinity. In spite of this the migration of bands

in separate lanes can often be compared on the basis of "trailing arms"

which extend from the lateral edges of the bands.

Each of the four cell fractions has a distinct and characteristic

molecular weight "fingerprint" of proteins. A meaningful separation

of intracellular regions seems to have been achieved because of this.

Some proteins were common to all compartments and others were shared

by only two or three, or were unique (at least in amount) to one.

Glycoproteins which Bind Con A in Vegetative Cells

Glycosylation is very common in cellular proteins in all sub-

cellular fractions. For example, in the supernatant from vegetative cells

(Fig. 1) fluorescent Con A labeling superimposes on top of 32 of the

44 most darkly staining protein bands determined by Coomassie Blue

staining (Fig. 1b). These were considered to be glycoproteins because

1) they stained with Coomassie Blue, 2) all plasma membrane Con A

receptors have been proven to be glycoproteins on the basis of sensi-

tivity to pronase (West and McMahon, 1977b) and 3) the Con A receptors

migrated in the gel as sharp bands as expected of proteins. As has

been well documented for the plasma membrane Con A binding glycoproteins

(West and McMahon, 1977a), the binding of Con A to all of the molecules

identified in Fig. 1b is carbohydrate-specific since it was blocked by

the presence of the hapten inhibitor α-methyl-D-mannoside (0.25 M) (not

shown).

Proteins from the 7700 g pellet fraction exhibited the least degree

of labeling with Con A, while plasma membranes exhibited the greatest

extent. Above 90,000 daltons, the only cellular glycoproteins seemed

to reside in the plasma membrane. The Con A binding in the >90,000 molecular weight region in the microsomal fraction, from which the plasma membrane was purified, was probably due to the plasma membrane contained in that fraction. These high molecular weight plasma membrane glycoproteins were detectable in whole cells (Fig. 1b).

Wheat Germ Agglutinin Receptors in Vegetative Cells

WGA identified a distinct smaller set of receptors in the cell than did Con A (Fig. 1c). WGA bound to its receptors due to their content of α- or β-linked N-acetyl-D-glucosamine, since the presence of this sugar (0.25 M), a specific hapten inhibitor for WGA, abolished all detectable binding of this lectin (not shown).

Nearly all, if not all, of the wheat germ agglutinin receptors are membrane bound or trapped within an organelle (Fig. 1c). The small amount of three receptors in the supernatant could be contaminant. In addition all of the WGA receptors with molecular weight of 28,000 or greater seem restricted with one exception to the plasma membrane. The occurrence of WGA receptors with apparent molecular weights greater than 30,000 in other cell fractions of the whole cells can probably be attributed to their plasma membrane content. A receptor with molecular weight ca. 45,000 seems to be microsomal as well as plasma membrane in origin. The 27,000 molecular weight receptor is probably mitochondrial in origin. The group of receptors with apparent molecular weights below 21,000 are probably shared by one or more microsomal components and plasma membrane. Several of the glycoproteins seem to bind both Con A and WGA, suggesting that both α-linked D-mannose (or glucose) and N-acetyl-D-glucosamine

Fig. 1. Identification of proteins, glycoproteins, and other carbohydrate containing molecules in D. discoideum cells.

Vegetative stage cells were lysed and separated into four sub-cellular fractions and these were electrophoresed into discontinuous SDS-polyacrylamide slab gels. Following fixation gels were stained either for protein using Coomassie Blue (A), or for carbohydrate using fluorescent derivatives of the lectins concanavalin A (B) or wheat germ agglutinin (C). Individual lanes are labeled according to the source of the protein they contained. Lanes C contained 145 μg of protein on the left-hand side of each section and 240 μg of protein on the right-hand side. Lanes S contained 100 μg of cell supernatant protein which is the amount of supernatant protein contained in 145 μg of whole cell material. Lanes Mt contained the mitochondrial pellet (100 μg), Mc the microsomal pellet (100 μg) and PM the plasma membrane (100 μg).

137

MOLECULAR WEIGHT × 10⁻³
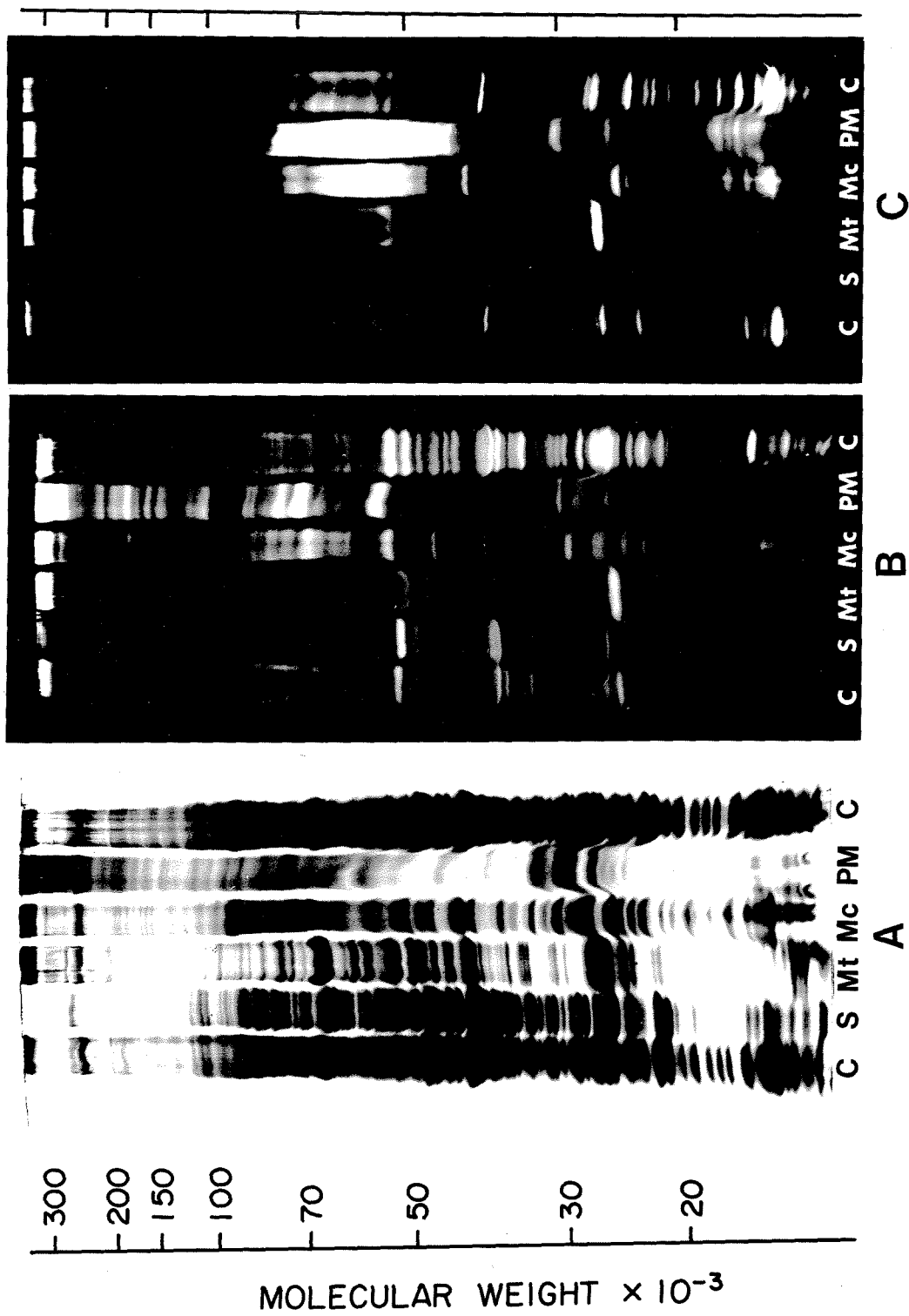
are present in these glycoproteins. For instance, the "mitochondrial" glycoprotein at apparent molecular weight 24,000 binds both of these lectins (Fig. 1b and Fig. 1c).

Pseudoplasmodial Stage Lectin Receptors

Glycoproteins were also studied in vegetative cells which had aggregated and formed pseudoplasmodia. Although the detectable pattern of proteins (Fig. 2a) was little changed as a consequence of development, the glycoproteins were very different (Fig. 2b and Fig. 2c). In general glycoproteins identified by WGA were changed more than those identified by Con A. The subcellular pattern of glycosylated proteins detected by both of the lectins was also modified as a result of development (Fig. 2). This was in spite of the relatively similar protein profiles between the two developmental stages.

Glycosylation of all cellular supernatant proteins apparently decreased or was modified considerably (Fig. 2b) and the amount of fluorescent Con A labeling in other subcellular fractions now appears to be nearly equal (Figs. 2b, and Fig. 2c). The increased labeling of the 7700 g pellet protein by Con A relative to the vegetative stage of development might be due to the occurrence of a new developmentally controlled membranous organelle in this fraction, the prespore vescicle (Ikeda and Takeuchi, 1971; Takeuchi, 1972). During sporulation these vescicles probably fuse with the plasma membrane to release their contents on the cell surface (Maeda and Takeuchi, 1969; Hohl and Hamamoto, 1969).

The appearance of new Con A binding glycoproteins near 115,000 daltons which seems to occur in all of the subfractions and the persistence

after development of Con A receptors in all subfractions at lower
molecular weights means that, because of interference, a study of the
whole cell Con A receptors will reveal unambiguous information about
plasma membrane glycoproteins only if they have an apparent molecular
weight of greater than 120,000.

Several WGA receptor glycoproteins which occur in the plasma
membrane of pseudoplasmodial cells, including those with apparent mole-
cular weights of 22,000, 80,000 and 85,000, also occur in the 7700 g
pellet of pseudoplasmodial cells in an even greater amount. Perhaps
these glycoproteins originate in prespore vesicles and are destined for
the cell surface. Most of the WGA receptors with apparent molecular
weights of less than 30,000 which are visible in the whole cell seem to
be primarily localized in the microsomal fraction. Consequently, analysis
of whole cell WGA receptors will reveal information about the plasma
membrane receptors at these molecular weights only insofar as they are
regulated in a manner similar to those in the other cell subfractions.
However, those plasma membrane WGA receptors with apparent molecular
weights of 105,000, 95,000 and 30,000-55,000 can be unambiguously analyzed
in the whole cell fraction.

When pseudoplasmodial cell plasma membranes were digested with
pronase in advance of SDS-polyacrylamide gel electrophoresis (as
described in West and McMahon, 1977b), all of the WGA receptors were
digested into low molecular weight material, except for the broadly
migrating species between 30,000 and 45,000 apparent molecular weight
(not shown). This is the same result as was obtained with vegetative
stage WGA receptors (West and McMahon, 1977b).

Fig. 2. Pseudoplasmodial cells from D. discoideum were analyzed

according to the legend to Figure 1.

MOLECULAR WEIGHT × 10⁻³
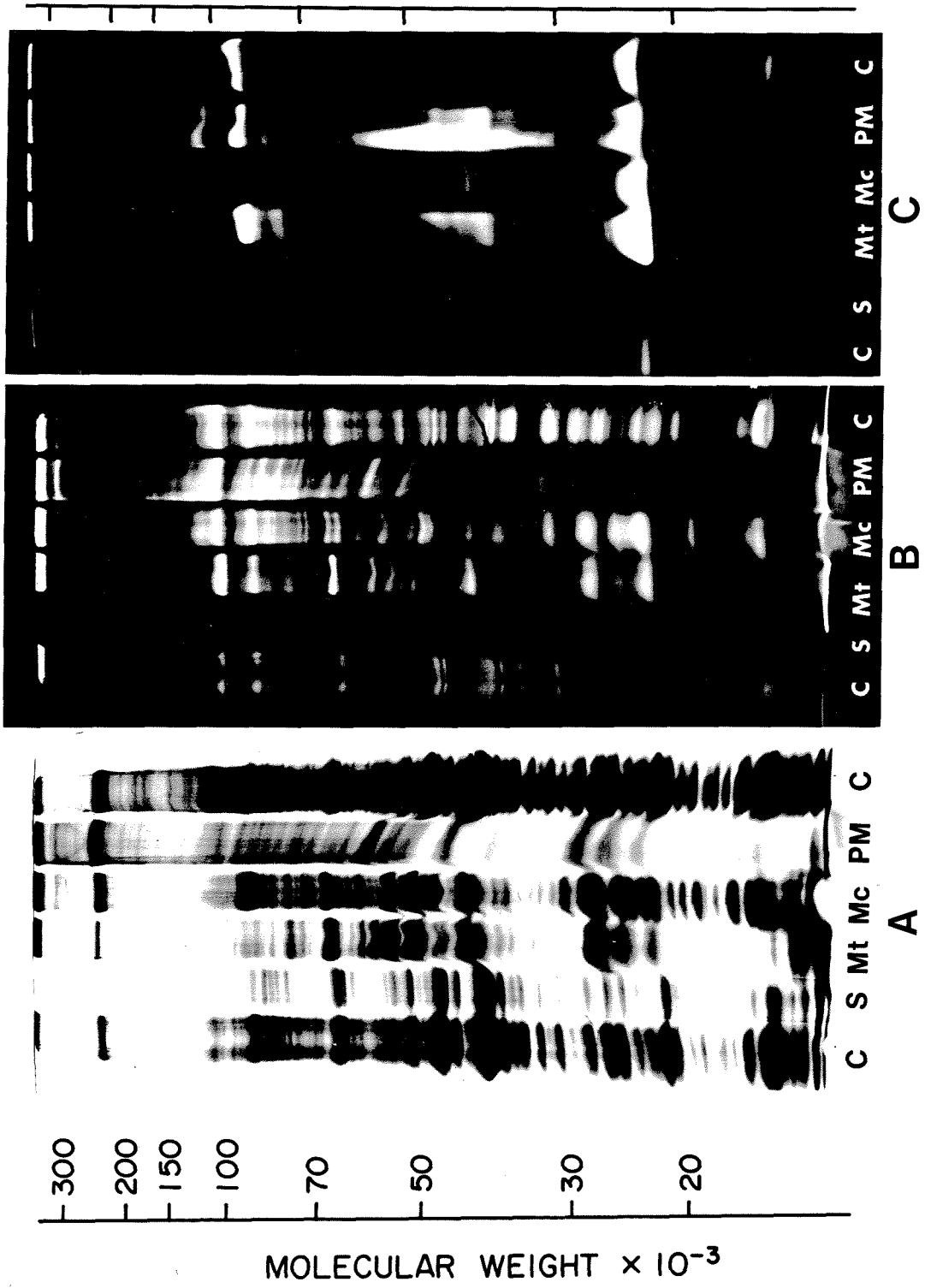
Glycoproteins of Prestalk and Prespore Cells

Cells located in the anterior one-fifth of the pseudoplasmodium are fated to become stalk cells and cells in the posterior four-fifths will become spores (Raper, 1940). Biochemical, histochemical and serological differences between the anterior and posterior cells are discernible at the pseudoplasmodial stage in correspondence with the different fates of these cells (Bonner et al., 1955; Bonner, 1949; Hohl and Hamamoto, 1969; Krivanek, 1956; Krivanek and Krivanek, 1958; Maeda and Maeda, 1973; Takeuchi, 1960, Takeuchi, 1963; Wright and Anderson, 1958). We undertook to examine the distribution of glyco-proteins in these two regions of the pseudoplasmodium.

The anterior tip, containing prestalk cells (Fragment 1 in Fig. 3), was sliced from slugs, deposited into SDS gel dissociation buffer containing a protease inhibitor and boiled. Prespore cells were cut from a portion of the slug between one-half and three-fourths of the distance from the anterior tip (Fragment 3 in Fig. 3) and treated similarly.

The apparent molecular weight profile of proteins and Con A binding glycoproteins (Fig. 4) is nearly identical between prestalk and prespore cells. One exception is that a glycoprotein with an apparent molecular weight of 22,000 is present in a larger amount in prespore cells. This glycoprotein also binds WGA (see below). Although the region where plasma membrane glycoproteins which bind Con A are separated from other cell glycoproteins, above 120,000 apparent molecular weight, is not labeled well due to the small amount of protein; at least one plasma membrane Con A receptor (215,000 daltons) is present in similar amounts in the two cell types.

Fig. 3. Dissection of a pseudoplasmodium.

One pseudoplasmodium was lifted with a scalpel from a filter so that it rested flat on the blade, covered with a drop of buffer and clamped to a microscope stage. Using a second scalpel the pseudoplasmodium was cut into four fractions. Parts labeled 1 through 4 are consecutive fragments beginning at the anterior tip. Part 1 was used as prestalk cells and Part 3 as prespore cells. Parts 2 and 4 were discarded. The bevel on the scalpel blade measured 0.85 mm.
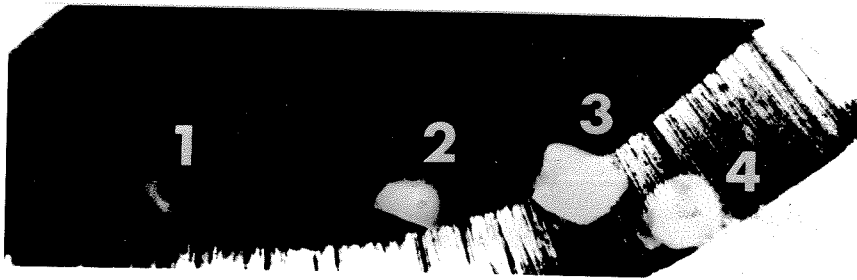
Fig. 4. Molecular analysis of prestalk and prespore cells.

Prestalk and prespore cells were obtained as shown in Fig. 3 and analyzed for protein and carbohydrate after electrophoresis on SDS-polyacrylamide gels. Equivalent amounts of prestalk and prespore cell protein, approximately 30-40 µg, were electrophoresed in adjacent or nearby lanes of the same gel. In each of the three pairs of lanes, prestalk cells are on the left and prespore cells on the right. In the left panel, gels have been stained for proteins with Coomassie Blue (CB), the gels in the center panel for carbohydrate with concanavalin A (CON A) and in the right-hand panel for carbohydrate with wheat germ agglutinin (WGA).
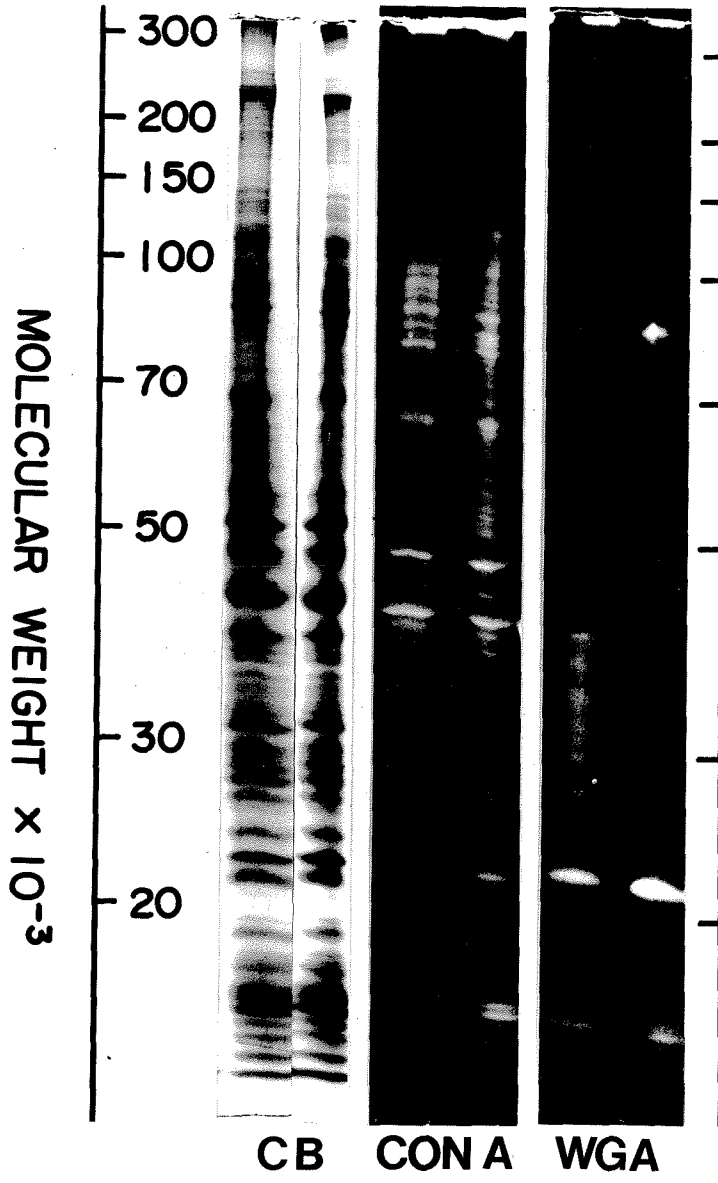
In contrast, WGA receptors are clearly different in prestalk and prespore cells (Fig. 4). Prestalk cells are distinguished by two glyco-proteins with apparent molecular weights of 95,000 and 105,000. These glycoproteins were found above to occur only in the plasma membrane (vide supra; West and McMahon, 1977b). In addition, prestalk cells are characterized by a broad band spreading between apparent molecular weights of 30,000 and 45,000 daltons which is not a protein. This molecule is also restricted to the plasma membrane. It may be a glyco-sphingolipid described by Gerisch and his coworkers to be present on the cell surface (Wilhelms et al., 1974).

Prespore cells are characterized by having higher amounts of glyco-proteins at apparent molecular weights of 85,000 and 22,000 (Fig. 4). These were shown before to be present in the plasma membrane and also in the mitochondrial pellet, possibly as prespore vescicles. Their possible occurrence in prespore vesicles is sensible as prespore vescicles are preferentially located in prespore cells. The other WGA receptors, some of which are known to be and all of which may be present in other membranous cell compartments, are present in similar amounts on a per protein basis in both types of cells.

Chronology of Differentiation

In order to interpret the possible significance of the differences in membrane molecules between prestalk and prespore cells, it was nec-essary to order these membrane differences in time with respect to other events of differentiation. Differentiation of the prestalk and prespore cells in the pseudoplasmodium was monitored using the calcofluor assay

Fig. 5. Analysis for differentiation in the pseudoplasmodium

Whole pseudoplasmodia were incubated in the fluorescent brightener Calcofluor. A preculmination pseudoplasmodia, at approximately 21 hrs of development, was viewed in bright field (A) and fluorescent (B) optics (x360). A stalk has formed and is fluorescent. In (C) and (D) a pseudoplasmodium taken at the time of dissection (18.5 hrs) for prestalk and prespore cells (see Fig. 3), was also analyzed in bright field (C) and fluorescent (D) optics (x240). No stalk was formed.
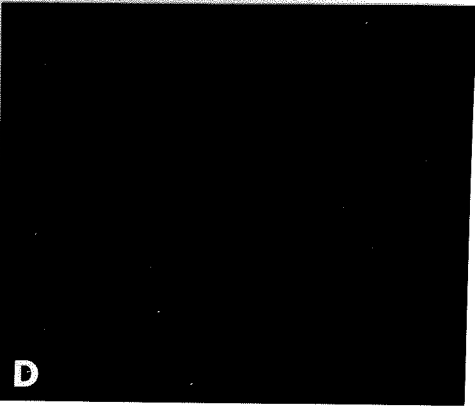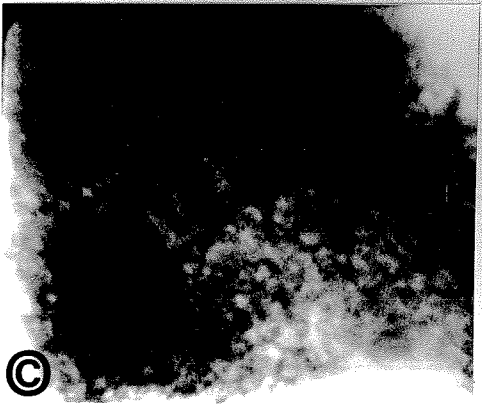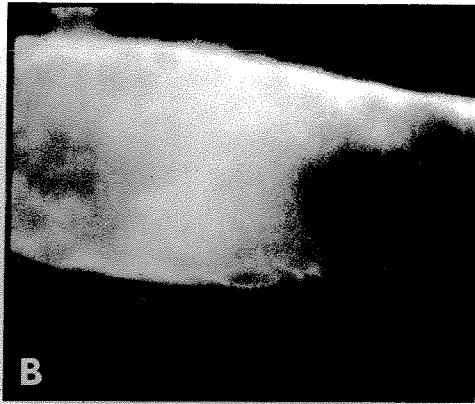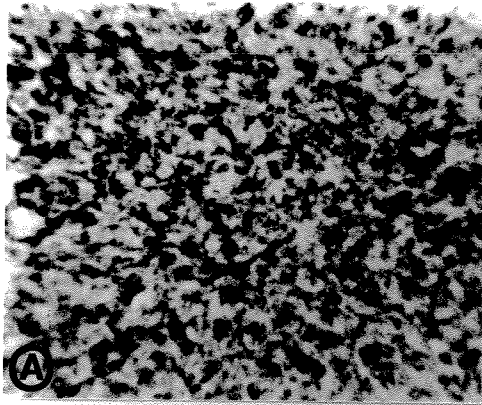
for cellulose (Harrington and Raper, 1968). Figure 5b shows the
appearance of a pseudoplasmodium containing a stalk in a whole mount
stained with calcofluor. The adjacent cells external to the stalk have
also begun to stain slightly by this stage. Eventually the spores will
also be very fluorescent. In contrast, slugs which were dissected for
prestalk and prespore cells show no stalk (Fig. 5d). This is consistent
with our failure to find a stalk during dissection (stalks are apparent
when present).

Biological Significance of Wheat Germ Agglutinin Receptors

In order to determine whether any of the wheat germ agglutinin
receptors were directly involved in cell physiology or differentiation,
wheat germ agglutinin was applied to developing cells. Cells which had
developed to stage 12 (Loomis, 1975) were harvested from filters,
dissociated into single cells and incubated in the presence of
appropriate substances for 15 min in the cold. These cells were replated
on blue millipore filters at a density of 1.0×10^8 cells/ml. Normally
the cells reaggregate within 1 hr and have formed vertical pseudoplasmodia
(stage 17) by 3-4 hr. These pseudoplasmodia are visible when viewed
from above in Fig. 6a after 5.5 hr. (These cells were incubated with 2.5
mg/ml of BSA which does not affect development.) In the presence of
2.5 mg/ml WGA, which was a sufficient concentration to cause some of
the cells to agglutinate (Fig. 6f), the cells formed shallow aggregates
on the filter but did not develop further into pseudoplasmodia (Fig. 6b).
One mg/ml of WGA did not inhibit development nor did it differentially
clump cells (not shown). When the WGA was supplemented with
20 mM NAG_5, a hapten inhibitor of WGA, these cells did not clump before

Fig. 6. Inhibition of development with a carbohydrate-binding

protein.

Pseudoplasmodia (18.5 hrs) were dissociated into single

cells and incubated for 15 min in 2.5 mg/ml of bovine serum

albumin (A,E), wheat germ agglutinin (WGA) (B,F), WGA plus a

hapten inhibitor, 20 mM NAG_5 (C,G), or IgG produced against

pseudoplasmodial plasma membranes (D,H). Following incubation,

cells were replated on millipore filters. After 5.5 hrs cells

were photographed to evaluate their ability to perform pseudo-

plasmodia (A-D). Immediately following incubation, a sample of

cells were also diluted and observed in a microscope for

agglutination (E-H). Scale for (A-D) is given in (C) and (D),

where grid bar is 0.22 mm wide. Magnification for (E-H) is x80.
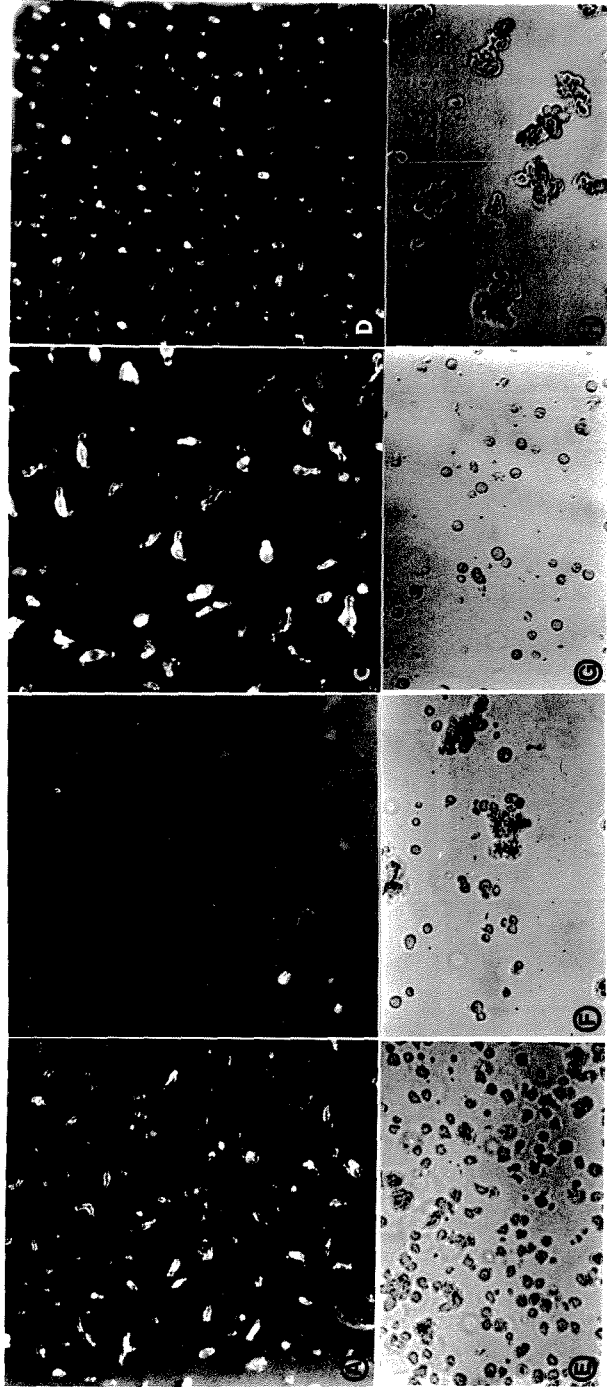
plating (Fig. 6g) and the cells developed normally (Fig. 6c). NAG$_5$ had

no effect on development when added alone without WGA (not shown). The

effect of WGA was not simply due to the agglutination which it caused,

because IgG purified from rabbit antiserum against pseudoplasmodial cell

plasma membranes, at the same concentration (2.5 mg/ml) caused the cells

to clump (Fig. 6h) but only delayed development somewhat (Fig. 6d).

DISCUSSION

Prestalk cells contained three WGA receptors which were not

detectable in prespore cells. Two of these were glycoproteins with

approximate molecular weights of 95,000 and 105,000 and isoelectric

points of 6.7 and 7.0 respectively (West, 1978). The other occurred as

a broad band with an apparent molecular weight range of 30,000 to 45,000

and had a relatively acidic isoelectric point of 5.5 (West, 1978). These

molecules were plasma membrane molecules because in addition to occurring

in plasma membranes they were not detectable in three other subcellular

fractions. On the other hand, prespore cells contained two WGA receptors,

both glycoproteins with apparent molecular weights of 85,000 and 22,000

and isoelectric points of 7.4 and 6.0, respectively (West, 1978). These

two glycoproteins were also detected in prestalk cells but at much lower

levels. Both of these glycoproteins were present in the plasma membrane,

but also in distinct material in the 7700 g cell-free pellet. These

glycoproteins, when they occur in the 7700 g pellet, may be associated

with a developmentally controlled organelle, the prespore vesicle, which

pellets at 7700 g, contains antigens which cross-react with the spore

cell surface (Takeuchi, 1972), and is present at low frequency in the

prestalk region of the slug (Farnsworth and Loomis, 1976). It is thought

that these vesicles fuse with the plasma membrane as a part of sporulation (Hohl and Hamamoto, 1969), and thus it would be expected for glyco-proteins to be shared between these organelles. In conclusion, these two glycoproteins which distinguish prespore cells are present at higher frequency in the plasma membrane of prespore cells than prestalk cells, if the same proportion of each glycoprotein is present in the plasma membrane in both cell types.

In contrast to the picture for WGA receptors, there were, with one exception, no detectable differences between Con A receptors and proteins (identified using the stain Coomassie Blue) between prespore and pre-stalk cells. The exception was the prespore-cell specific glycoprotein at 22,000 daltons. Although this does not rule out relatively minor differences between these two types of cells in protein and Con A receptor composition, the most major differences analyzed for were in the WGA receptors. In general, it can be concluded that the protein compositions of prestalk and prespore cells are remarkably similar. It is remarkable that all of the detectable differences occurred in the plasma membrane.

Plasma membrane differences can be added to the list of several other properties which distinguish cells at the anterior end (prestalk cells) from those at the posterior end (prespore cells) of the pseudo-plasmodium. Prestalk cells have been found to contain higher levels of calcium ions (Maeda and Maeda, 1973) and cyclic AMP (Bonner, 1949; Pan et al., 1974; Brenner, M., personal communication). Prespore cells have been distinguished by prespore vescicles (Hohl and Hamamoto, 1969), PAS

staining (Bonner, Chiquoine and Kolderie, 1955), and higher levels of other

enzyme activities (Wright and Anderson, 1958; Takeuchi, 1960; Mine and

Takeuchi, 1967; Newell, Ellingson, and Sussman, 1969). However, the

molecules or properties of pseudoplasmodial cells which actually regu-

late differentiation into either prestalk or prespore cells and/or sorting

out of these cells are unknown.

It is likely that the plasma membrane lectin receptors are on the

cell surface rather than on the cytoplasmic face of the plasma membrane,

according to histological and microscopic studies in yeast (Boller,

Durr, and Wiemken, 1976) and mammalian cells (Nicolson and Singer, 1974;

Walsh, Barber and Crumpton, 1976). In addition, WGA and Con A bind

to intact cells of D. discoideum (Molday, Jaffe, McMahon, 1976) and

many of the Con A receptors have been demonstrated to be present on

the cell surface by lactoperoxidase labeling (Geltosky, Siu, and

Lerner, 1976). It is not proven, however, that all plasma membrane

lectin receptors reside on the cell surface, since in an amoeba a Con A

receptor seems to lie on both sides of the plasma membrane (Bowers

and Korn, 1974).

These presumed cell surface molecules which are different between

prestalk and prespore cells are potential candidates for creation or

regulation of the pattern of cell types in the pseudoplasmodium. The

molecules are absent from vegetative cells and are all except one

present in plasma membranes of cells from relatively young cell aggre-

gates (12 hr of development) (West and McMahon, 1977b). The exception

is a prestalk cell specific WGA receptor at 30,000-45,000 daltons,

which has a lower mobility in aggregation stage plasma membranes (West

and McMahon, 1977b). Thus these molecules, which may even be cell-type

specific in aggregates (which have not yet formed pseudoplasmodia), occur

prior to most other markers distinguishing the two cell types. As cell-

type specific molecules, they have been shown to occur prior to actual

formation of the stalk. The occurrence of cell type-specific cell

surface molecules are key to models for formation of cell patterns

such as sorting out of cells (Garrod and Forman, 1977; Sternfeld and

Bonner, 1977), the cell contact model (McMahon, 1973), the regional

model (McMahon and West, 1976), and others (reviewed in McMahon and

West, 1976).

This study has revealed other interesting information about glyco-

proteins in D. discoideum. The number of detectable glycoproteins in

the whole cell is at least as great as half the number of detectable

proteins, which is consistent with what has been found with plasma

membranes. Many of the glycoproteins in both vegetative and pseudo-

plasmodial cells are not present in the plasma membrane. Some are

apparently unique to the soluble fraction of the cell, while others

are unique to the mitochondrial pellet (which is contaminated with

other organelles) or the microsomal pellet. Studies made with purified

proteins or histochemical studies in other cell systems corroborate

these findings (Howard and Schnebli, 1977; Bosmann and Myers, 1974;

Nicolson, Lacorbière, and Delmonte, 1972; Michaels et al., 1977;

Virtanen and Wartiovaara, 1976). In addition, a number of intracellu-

lar glycoproteins are modified or lost, and others created, during

the course of development. In contrast, the protein pattern as deter-

mined by Coomassie blue is relatively constant in the whole cells and

subcellular fractions. This is also consistent with our observations
of the plasma membrane. The wide variety of glycoproteins identified
inside the cell seems to be in conflict with the hypothesis that glyco-
protein marks a protein for export to outside the cell or to the
plasma membrane (Eylar, 1965). If this hypothesis were true, all of
the glycoproetins identified in Fig. 2 or Fig. 3 would be at some
stage in the process of being exported from the cell or moved into the
plasma membrane.

While Con A receptors seem to be distributed throughout all parts
of the cell, WGA receptors are predominantly membrane bound and many
are strictly plasma membrane bound. This observation has been made in
other types of cells (Cautrecasas, 1974). In vegetative cells, 60%
of the detectable WGA binding species were restricted to the plasma
membrane. Although there were more WGA receptors in the mitochondrial
and microsomal pellets which could not be explained as plasma membrane
contaminants in pseudoplasmodial cells, WGA receptors were still pre-
dominantly localized in the plasma membrane. These findings regarding
the intracellular distribution of WGA receptors are suggestive of a
relatively high purity of the plasma membranes used in this and previous
studies.

EXPERIMENTAL PROCEDURES

Materials

FITC-WGA and FITC-Con A were obtained from Vector Laboratories

(Ignacio, California), and WGA (Grade A) was obtained from Calbiochem

(La Jolla, California). All monosaccharide hapten inhibitors were

supplied by Sigma Chemical Co. (St. Louis, Missouri), and NAG_5 was

the generous gift of Dr. M. Raftery (Caltech, Pasadena, California).

Calcofluor White ST was the product of American Cyanamide Co. (Bound

Brook, New Jersey).

Cells and Cell Fractionation

D. discoideum A3 cells were axenically grown. Vegetative cells

were collected as logarithmically growing cells and pseudoplasmodial

cells were vegetative cells which had developed on filters for 18 hrs

(McMahon et al., 1975). The pseudoplasmodia were comparable in

morphology to those pictured in panels 12 and 17 of Fig. 1.6 in Loomis

(1975). In preparation for cell fractionation, pseudoplasmodial cells

were scraped from filters into 1/2 PDF (McMahon et al., 1975) and both

vegetative and pseudoplasmodial cells were pelleted and resuspended

in a buffer containing 0.25 M mannitol, 0.01 M Tris buffer (pH 7.6),

and 0.01 M EDTA. A mitochondrial pellet was collected as described

in West and McMahon (1977a) as modified from Stuchell et al. (1975).

Ther supernatant from the first 7700 g pellet (Stuchell et al., 1975)

was centrifuged again at 7700 g for 15 min and the resulting supernatant

was centrifuged at 100,000 g for 65 min. This supernatant was recen-

trifuged identically to yield the cell supernatant, and the pellet

from the first 100,000 g spin was resuspended in the buffer containing

mannitol and also centrifuged again identically to yield the micro-

somal pellet. Plasma membranes were purified from the supernatant

above the mitochondrial pellet as described in West and McMahon (1977a)

(as modified from McMahon et al., 1977). All operations were performed

in the cold and the protease inhibitor phenylmethylsulfonyl fluoride

was added to saturation prior to cell lysis. Protein was measured

according to the method of Lowry et al. (1951).

In some experiments pseudoplasmodia were dissected to obtain prestalk

and prespore cells during the time period from 17.5 to 19 hrs of develop-

ment. Pseudoplasmodia were scooped from the filter in sets of five

with a scalpel blade. The pseudoplasmodia were covered with a drop

of 1/2 PDF and the scalpel was clamped to the stage of a dissecting

microscope. A second scalpel was used to cut each pseudoplasmodium

into four parts as shown in Fig. 3. As each part was cut off it was

either pushed to an extreme corner (parts 2 and 4, Fig. 3) of the blade

or drawn into a capillary tube mounted at the end of a closed piece of

plastic tubing whose internal volume was regulated by finger pressure

(parts 1 and 3, Fig. 3). Separate capillary tubes were used for parts

1 and 3 (Fig. 3). Cells drawn into the capillary tube were immediately

discharged into a denaturing solution containing 4.0% sodium dodecyl

sulfate (SDS), 10% (v/v) β-mercaptoethanol, 0.125 M Tris buffer (pH

6.8), 20% (v/v) glycerol and saturated with phenylmethyl sulfonyl

fluoride. As a result of discharging cells into this buffer it became

diluted about 2-fold with 1/2 PDF. Following dissection, the cell

suspension was incubated in boiling water bath for 5 min and then

frozen. In trial experiments, prestalk and prespore cells were solubilized in 2% sodium dodecyl sulfate and 20 mM Tris buffer (pH 6.8) and absorbance was measured at 280 nm. It was found that 4.0 prestalk parts contained the same amount of protein as 1.0 prespore part.

Solutions containing prestalk or prespore cells were concentrated under a stream of dry filtered N_2 at 30° prior to SDS-polyacrylamide gel electrophoresis. Gel lanes (2.9 mm x 1.0 mm) were loaded with approximately 50 prestalk regions or 12 prespore regions, or about 30-40 µg or protein each.

Electrophoresis and Carbohydrate Staining

Protein samples were solubilized in SDS and β-mercaptoethanol, and electrophoresed on discontinuous polyacrylamide gels ranging exponentially in acrylamide content from ten to fifteen percent as previously described (Hoffman and McMahon, 1977a).

SDS-polyacrylamide gels were fixed in methanol followed by glutaraldehyde and unreacted aldehydes were reduced with sodium borohydride as previously described (Tanner and Anstee, 1976). Fluorescein-derivatized lectins were diffused into the gels in the presence of carrier hemoglobin and washed out as described in West and McMahon (1977b) with the exception that lectin solutions were 4- to 5-fold more concentrated than previously described for the gels shown in Fig. 4. This permitted a higher sensitivity (the increased fluorescence remained sensitive to hapten inhibitors). Photography was as described (West and McMahon, 1977b) using a type 61 Wratten filter. Gels shown are representative of at least three independent experiments.

Histochemistry

Cellulose was assayed in intact pseudoplasmodia, using the fluorescent brightener Calcofluor, as described in Harrington and Raper (1968). The slug sheath did not present a barrier to diffusion to Calcofluor as similar results were obtained when it was torn first. Photography was in a Zeiss photomicroscope III equipped with eip-fluorescent optics.

Inhibition of Development

Pseudoplasmodia at 18 hrs of development were dissociated into single cells as described in McMahon et al. (1975) and incubated at 1.0×10^8 cells ml^{-1} for 15 min, on ice, in the presence of proteins or oligosaccharides dissolved in 1/2 PDF. The cells were then replated on filters and their morphological development was monitored hourly. A sample of cells was also diluted 25-fold in 1/2 PDF and observed in Zeiss photomicroscope III.

Acknowledgments

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CHAPTER V

A PHYSICAL EXPLANATION FOR MULTIPLE CELL-CLASSES AFTER

CENTRIFUGATION IN COLLOIDAL SILICA GRADIENTS

A Physical Explanation for Multiple-Cell Classes after Centrifugation in Colloidal Silica Gradients

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When cells of *Dictyostelium discoideum* were centrifuged to density equilibrium in linear gradients of colloidal silica (Ludox), approximately 40 discrete bands appeared. This suggested that there could be at least 40 different cell types. A similar result was found for formalinized red blood cells and plastic beads. Isolated bands of cells rebanded faithfully in new gradients and band spacing depended upon gradient steepness. However, the cell bands resulted from microscopic modifications in the linear gradients of Ludox caused by centrifugation. When the gradients were analyzed in the analytical ultracentrifuge, absorbance scans revealed that cell bands coincided with "bands" of Ludox, which formed even without cells. Evidence ruling out other possible causes for cell bands is presented. Additionally, procedures which avoid this condition are described.

Isopycnic centrifugation of cells in linear density gradients has often been successfully applied to the separation of cell types (1–4). While bovine serum albumin (BSA), Ficoll, colloidal silica (Ludox), and Renografin and related substances such as metrizamide have been usefully employed as gradient media for this purpose, Ludox seems to be best suited because of its low viscosity, lack of cell toxicity under proper conditions, extremely low cost, ease of preparation, and stability in the presence of divalent cations and polymers. Additionally, it is straightforward to make a constant and physiologic osmotic balance throughout the gradient.

Our initial experiments using colloidal silica gradients in an attempt to separate different cell classes indicated that there were 15 to 40 discrete cell types in populations of *Dictyostelium discoideum*. Similar results have been obtained with lymphocytes by Bach and Brashler using Ficoll gradients (21) and by Shortman using gradients of BSA (13,20). In our system, live or formalinized red blood cells (frbcs) or styrene–divinylbenzene (SDVB) latex beads also were separated into multiple bands after centrifugation to density equilibrium in Ludox gradients. Particle–particle associations, adherence of Ludox to the particles, and/or vibrations in the centrifuge were not responsible for the cell or particle bands. Further investigation revealed that the Ludox gradients were microscopically unstable above a critical $\omega^2 t$ of centrifugation and

TABLE 1

GRADIENT CHARACTERISTICS AND COMPOSITIONS[a]

(Gradient)	Figure		Density (g/ml)	mOsm	NaCl (mg)	Volume (ml)						
						H₂O	RG	0.11 M Na₂EDTA	HS-30 (D, dialyzed)	1 N HCl	12% PVP	KP
a	1a	H	1.075	185	0	11.99	0	2.80	49.40 (D)	0.82	26.90	7.75
		L	1.032	185	0	42.32	0	3.50	18.03 (D)	0.30	26.90	8.95
b	1b,c	H	1.114	300	0	11.36	0	0	52.40	3.23	26.90	6.11
		L	1.075	300	109	28.73	0	0	34.93	2.15	26.90	7.29
c	1d	H	1.105	300	44	18.39	0	0	45.33	2.79	26.90	6.58
		L	1.085	300	79	23.86	0	0	39.82	2.46	26.90	6.96
d	3a	H	1.265	770	0	38.30	61.70	0	0	0	0	0
		L	1.215	770	0	40.40	51.00	0	0	0	0	8.60
e	3b	H	1.069	300	0	34.17	0	3.64	42.10 (D)	1.55	0	17.5
		L	1.054	300	0	43.46	0	3.64	33.48	1.19	0	18.2
f	3c	H	1.210	300	43	4.5	0	0	84.44	11.06	0	0
		L	1.160	300	263	27.25	0	0	64.32	8.43	0	0
g		H[b]	—[c]	—	0	0	0	10.04	76.17	5.46	0	6.95
		L[b]	—	—	127	27.72	0	9.87	50.63	3.57	0	6.84
h	3d	H	1.209	—	0	12.80	27.80	0	34.50	1.50	25.00	0
		L	1.124	—	0	32.90	7.55	0	34.50	1.50	25.00	0
i	3e	H	1.168	—	0	19.15	17.50	0	41.15	2.50	20.00	0
		L	1.118	—	0	41.35	17.50	0	20.00	1.15	20.00	0
j			—	300	55	20.05	0	0	43.67	2.69	26.90	6.70
k	5		—	—	0	29.25	0	0	60.00	4.10	0	6.65
l	6		—	—	0	15.90	0	0	72.50	4.95	0	6.65

[a] Abbreviations: RG, Renografin-76; HS-30, Ludox HS-30; PVP, polyvinylpyrrolidone; KP, KCl-NaPO₄ buffer; H, high density component of the gradient; L, low density component of the gradient.

[b] Plus 10.96 g of recrystallized Eastman acrylamide and 0.41 g of recrystallized Eastman bisacrylamide, added first, and 103 μl of TEMED (Bio-Rad) and 1.29 ml of 30% (w/v) ammonium persulphate added following the HS-30.

[c] Not measured.

that this caused the multiplicity of cell bands. Once assured that the cell bands were not reflecting a biologically significant property, we found centrifugation conditions suitable for buoyant density cell separations in Ludox which were free from this artifact.

MATERIALS AND METHODS

Solutions. Ludox HS-30 (Dupont, 30% w/w) is a colloidal silica (5,6) consisting of particles 10 to 20 nm (mean, 15 nm) in diameter with an average molecular weight of 1.8×10^6 daltons. The suspension has a density of 1.221 g/ml and a milliosmolarity of 73 (freezing point depression measurement determined with an osmometer, Model 3L, from

Advanced Instruments, Newton Highlands, Mass.). Ludox HS-30 was occasionally dialyzed for 3 hr against a solution of 4 mM Na_2EDTA and 10 mM $NaPO_4$ (pH 7.2) followed by dialysis, twice for 8 hours apiece, against deionized distilled water to remove sodium and reduce the milliosmolarity (to 12). Polyvinylpyrrolidone (PVP; MW, 4×10^4 daltons) was dialyzed with the same procedure and then diluted to 12 or 20%, w/v, with water. KP buffer was 0.20 M KCl and 0.13 M $NaPO_4$ (pH 8.0) and 1.56 osM. Renografin-76 (RG) from Squibb had a density of 1.422 g/ml and an osmolarity of 1.25. The gradient properties and compositions are described in Table 1. For each gradient, there are entries H and L, the solutions used to form the gradients. The solutions were made at room temperature (except those for the acrylamide gradients, which were made at 5°C) immediately prior to use. The order of addition of components is important and corresponds to the order given by reading the columns of Table 1 from left to right. Additions subsequent to the Ludox required vigorous shaking for adequate mixing and to prevent gelling.

Cells and particles. *D. discoideum* strain A3 was raised on shakers in HL5 medium (7) at 22°C. The cells were harvested in log phase by centrifugation at 750g for 1 min in the cold, were washed and recentrifuged in a solution of 0.115 × KP and 0.011 M Na_2EDTA, and were resuspended in the same buffer. Human and sheep blood were collected into Alsever's solution. Sheep erythrocytes were formalinized by the method of Butler (26). Styrene–divinylbenzene (SDVB) latex beads were from Dow Diagnostics and were 5.7 ± 1.5 (standard deviation) μm in diameter. Blood cells and SDVB beads were suspended in a dilution of KP whose osmolarity equalled that of the L solution of the gradient in which the particles were spun.

Preparation and centrifugation of gradients. Gradients (11.4 ml) were prepared with a linear gradient former, unless otherwise stated. The gradient former was similar to that described in Ref. [12]. Solution L was pumped into the mixing chamber, which initially contained an equal volume of solution H. The contents of the mixing chamber were pumped into a gradient tube at a rate twice that of the entering solution L. Gradients with PVP constructed in this way were macroscopically linear after 45 min of centrifugation at 12,000g as determined by measuring their refractive index. The refractive index of the Ludox solutions was linearly related to density as measured pycnometrically (accurate to 3 mg/ml). One milliliter of cell suspension (concentrations ranging from 5×10^6 to 5×10^8 cells/ml, did not affect the results) was layered on the gradient or mixed with the H solution, which was modified in composition to compensate for the dilution, before making the gradient. Centrifugation was in a Beckman SW 41 (swinging bucket) rotor in a Beckman L2-65B or Sorvall OTD-2 ultracentrifuge; deceleration was without

brake from 3000 rpm down. Experiments with live cells were at 2°C, but at 20°C in other cases.

Analytical ultracentrifugation was in a Beckman Model E ultracentrifuge equipped with schlieren optics and a Beckman DU spectrophotometer. A Ludox solution was placed in the sample sector of a two-sector cell and water was placed in the other. Centrifugation was at 14,400g and 20°C, and absorbance measurements were made at 410 nm.

Analysis of gradients. Gradients were photographed immediately after removal from the centrifuge. An ISCO Model 183 gradient fractionator, in conjunction with an ISCO spectrophotometer flow cell apparatus, was used to fractionate the gradients. Occasionally a refractometer flow cell system (Waters Associates, R-401) was connected in series with the spectrophotometer. The reference solution for the refractometer was the L solution of the gradient being analyzed. The temperature of the refractometer optical system was controlled at 31.2 ± 0.1°C with a Lauda water circulator, and the refractometer was powered through a voltage regulator.

Microscopy. Thick (40 μm) and thin (0.06 μm) sections were observed in the light microscope, and thin sections were observed in the electron microscope. Thick sections were cut from frozen polyacrylamide gradient blocks with a Slee microtome (London) so that transverse bands were visible in the section. Polyacrylamide gradient blocks for thin sections were fixed in an aqueous solution of 5% glutaraldehyde and 0.04 M KPO$_4$ (pH 7.0) at room temperature. After 80 min, the blocks were rinsed in 0.2 M sodium cacodylate (pH 7.4) buffer and fixed further in cacodylate buffer containing 1% OsO$_4$ at 5°C. An hour later, the blocks were dehydrated through the following series of solutions: 90 sec in cold 50%, v/v, ethanol; 60 sec in cold 75% ethanol; 60 sec in cold 90% ethanol; 60 sec in cold 95% ethanol; 20 min in absolute ethanol at 22°C; and two more changes of absolute ethanol, 20 min apiece. The ethanol was decanted away and the sample was rinsed three times with propylene oxide (PPO). The blocks were kept for 18 hr at 22°C in a desiccator in a solution of PPO:epon:nadic methyl anhydride:dodecyl sulfonic anhydride:DMP-30 (50:11:11:27:1). They were transferred to the same solution lacking PPO for 12 hr at 22°C and then were polymerized for 72 hr at 60°C in an oven in this solution. Thin sections, cut with a manual ultramicrotome, were placed on perloidinized grids and post-stained with neutralized 0.2%, w/v, lead citrate. Electron microscopy was on a Philips 201 electron microscope at 60,000 V.

RESULTS AND DISCUSSION

When living *D. discoideum* cells were centrifuged into linear isotonic Ludox–PVP gradients (Table 1a) for 30 min at 12,000g, the cells distributed
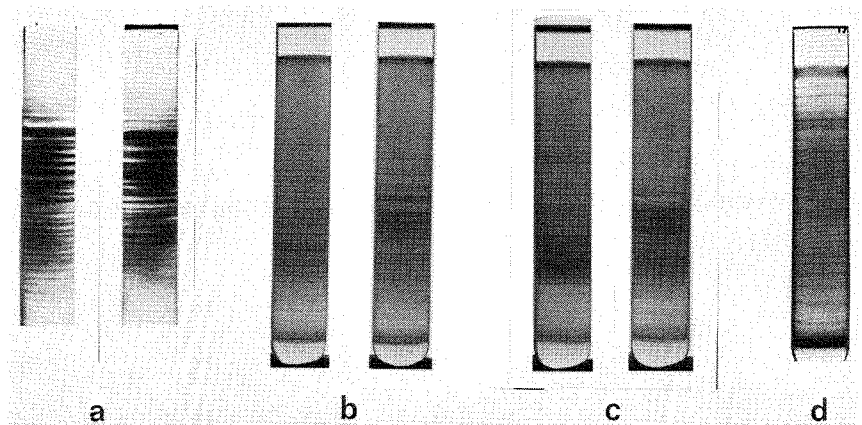
FIG. 1. Multiple bands of cells on linear Ludox–PVP gradients. (a) *Dictyostelium discoideum* cells were centrifuged at 12,000g for 30 min on duplicate gradients (composition in Table 1a). (b, c) Formalinized sheep red blood cells (frbcs) were centrifuged at 12,000g for 30 min (b) or 45 min (c) on duplicate steep gradients (composition in Table 1b). (d) frbcs were centrifuged for 30 min at 12,000g on a shallow gradient (composition in Table 1c). Gradients in all figures were formed on a linear gradient maker.

into about 40 distinct, visible, equally spaced bands (Fig. 1a), occupying a range of density from 1.045 to 1.070 g/ml. There were small differences in band spacing and relative band intensities between duplicates. The large number of bands was unexpected because other published reports describe at most four density classes of *D. discoideum* cells in sodium diatrizoate (8,9), metrizamide (10), or Ludox (11) gradients. Figure 2a shows an absorbance scan of a typical gradient when its contents were pumped upward through a spectrophotometer flow cell. This procedure also revealed the multiple bands. This population of cells formed a single band when centrifuged in renografin gradients (12,000g, 30 min; Fig. 3a; Table 1d).

We attempted to find differences between the populations of cells which composed different bands. Cells drawn from different regions of the gradient with a Pasteur pipet showed no distinguishable differences in size or morphology, and there was no detectable difference in aggregation of cells.

In other gradient media (12), red blood cells (rbcs) have a unimodal density distribution, so we examined their behavior in Ludox gradients. Live human rbcs also were separated into at least 35 clearly resolved bands. To avoid any difficulties which might be associated with living cells we also centrifuged formalinized sheep rbcs (frbcs). They had about the same average density and band spacing, and were separated into as many bands as live rbcs. Figures 1b and 1c show that frbcs centrifuged into duplicate linear isotonic Ludox–PVP gradients (Table 1b) for 30
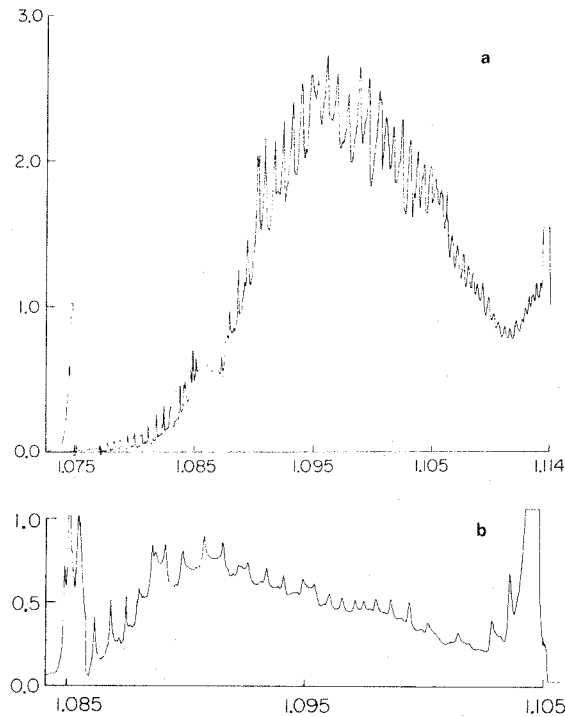
Fig. 2. Abscissa: density (g/ml); ordinate: absorbance (405 nm). Multiple bands of cells in density gradients of different slopes. These absorbance scans were obtained by pumping gradients centrifuged with frbcs through a recording spectrophotometer. (a) Steep gradient, shown in Fig. 4a. (b) Shallow gradient, as in Fig. 1d.

min (Fig. 1b) or 45 min (Fig. 1c) at 12,000g formed about 35 bands distributed over the density range 1.090 to 1.110 g/ml. The cells were deemed to have reached equilibrium buoyant density because the average position of the population did not change significantly on recentrifugation under the same conditions (see Fig. 1b,c). With longer times, the mean position of the cells slowly moved toward the bottom of the tube as the Ludox sedimented. In some duplicates, the bands seemed to match in one-to-one correspondence, but with others this was not so (Fig. 1b,c).

We varied several of the physical conditions in order to determine whether the pattern of bands would be disrupted. A similar multiplicity of bands was obtained when the cells were premixed with the dense solution of a linear gradient and then centrifuged. Centrifugation at a different temperature (2°C) had no effect on the number of bands or their average density. Removal of the PVP decreased the number of bands to about 15 but had no effect on the average density of the cells. Decreasing the pH to 3.6 in the absence of PVP, where the Ludox
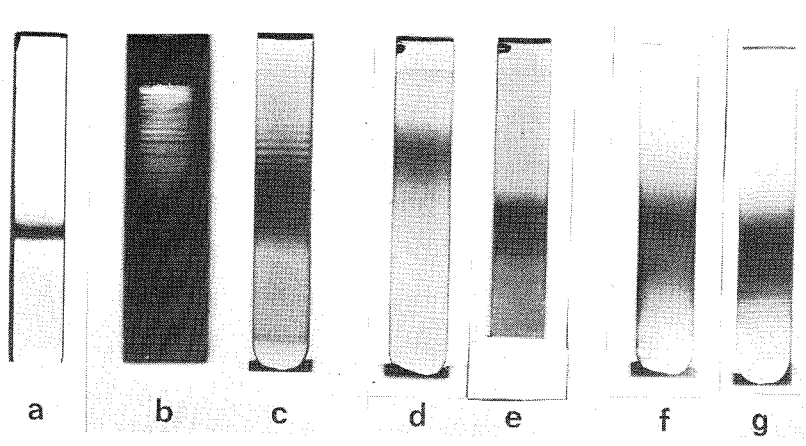
FIG. 3. Effects of miscellaneous conditions on the banding pattern. (a) frbcs centrifuged at 12,000g for 30 min in a linear Renografin gradient (composition in Table 1d) formed only a single band. (b) Plastic beads centrifuged at 25,000g for 20 min in a linear Ludox gradient (Table 1f) formed many bands. (c) frbcs centrifuged at 12,000g for 30 min in a linear Ludox (no PVP) gradient at pH 3.6 (Table 1e) formed fewer bands. (d) frbcs centrifuged on hybrid gradients of Renografin, Ludox, and PVP (see Table 1i), where only the Renografin concentration varied, did not form multiple bands. (e) frbcs centrifuged at 12,000g for 30 min in a hybrid Renografin–Ludox–PVP gradient where only the Ludox concentration varied (Table 1h). Renografin does not prevent bands from forming. (f) frbcs were centrifuged on a gradient like that in Fig. 1b at 3000g for 12 min. There is a broad distribution of cells but no bands. (g) frbcs were centrifuged in a gradient like that in Fig. 3f which was precentrifuged without cells at 12,000g for 15 min. Cells formed familiar multiple bands.

beads are essentially neutral in charge (5,6), increased the average density of the cells but did not change the number of bands (Table 1f; Fig. 3c). Decreasing the salt concentration decreased the average density of the frbcs but did not change the number of bands (data not presented). Finally, centrifugation of frbcs at 12,000g for as short a time as 4 min still resulted in multiple bands of cells (data not shown). Therefore this phenomenon was amazingly resistant to changes in the gradient composition and centrifugation conditions.

We were curious whether inanimate particles would behave similarly. To test this we centrifuged spherical SDVB latex beads under similar conditions. They formed about 17 bands when centrifuged to equilibrium on linear Ludox gradients (Table 1e) at 27,000g for 20 min (Fig. 3b).

A variety of mechanisms could potentially form the bands: (a) They could result because the population of cells (or latex beads) consists of multiple buoyant density classes; (b) interparticle adhesions might decompose a naturally smooth distribution of particles into bands; (c) the population could consist of particles which have the same density but bind (in a quantized manner) different amounts of a component of the

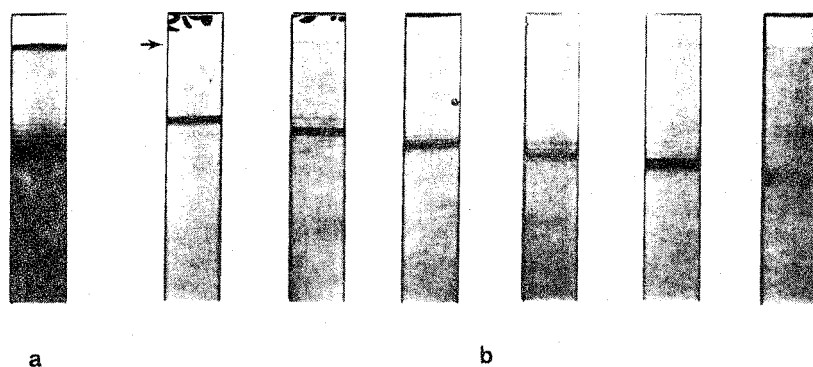a   b

FIG. 4. Isolated cell bands reband at a similar density. Gradient in (a), prepared and centrifuged as in Fig. 1b, was fractionated and alternating bands were centrifuged on new gradients. The order of gradients in (b) from left to right corresponds to increasing density of applied bands. The arrow refers to the buffer–gradient meniscus.

gradient, such as Ludox; (d) the gradients may be microscopically nonlinear in density. If proposition d is true, the macroscopic positions of cells are governed by their intrinsic buoyant densities, and microscopic discontinuities of density in the gradient distribute cells of a broad smooth density distribution into bands.

We initially examined the first possibility. If different bands reflect different density classes of cells, then they should be spaced farther apart in a shallower density gradient. In Fig. 1d, frbcs were spun on a gradient (Table 1c) whose range of density was one-half that seen in Fig. 1b, and the bands were about twice as far apart from one another. The increased band spacing was also evident when the contents of the gradient were analyzed by pumping them through a spectrophotometer flow cell to detect bands (compare Fig. 2b with 2a).

This potential property of bands, that they reflect discrete density classes of cells, was tested in another way. Single bands were isolated from one gradient and centrifuged into similar gradients. A gradient (Fig. 4a; Table 1b) was fractionated so that each fraction contained cells between adjacent minima in the A_{405} scan (Figs. 2a, 4a). Six fractions, selected from alternate bands, were diluted with isotonic KP and recentrifuged on six identically constructed gradients (Fig. 4b). The bands maintained their relative positions. However, the recentrifuged bands resolved into two or three approximately equal bands and additional minor ones. The possibility that there was contamination by cells from adjacent bands was further investigated in a gradient where the width of the bands was less than the interband distance (Figs. 1d, 2b). The gradient was fractionated so that each band was separated from adjacent interband regions as well as from adjacent bands. Each band
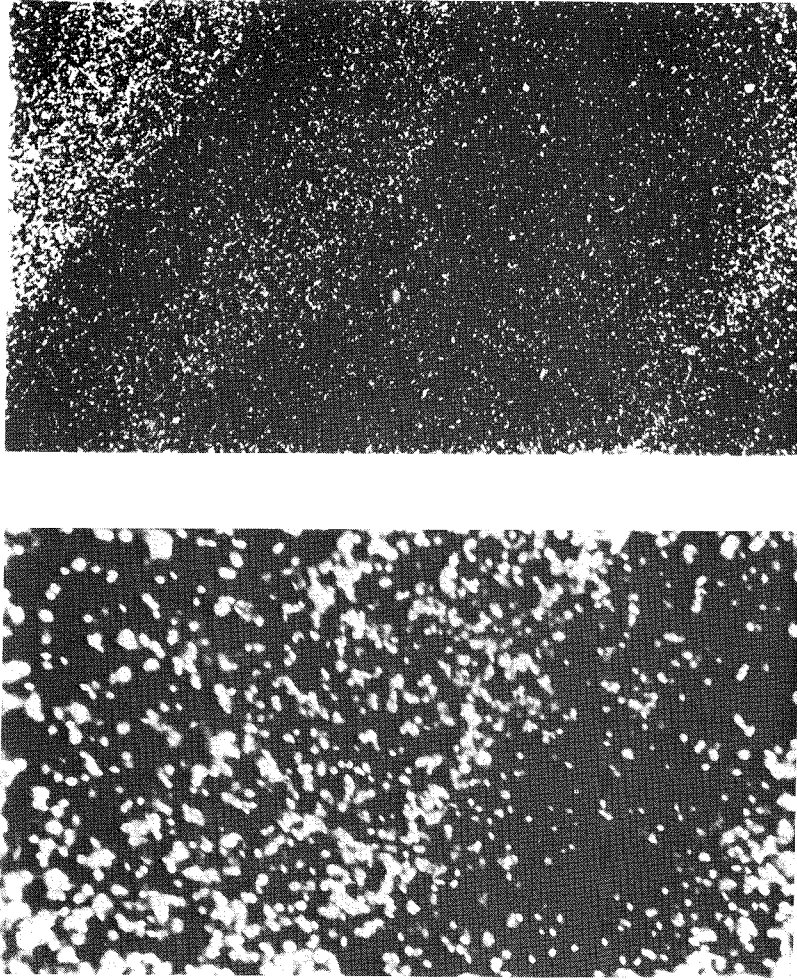
FIG. 5. Bands of cells in a Ludox gradient solidified with polyacrylamide, as seen in an optical microscope (dark field). In the upper panel (×60) parts of three bands of cells are presented in a thick section obtained from a gradient which was polymerized after centrifugation, with acrylamide and bisacrylamide which had been included in the gradient. The lower panel (×240) is an enlarged view.

returned to near its original position, but again resolved into a small group of bands.

This experiment allows two conclusions. It shows that, although the macroscopic position of a band of cells was preserved on recentrifugation, there was not a one-to-one correspondence between groups of cells and cell bands. The same group of cells which, after the first centrifugation was associated with a single band, distributed into several bands after

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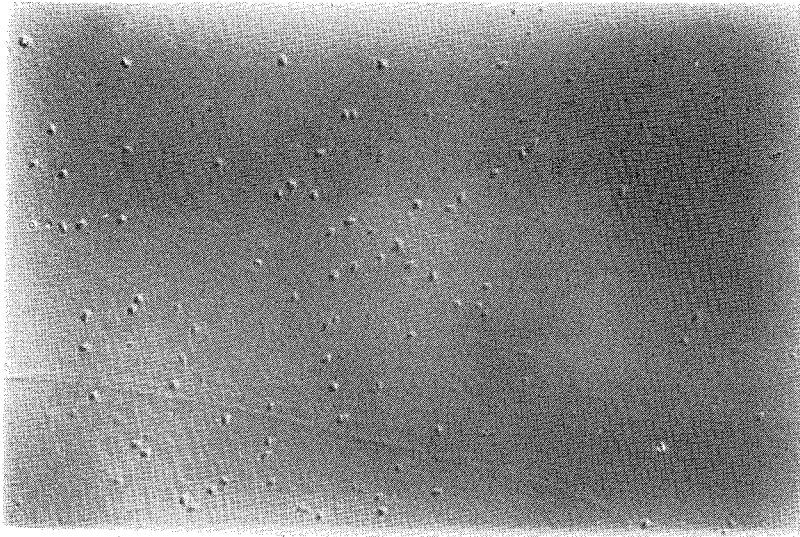
FIG. 6. Cells in a thin section cut from the gradient illustrated in Fig. 5 (×240), as seen with Nomarski optics.

the second centrifugation. Since it was unlikely that the density of a formalinized rbc would change on recentrifugation, multiple bands of cells did not reflect multiple density classes of cells. Acceptance of this conclusion requires that whatever is responsible for causing the bands spaces them by a mechanism dependent on the steepness of the density gradient.

According to the second hypothesis, cell-to-cell association could agglutinate cells and thus oppose the tendency for cells to achieve their true buoyant density. Such an effect could create local concentrations of cells and deplete adjacent regions of cells. The existence of intercell association was investigated directly by microscopic examination of gradients solidified with polyacrylamide. Acrylamide and appropriate catalysts and cross-linkers were premixed with the gradient components (see Table 1g) and their polymerization was stimulated during centrifugation by raising the temperature from 2 to 20°C. Under these conditions, there were eight or nine bands. But bands were observed even when the gradient was polymerized during the run. This result showed that the banding was not produced by some artifact such as vibration during deceleration. These solidified gradients were examined with a microscope for evidence of cellular aggregation. Thick sections (40 μm) examined in a light microscope revealed that the ratio of cells in a band to cells in an interband ranged from 2.5 to 4.0 (Fig. 5). Observations of several regions of the gradient showed that it was uncommon to see cells close enough to be touching. The same results were obtained with thin sections (Fig. 6). Therefore the cells are not agglutinated.

These thin sections were also examined in the electron microscope (Fig. 7) to test whether the position of a cell was obviously correlated to the amount of Ludox which it bound. The Ludox was clumped in these sections and was excluded from the cells. Most of the surfaces of the cells were covered with several layers of Ludox beads, but occasional patches of bare membrane were evident. There was no apparent correlation between the extent of Ludox binding and the position of cells in the gradient.

In order to test the possibility that the bands of cells represented classes of cells which bound varying quantal amounts of Ludox, Ludox was added to Renografin gradients (Table 1h) so that its concentration was equal throughout the gradient and similar to the concentration which occurs in Ludox–PVP gradients at the density at which the frbcs band. Although there was a much broader distribution of frbcs, compared to that in gradients of Renografin alone, multiple bands of cells did not form (Fig. 3d). Since Renografin might interfere with the postulated binding of Ludox, Renografin was added to Ludox gradients (Table 1i; Fig. 3e) and bands still formed. These results indicate that differential binding of Ludox is not responsible for forming bands, although it may alter the buoyant density of the cells.

To test whether the gradients contained density discontinuities introduced during mixing of the gradients, we first formed gradients by centrifuging an initially uniform solution of Ludox (Table 1j) for 90 min at 50,000g. Generating the gradients in this way should eliminate any potential artifacts of mixing, etc. Cells spun on these gradients still gave many bands (data not presented). Second, preformed gradients centrifuged with or without cells were pumped through a spectro-photometer flow cell, so that the refracteretogram could be analyzed for deviations from linearity at the positions of the bands. The results indicated that the gradient was linear. However, since mixing during analysis might have obscured microscopic discontinuities, we resorted to a technique of greater sensitivity.

A Ludox solution was sedimented at 14,400g in an analytical ultra-centrifuge and was observed with schlieren optics. When the solution (Table 1k) was centrifuged until a gradient formed, the Schlieren image dramatically demonstrated that the Ludox gradient was microscopically discontinuous (Fig. 8a). During the run, bands of Ludox appeared as a gradient began to form (Fig. 8b). The bands moved so that the pattern of bands changed considerably after several minutes. Throughout the run, the gradient has a larger slope near the Ludox sedimentation boundary, and in this region the bands of Ludox were more closely spaced. Cells were run on these gradients in order to confirm that conditions which formed Ludox bands also formed bands of cells. Figure 9 compares absorbance scans of Ludox gradients with and without frbcs (Table 1i). The number of cell and Ludox bands was similar, but the position of the
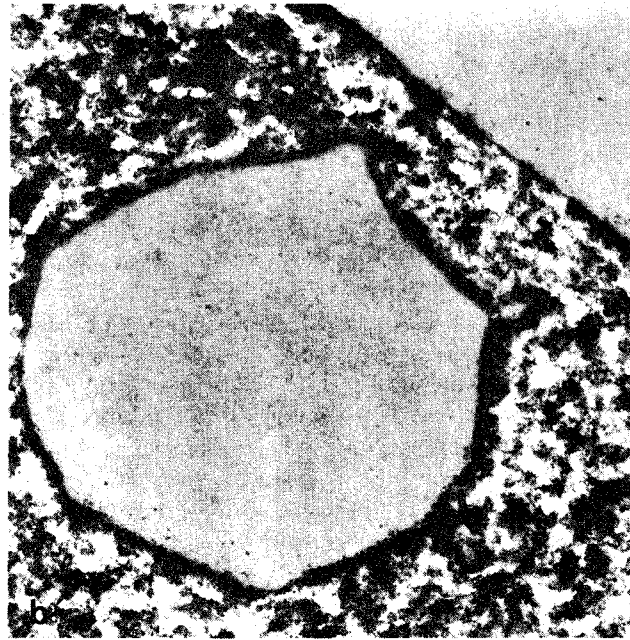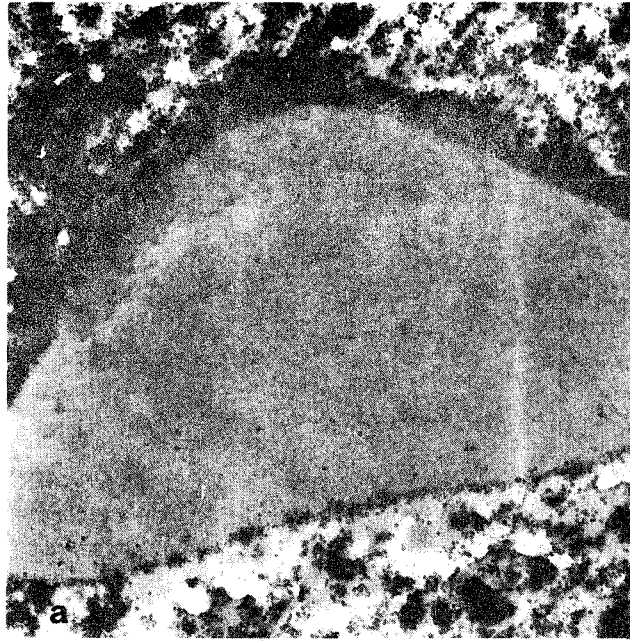
FIG. 7. Electron micrographs of thin sections of cells from the gradient presented in Fig. 5. Magnifications, (a) ×27,000; (b) 18,000; (c) ×4,500; (d) ×13,000.

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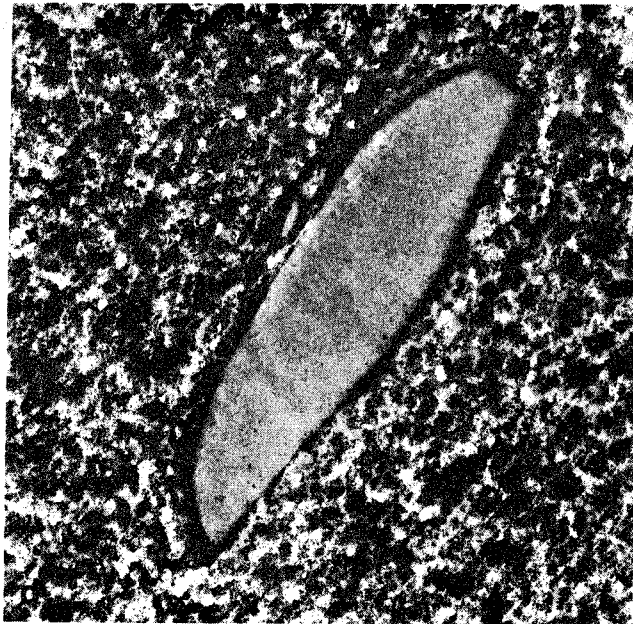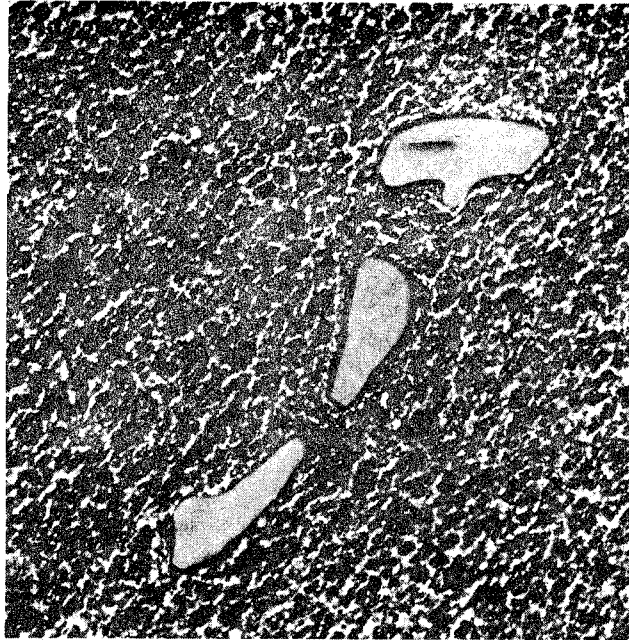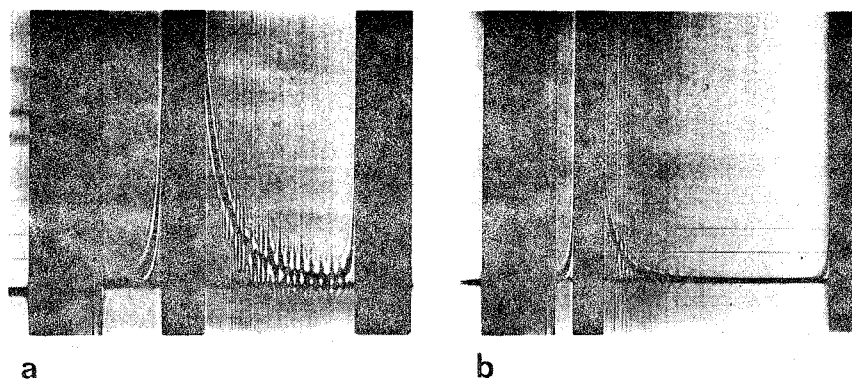Fig. 7 (*Continued*).

a   b

FIG. 8. Ludox discontinuities in the analytical ultracentrifuge. A Ludox solution (Table 1k) was centrifuged and photographed using Schlieren optics. Centrifugation was at 14,400g for 70 min (a) and 29 min (b), and the bar angle was 70° and 80°, respectively.

Ludox sedimentation boundary was somewhat affected by the presence of cells.

The properties of the Ludox bands allow us to explain some of the earlier results. The exact positions of the bands of cells did not match in position between identically made gradients because of the motion of the Ludox bands. Isolated frbc bands did not form single bands on recentrifugation because they may spread over more than one Ludox interband. Cells in Renografin gradients containing Ludox may not have formed bands because it seems that Ludox must be in a concentration gradient to form the Ludox bands (Fig. 8b). Cell bands were closer together in steeper gradients, and this was also true for Ludox bands observed in the analytical ultracentrifuge (Fig. 8a). In general, the number of bands a particle population forms seems to depend on the width of its intrinsic density distribution and on the spacing of the Ludox bands.

Our working hypothesis was confirmed in another way. When a gradient is centrifuged, there is a short interval between the time when cells reach their final average position and when they form bands. The delayed formation of the bands of cells is consistent with the requirement for the prior appearance of discontinuities of Ludox. Thus we determined whether conditions favorable for the formation of multiple bands could be produced in a gradient which did not contain cells. If frbcs were centrifuged at 3000g for 12 min, they seemed to reach equilibrium but not form bands (Fig. 3f; Table 1b). However, if cells were added to a gradient which had been precentrifuged at 12,000g for 15 min (without cells) and then centrifuged with cells at 3000g for 12 min, the cells formed bands (Fig. 3g). Thus the appearance of bands depended upon some change in the gradient itself produced by precentrifugation. We would expect this result if the bands of cells are generated by the microscopic discontinuities which appear in the Ludox gradient when it is centrifuged.

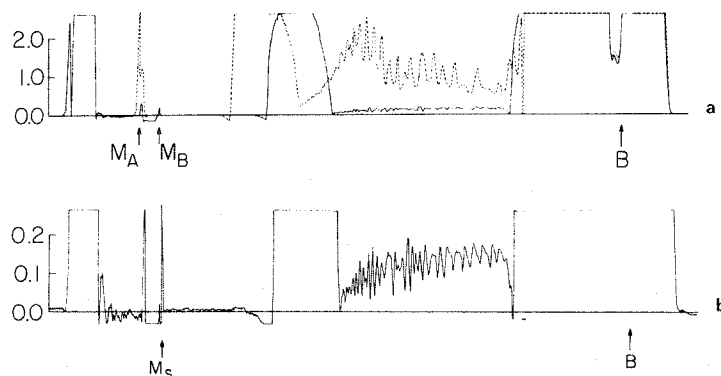MULTIPLE BANDS OF CELLS IN COLLOIDAL SILICA GRADIENTS   603

FIG. 9. Ordinate: absorbance at 410 nm. Cell bands and Ludox bands in the analytical ultracentrifuge, illustrated with absorbance scans. Centrifugation is from left to right at 14,400g. (a) A Ludox solution (Table II) was centrifuged with cells (– – –) and without cells (———) for 90 min; M_A and M_B denote the respective menisci, and B indicates the bottom of the cell. (b) The Ludox solution (without cells) illustrated in (a) was scanned on a more sensitive absorbance scale. M_S and B are the meniscus of the gradient and the bottom of the cell, respectively. The absorbance is large (offscale) in the region of the Ludox sedimentation boundary because the gradient of refractive index deflects light.

Similar discontinuities have been observed when solutions of serum albumin (14) and insulin (15) are centrifuged. In addition, microscopic banding of pseudoglobulin A (16), hemocyanin (16), lipoprotein (17), and TMV virus in Ludox (18) was visible in photographs contained in these references but not discussed by the authors.

What causes this behavior of macromolecules in a centrifugation field? Schachman and Harrington (15) found a similar phenomenon when a sharp synthetic boundary was formed between two concentrations (0.6% above a 0.9% solution) of insulin. Furthermore, they showed that the multiple bands formed only at an ionic strength (0.4), which permits reversible polymerization of insulin. The hypersharp boundaries (bands) did not occur at lower ionic strengths such as 0.1 and 0.2, where insulin exists mainly in a nonpolymerized form. This suggests that insulin molecules underneath the synthetic boundary sedimented faster than those above the boundary, which created an interface region where the density was less than that above. The result would probably be a convective disturbance to relieve the density inversion. The molecules of insulin in the lower solution probably sediment faster because of greater polymerization (due to the higher concentration) and a higher g force. Schachman and Harrington substantiated this interpretation by layering a solution of lactoglobulin (sedimentation constant, 2.8S) at 0.6% over a 0.9% solution of bovine serum albumin (sedimentation constant, 4.1S) and finding an additional Schlieren spike (band).

Our results with Ludox can apparently be interpreted in the same way. The Ludox must be in a concentration gradient for bands to form, even when Ludox is dispersed in a gradient formed by another molecular

species, Renografin (see Figs. 3e and 8b). We have not examined the properties of Ludox under conditions where we know that there is no association of the particles, but under our conditions they must associate with each other, since after several days the Ludox suspension gels.

These results indicate that some previously published cell separation experiments in the centrifuge should be reevaluated. When a cell population separates into several or more bands, it is necessary to establish the linearity on a microscopic scale of the gradient before it can be assumed that the bands represent qualitatively different cell density classes, and by implication qualitatively different cell types on the basis of physiology. As we have learned, even a rather thorough investigation of the microscopic properties of the gradients may be misleading. Since discontinuities generate bands, it is probably safe to use rbcs or plastic beads to assess lack of linearity or other anomalous behaviors when they are tested under the *same* conditions. These criteria have not been adequately applied in several experiments which have concluded that many discrete cell density types exist because many discrete bands of cells formed when the cells were centrifuged on density gradients (8,9,13,19–24,27).

Thus a population of cells with a relatively wide distribution of densities can behave as a number of discrete classes when centrifuged in a density gradient at a g force similar to that used in many cell separation experiments (8,9,12,13,19–21,23). When such multiple bands of cells form, it seems wise to examine the gradients for density discontinuities. If discontinuities are observed, they may be prevented by centrifugation at an $\omega^2 t$ beneath the critical threshold for gradient instability (3000g for 12 min was suitable for frbcs in Ludox gradients (Fig. 3f)), which must be determined experimentally.

ACKNOWLEDGMENTS

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