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THE UNIVERSITY OF ALBERTA

LECTIN-INDUCED AGGLUTINABILITY AND RELATED SURFACE PROPERTIES OF EMBRYONIC AMPHIBIAN CELLS

by

© BROCK R. FRASER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

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EDMONTON, ALBERTA

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Lectin-induced agglutinability and related surface properties of embryonic amphibian cells," submitted by Brock R. Fraser in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The purpose of this study was to examine the surface properties of embryonic amphibian cells just prior to gastrulation. Lectins were used as investigative probes. The effects of lectin dosage, cell fixation, and cell treatment with neuraminidase and a series of drugs upon lectin-mediated cell agglutinability were investigated.

Cells of early embryos of *Xenopus laevis* are agglutinable with wheat germ agglutinin, *Ricinus communis* agglutinin, soya bean agglutinin, and concanavalin A, but not with fucose-binding protein. This agglutinability is not affected by treatment of cells with local anaesthetics, cytochalasin B, colchicine, or with both cytochalasin B and colchicine in combination. Phenothiazine tranquillizers inhibit lectin-induced cell agglutination, this inhibition being drug-dose dependent. Treatment of cells with neuraminidase renders them more agglutinable with *Ricinus communis* and soya bean agglutinins, but does not alter their agglutinability with the other three lectins. Fixed cells are not agglutinable with wheat germ, *Ricinus communis*, or soya bean agglutinins, nor with fucose-binding protein. Concanavalin A agglutinates fixed cells slightly, as well as yolk platelets present in fixed cell suspensions.

Dissociated cells of embryonic *Xenopus* are active in the extrusion of large lobopodia which move about their smooth peripheries. Neither local anaesthetics nor colchicine alters this surface morphology. Fixed cells, cells treated with cytochalasin B, and cells treated with concentrations of phenothiazine tranquillizers which inhibit agglutination lack lobopodia and appear contracted at their peripheries.

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INTRODUCTION

Cells show selectivity in their interactions with their environment, whether the latter is cellular or non-cellular, artificial or natural. This selectivity is particularly evident during embryonic development, where it occurs in a highly controlled manner. Selectivity of cellular interactions is expressed in morphogenetic movements, the processes crucial to embryonic development whereby groups of cells are reorganized within the embryo. Morphogenetic movements demand locomotory as well as discriminatory capabilities of the cells involved, capabilities which must be closely regulated to ensure the successful completion of these processes. Since morphogenetic movements entail the exchange of pre-existing cell contacts for new ones of distinctly different specificities, the cells involved must be able to discriminate between like cells and unlike cells and between dissimilar types of unlike cells.

Gastrulation is the first and most extensive of the morphogenetic movements occurring during vertebrate embryogenesis. It has been studied in amphibians and represents a system wherein the molecular basis for cellular interactions can be investigated. Prior to gastrulation, the amphibian blastula can be described as a ball of cells surrounding a fluid-filled cavity. The cells that constitute the blastula differ from one another in morphological and biochemical characteristics such as cell size, yolk and RNA content, and protein synthetic activity (Balinsky, 1970). In general, for any given characteristic, the degree of expression either increases or decreases progressively along the animal-vegetal axis

of the embryo. Gastrulation transforms this embryo into a three-layered entity composed of outer ectodermal and inner endodermal cell layers, with mesoderm lying between the two. As gastrulation proceeds, the embryo acquires anterior-posterior and lateral axes, as well as an archenteron, or primitive gut. The movements of groups of cells during gastrulation are highly specific and, in at least some cases, are the result of intrinsic properties of the cells involved (Holtfreter, 1939; Cooke, 1975).

As the positions of groups of cells change relative to one another, the interactions between groups of cells must also change. Morphogenetic movements and the changes in cell-to-cell interactions that accompany them occur in a precise, non-random fashion with respect to time and space. Cells recognize other groups of cells and respond to them predictably, the response depending upon the types and specific stages of development of the cells concerned. The capacity for cellular recognition was clearly demonstrated by the classical investigations of several developmental biologists. Wilson (1907), studying the reaggregation of physically dissociated sponge cells, noted that this phenomenon is species specific. When interspecific mixtures of dissociated cells are allowed to aggregate, the cells reassociate into small aggregates composed exclusively of cells of one species or the other. A similar species specificity has been reported by Spiegel and Spiegel (1975) with respect to the reassociation of chemically dissociated cells derived from 16-cell sea urchin embryos. When cells of *Arbacia punctulata* and *Lytechinus pictus* are combined, initial reaggregation is non-specific. Within each aggregate, initially composed of a random mixture of cells of both species, the process of sorting out quickly begins. Single cells and cell clusters actively move about within the composite aggregates, "seeking

out" and adhering to those cells encountered which are of the same species. Spiegel and Spiegel also reported the detachment of species-specific groups of adherent cells from interspecific aggregates, the former moving and usually attaching to clusters of like cells elsewhere. The end result of this sorting out process is the formation of multicellular aggregates, each composed exclusively of cells of only one species. These aggregates may, under the appropriate culture conditions, continue to develop and differentiate into plutei.

Experimental evidence indicates that cells derived from a wide range of vertebrate tissues are capable of discriminating between different cell types. However, in contrast to the above described intercellular recognition based on species-related compatibilities, cells and tissues of vertebrate embryos more commonly exhibit recognition based on tissue-related compatibilities. This was first clearly demonstrated by Holtfreter (1939), using amphibian embryos at the gastrula and neurula stages. Excised pieces of tissue from different regions of the embryos, for example the marginal zone, neural plate, or presumptive ectoderm or endoderm, were cultured either alone or in various combinations with each other. Observations were then made of how individual tissue fragments reacted to the presence or absence of other tissues. These experiments established that inherent differences exist between different embryonic amphibian tissues with respect to their mutual adhesive properties. Holtfreter introduced the term selective "affinity" to describe these differences; he envisaged both positive and negative affinities operating between different tissue types, resulting in adhesive or repulsive forces generated between tissues placed in apposition. His investigations also demonstrated that over time such tissue affinities can change relative to

one another. Holtfreter hypothesized that these affinities were responsible for the responses elicited in his heterotypic tissue combination experiments. He further suggested that the tissue affinities observed *in vitro* may be of functional significance during normal development, more specifically when cells undergo morphogenetic movements.

In 1955, Townes and Holtfreter expanded these investigations, but with an important technical innovation which allowed them to extend the concept of selective affinity from the tissue level to the cellular level; the tissues being recombined were first dissociated into single cells. Dissociated cells of different tissue types were then mixed in various homotypic and heterotypic combinations and allowed to reassociate. It was found that heterotypic cell combinations reaggregate to form cohesive aggregates composed of random mixtures of cell types. Upon further incubation, cells within the aggregates sort out histotypically and frequently differentiate into structures similar to those normally formed by those tissues *in vivo*. These experiments demonstrated that the tissue affinities previously observed with intact tissue fragments were, in fact, the combined results of the affinities of the individual cells making up a particular tissue. Once again it was possible to correlate the temporal and spatial relationships observed between tissue types reaggregating *in vitro* with the corresponding relationships observed between the same tissues in the intact embryo.

Following the introduction of cell dissociation-reaggregation techniques to the study of cellular interactions, much additional evidence has been obtained supporting the concept of specific recognition between dissociated cells obtained from differentiating embryonic tissues. That even relatively undifferentiated cells from very early chick embryos

possess the discriminative capabilities necessary for tissue-specific sorting out following reaggregation has been shown by Zalik and Sanders (1974). In all such studies, the sequence of events observed is basically the same. Cells first recombine into aggregates of randomly associated cells; they then sort out into homotypic groups within the larger aggregates; the cells in these homotypic groups frequently reorganize and differentiate into tissue-specific structures.

It is thus evident that cells possess capabilities which allow them to recognize and respond in a predetermined manner to different kinds of cells. In the case of invertebrates, this specificity is often species-specific, while with cells of vertebrate embryos these differences are more often tissue-specific.

To obtain a more complete understanding of the nature of cell-to-cell interactions, it is necessary to investigate where in the cell this specificity resides and by what mechanism(s) cells express it. As for the question of the location of this specificity, experimental evidence suggests that it resides at the cell surface, a reasonable possibility considering that it is the surface of a cell which is in direct contact with the cell's environment.

Evidence implicating components of the plasma membrane in cell recognition and intercellular adhesion has been presented by Merrell and Glaser (1973). Studying the reaggregation of dissociated cells of various embryonic chick tissues, these investigators found that isolated plasma membrane vesicles can inhibit cell aggregation. This inhibition has been interpreted as resulting from the competition by membrane vesicles for the adhesive sites present on the surfaces of dissociated cells. The inhibition displays tissue specificity; the reaggregation of

neural retina cells is inhibited by plasma membrane vesicles isolated from neural retina cells, but not by similar vesicles from liver or cerebellum. In like fashion the aggregation of cerebellar cells is considerably inhibited by isolated plasma membrane vesicles from cerebellar cells, but is only slightly inhibited by neural retina plasma membrane. Using radiolabelled membrane preparations, these same investigators found that the tissue specificity of the inhibition of aggregation reflects a similar specificity in the binding of plasma membrane vesicles to dissociated cells. When compared to dissociated cerebellar cells, cells derived from neural retina bind four to five times the amount of labelled plasma membrane material isolated from neural retina. Recently, two trypsin-sensitive factors have been extracted and partially purified, one from plasma membrane preparations of neural retina cells, the other from similar preparations of optic tectum cells (Merrell *et al.*, 1975). Each factor exhibits a homotypic specificity in the inhibition of aggregation.

Among the most intensively studied cellular recognition phenomena are those involved in the immune response. Immunologically detectable differences exist between the cells of organisms of different species, as well as between the cells of organisms of the same species bearing different antigenic determinants. Examples of such differences are the histocompatibility antigens. In homeotherms, these antigens function in the immunological rejection of tissue transplants and are present at the cell surface (Nathenson, 1970). Similarly, the ABO(H) and Lewis blood group antigens which can elicit immunological reactions of great medical significance are found on the surfaces of erythrocytes. The immunospecificity of these antigenic molecules is known to reside in the exposed

terminal carbohydrate residues of the membrane glycoproteins and/or glycolipids (Ginsburg, 1972; Cook and Stoddart, 1973). Transformed cells are also believed to possess surface antigens capable of eliciting an immune response in the host organism. However, evidence suggests that these antigens may be masked so as to render the cells carrying them non-immunogenic (Braun, 1974). Simmons and Rios (1974) have reported that neuraminidase treatment of dissociated tumor cells can retard the growth and in some cases elicit the regression of established tumors when the treated cells are injected into syngeneic tumor-bearing hosts. This deleterious effect on the host tumor is immunospecific. The neuraminidase-treated cells must be of the same tumor type as the tumor borne by the host, and the tumor cells and the tumor-bearing host must be immunologically compatible. These experiments suggest that removal of exposed sialic acid residues from the surfaces of individual tumor cells in some way increases their antigenicity and enhances the immune response of the tumor-bearing host against injected neuraminidase-treated cells. This response is presumably cross-reactive with the cells of the established tumor in the host and may result in retardation of tumor growth and in some cases tumor regression.

These investigations on blood group antigens and tumor immunogenicity are cited not only to indicate the importance of cell surface moieties in the specificity of cellular interactions but also to show the importance of carbohydrate-containing groups in this specificity.

Many studies have implicated cell surface carbohydrates in cell aggregation and in the capacity of cells to recognize one another. Lloyd and Cook (1974) found that neuraminidase treatment of trypsin-dissociated malignant fibroblasts enhances the subsequent reaggregation of the cells.

Neuraminidase treatment also increases the agglutinability of these cells with *Ricinus communis* agglutinin (RCA) and soya bean agglutinin (SBA), lectins known to bind to exposed beta-galactosyl and N-acetyl-D-galactosaminyl residues respectively. To test whether these specific sugar residues, presumably exposed by sialidase treatment and capable of binding RCA and SBA, are directly involved in the enhanced cell aggregation, a series of glycoproteins differing in the nature of their terminal sugars were added to the reaggregating, neuraminidase-treated cells. These oligosaccharide residues, by interacting with surface sites which would otherwise be available for cell-to-cell adhesion, could perhaps inhibit the neuraminidase-enhanced aggregation. Among those glycoproteins tested were desialysed bovine submaxillary mucin, which has terminal N-acetyl-D-galactosaminyl residues, and desialysed, agalacto fetuin, which has terminal N-acetyl-D-glucosaminyl residues. Both molecules can inhibit the neuraminidase-enhanced cell aggregation. Desialysed fetuin and ovalbumin, which possess terminal beta-galactosyl and N-acetyl-D-glucosaminyl residues respectively, do not inhibit the enhanced aggregation. These results not only implicate specific sugar residues in intercellular adhesion in this system, but also imply that factors other than the presence of specific terminal sugars may be involved in this process. One exogenous glycoprotein bearing terminal N-acetyl-D-glucosaminyl residues inhibits aggregation while another glycoprotein carrying the same terminal sugar does not. Factors such as steric conformational differences between the two glycoproteins, or differences in the nature of the subterminal sugars or protein cores of these molecules may be important in glycoprotein-mediated cellular interactions and may explain this discrepancy.

A similar enhancement of cell aggregation resulting from treatment of cells with sialidase has been reported by Vicker and Edwards (1972). Working with freshly trypsinized hamster fibroblasts, these investigators found that upon transformation with polyoma virus, the cells lose much of their capacity to aggregate. While neuraminidase treatment greatly enhances the size and number of aggregates formed by non-transformed cells, removal of sialic acid residues from the surface of the transformed cells increases aggregation only slightly. The decreased aggregative capacity of the transformed cells does not, in this case, appear to be the result of an increase in the number of neuraminidase-susceptible sialic acid residues at the surface of the transformed cells. However, many other surface-related alterations are known to accompany cell transformation such as the shortening of the oligosaccharide chains of membrane glycolipids (Hakomori, 1975). Such alterations may be involved in the decreased capacity to aggregate and its neuraminidase insensitivity in this particular system.

In 1967, Roth and Weston introduced the collecting aggregate assay for the quantification of intercellular adhesion. Using such a technique, Roth (1968) and Roth *et al.* (1971) have provided evidence for tissue specificity in the adhesion of suspended cells to pre-formed cell aggregates. In the latter study, it was also found that treatment of aggregates of neural retina cells with beta-galactosidase enhances non-specific adhesion of cells to these aggregates, thus again supporting a possible role for cell surface carbohydrates in intercellular recognition and tissue-specific adhesion.

Carbohydrates have also been implicated in the adhesion of cells to non-cellular substrates. Chipowsky *et al.* (1973) studied the adhesiveness

of 3T3 cells, SV40-transformed 3T3 cells, and BHK-21 cells to Sepharose beads to which different sugars had been covalently bound. It was hypothesized that sugars so displayed to dissociated cells might mimic the carbohydrates exposed at the surfaces of potentially adhesive cells. These workers found that 3T3 and SV40/3T3 cells adhere to beads derivatized with galactose, but not to control beads or to beads carrying glucose or N-acetyl-D-glucosamine residues. BHK-21 cells did not adhere to any of the beads tested. These observations again implicate carbohydrates in the specificity of cellular recognition and adhesive properties.

In 1970, Roseman proposed a possible mechanism for intercellular adhesion implicating both carbohydrates and specific enzymes at the cell surface. Sites of adhesion are envisaged as enzyme-substrate complexes formed between complementary molecules present on the surfaces of aggregating cells. The enzymes are proposed to be glycosyltransferases, the substrates to be specific oligosaccharide moieties of exposed glycoproteins or glycolipids. Such complexes could be relatively stable, but easily alterable by the completion of the enzyme-catalysed reaction upon the addition of the appropriate sugar nucleotide. Catalysis entails the transfer of the sugar from the sugar nucleotide to a position on the oligosaccharide chain, the position occupied being specified by the particular glycosyltransferase involved in the reaction. This proposed mechanism is attractive as a testable hypothesis because it can account for many of the observed characteristics of intercellular adhesion such as specificity. A high degree of substrate specificity is inherent in several glycosyltransferases themselves (Cook and Stoddart, 1973). The model can also account for changes in specific cellular interactions, such as are common

during embryogenesis. The formation of enzyme-substrate complexes is not irreversible, and the completion of the catalysed reaction results in altered chemical properties of the cell surfaces involved, a factor possibly important in the alteration of specificity. The validity of this hypothesis is contingent upon the demonstration of plasma membrane-associated glycosyltransferases at the cell surface. Much evidence supporting the existence of such enzymes at the surface of a wide variety of cell types, both embryonic and otherwise, has recently been reviewed by Shur and Roth (1975).

The above cited studies are only a few of many investigations into the role of surface components in cell recognition and other cellular interactions. In particular, they implicate carbohydrate moieties in these processes. Cell surface carbohydrates, especially membrane-associated glycoproteins, have themselves been the focus of a great many investigations utilizing a wide variety of techniques. Among the more productive of these techniques has been the use of plant lectins to probe the nature of cell surface carbohydrates.

The lectins are a diverse group of proteins and glycoproteins of biological origin, but of unknown biological function. They have been isolated from a wide variety of plant and animal tissues (Lis and Sharon, 1973), with new additions to the list coming from increasingly diverse sources. Lectins are distinguished by their capability to reversibly bind specific sugar residues, different lectins having different sugar specificities. Lectins can bind to and precipitate dissolved polysaccharides and glycoproteins which bear the appropriate exposed sugars.

Each lectin molecule has two or more saccharide binding sites

making lectins bivalent or multivalent. It is this property which is largely responsible for the utility of these molecules in studying cell surface carbohydrates. The phytohemagglutinins, as lectins used to be called, have long been known for their capacity to agglutinate erythrocytes. This lectin-induced agglutination is believed to result from the binding of two or more sugar residues present on the surfaces of two opposing cells by a single lectin molecule. The lectin thus forms a reasonably stable cross-linkage between the two cells. The formation of many such linkages between cells is envisaged as the basis for the agglutination of cells.

Interest in lectins was greatly stimulated by two separate discoveries, both of which suggested the possibility of medically important uses for these molecules. Renkonen in 1948 found that extracts from the seeds of certain legumes exhibited blood type specificity in the agglutination of human erythrocytes. Subsequent investigations revealed several lectins specific for the human blood types A, AB and O(H).

The other significant discovery was the finding that a component present in wheat germ lipase preparations preferentially agglutinated transformed rat cells but did not have this effect on their normal counterparts (Aub *et al.*, 1963). This component was subsequently partially purified and named wheat germ agglutinin (WGA) and its preferential agglutination of transformed cells was confirmed (Burger and Goldberg, 1967). The agglutination of transformed cells by WGA could be inhibited by the addition of N-acetyl-D-glucosamine or its dimer, chitobiose. This inhibition was found to be specific for these sugars; other sugars at the same concentration were not inhibitory. In 1969, Burger demonstrated that non-transformed cells could be rendered agglutinable with WGA by

prior treatment of the cells with trypsin. Inbar and Sachs (1969) reported similarly enhanced agglutinability of transformed cells as compared to normal cells with another lectin, concanavalin A (Con A). As with WGA, non-transformed cells could be rendered agglutinable with Con A by trypsinization.

Because of the potential use of lectins as tumor-specific cell surface probes, research on the biological effects of these agglutinins on a wide variety of cell types has rapidly progressed and expanded. It was found that although the binding of lectins to cell surface sugar residues is required for the agglutination of cells, this binding in itself does not necessarily result in cell agglutination. It has been suggested that membrane fluidity and the mobility of lectin receptors within the membrane may be important in the mechanics of the agglutination reaction (Nicolson, 1974; Sachs *et al.*, 1974). Most recently, interactions of cell surface lectin receptors with sub-plasma membrane cytoskeletal structures sensitive to cytochalasin B and colchicine have been implicated in surface-related phenomena such as agglutination and cell capping (Edelman, 1976). It has also been suggested that intracellular levels of cyclic AMP may influence cell surface morphology, which may in turn determine the agglutinability of cells (Willingham and Pastan, 1975). However, in spite of this uncertainty as to the exact mechanism of lectin-mediated agglutination, the use of lectins is still a useful and sensitive experimental technique for studying cell surface characteristics.

Most lectin-related research in cell biology has utilized cells from adult tissues or cultured cell lines, comparing transformed to non-transformed cells. Developmental biologists have been relatively slow in

applying these probes to the investigation of embryological cell surface-related problems. However, in a few experimental systems, lectins have been utilized to study cell surface changes occurring during early development and differentiation. Weeks and Weeks (1975) have observed a steady decrease in the Con A agglutinability of cells of *Dictyostelium discoideum* as these cells proceed from the exponential growth phase to the stationary growth phase, and finally to the aggregation phase. Studying the specific binding of radiolabelled Con A to *D. discoideum* cells at different differentiative stages, Weeks (1975) has described a slight increase in the number of Con A receptors at the surfaces of cells approaching the aggregation phase. As well, he has noted a decrease in the affinity of surface receptors for Con A on aggregating cells. These changes in cell surface properties, as well as those detected by other techniques, may be involved in the changes in cellular interactions observed during slime mold differentiation.

Lectins have also been used to detect differences at the surfaces of embryonic sea urchin cells at various stages of development. Krach *et al.* (1974) showed that dissociated cells from embryos of *Strongylocentrotus purpuratus* are more agglutinable with Con A than with RCA, and that with both of these lectins agglutinability is greater for cells from gastrulae than from later stage embryos. Cells from embryos at any of the stages studied were not agglutinable with WGA. Trypsinization of embryonic cells could render them slightly agglutinable with this lectin. Roberson and Oppenheimer (1975), comparing the Con A-induced agglutinability of isolated micromeres and isolated mesomeres and macromeres from 32-cell sea urchin embryos found that the micromeres were significantly more agglutinable. Subsequent investigation of the interactions between Con A

and each of the three cell types making up the early sea urchin embryo have shown that differences exist in the distribution of Con A-bound receptors. The distribution of fluorescent-labelled Con A on mesomeres and macromeres is diffuse, while on micromeres the lectin is localized in large patches or caps on the surfaces of intact cells. This capping or clustering of Con A-bound receptors can be inhibited by prefixation of the cells (Roberson *et al.*, 1975).

Lectins have been used to investigate the surface properties of differentiating embryonic cells of developing chicks. Moscona (1971) showed that dissociated neural retina and liver cells from 10-day chick embryos are agglutinable with Con A, regardless of whether they have been dissociated by trypsin treatment or by calcium chelation. However, dissociated neural retina cells from the same embryos are not agglutinable with WGA unless they have been trypsinized. Subsequently, Kleinschuster and Moscona (1972) studied the comparative agglutinability of freshly dissociated, non-trypsinized embryonic cells from 8, 12, 16, and 20-day embryos. They were able to show that 8-day retina cells are massively agglutinated by Con A. However, as the age of the embryo increases, the Con A agglutinability of the cells decreases such that cells from 20-day embryos are agglutinated by Con A only after they have been trypsinized. Neural retina cells from embryos of all ages examined are not agglutinable with WGA unless they are first treated with trypsin. The effect of trypsinization on WGA-mediated agglutinability of neural retina cells increases with embryonic age. Neural retina cells from 8 to 20-day embryos are massively agglutinated by RCA with or without prior treatment with trypsin.

Zalik and Cook (1976) characterized the surface properties of embryonic chick cells using a greater variety of lectins and cell types. They tested the agglutinability of dissociated cells derived from unincubated blastoderms, from young embryos undergoing the early morphogenetic movements associated with avian gastrulation, as well as from differentiating embryonic tissues. Cells of early embryos dissociated with calcium-magnesium-free saline or with ethylene-diamine tetra-acetic acid (EDTA) are readily agglutinable with Con A, WGA, and RCA, but not with soya bean agglutinin (SBA) or fucose-binding protein (FBP). SBA-mediated agglutination can be induced by pre-treatment of the cells with neuraminidase; no effect upon RCA- or Con A-mediated agglutination is observed. EDTA-dissociated cells of 8-day liver or brain tissue are only slightly agglutinable with WGA, while liver cells similarly obtained from 10- or 12-day embryos show no agglutinability with this lectin. However, trypsin treatment of cells obtained from differentiating tissues increases their WGA-induced agglutinability to levels near or equal to that observed with cells derived from early chick blastoderms. Furthermore, Zalik and Cook, using radiolabelled WGA, found that EDTA-dissociated liver cells from 12-day embryos bind more lectin than do the same cells after treatment with trypsin, even though the former are not agglutinable with WGA.

The studies on development cited above clearly illustrate three points. The first is that the changes in the interactions between cells which occur during early embryogenesis and tissue differentiation are accompanied by changes in carbohydrate-associated components located at the cell surface. Secondly, these studies make clear the usefulness of lectins as sensitive probes in the detection of such changes at the surfaces of cells, and demonstrate the potential value of lectins in

elucidating the mechanism(s) of these changes. Finally, these studies illustrate the suitability of embryonic systems for investigations into the nature of cell surface changes.

For these three reasons the present study was undertaken to investigate the surface properties of early embryonic cells. The present state of knowledge concerning the morphological features of early amphibian development is relatively advanced; in no other vertebrate system are these features so well documented. *Xenopus laevis* has in particular been a favored organism for research on development. In addition, cells of the early *Xenopus* embryo which are following different differentiative pathways, for example the presumptive ectodermal and endodermal cells of the blastula, are distinguishable from one another morphologically. These factors led to the choice of embryonic cells of *X. laevis* for use in this study. Cells from late blastulae were used since at this stage of development, the embryos and presumably the cells composing them are preparing to undergo gastrulation. A number of plant lectins were used as investigative probes to study the nature of the carbohydrate moieties exposed at the surfaces of these cells. Cells were also fixed, treated with neuraminidase, or incubated in the presence of specific membrane active drugs to investigate the possible effects of such treatments upon lectin-cell interactions. The final goal of these investigations was to gain a better understanding of the biochemical and organizational nature of the surface components of cells and of the regulatory mechanism(s) involved in their functional control.

MATERIALS AND METHODS

A. PREPARATION OF SOLUTIONS

Four stock solutions were prepared as follows and designated A, B, C, and D.

A	NaCl	17.0 g + 100 ml distilled water
B	KCl	0.5 g + 100 ml distilled water
C	$Ca(NO_3)_2 \cdot 4H_2O$	0.8 g + 100 ml distilled water
D	$MgSO_4 \cdot 7H_2O$	2.05 g + 100 ml distilled water

All physiological salines were prepared as follows, combining the above stock salt solutions in the indicated proportions.

1. Steinberg's Physiological Saline (S.S.)

Prepared as follows:

A	10 ml
B	5 ml
C	5 ml
D	5 ml

Distilled water to 500 ml

TRIZMA pre-set pH crystals pH 7.8 (Sigma Chemical Company, U.S.A.) 0.280 g

pH adjusted to 7.8 with 0.1 N HCl when necessary

The molar concentration of this saline is 58.18 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.83 mM $MgSO_4 \cdot 7H_2O$, and 4 mM TRIZMA.

2. Calcium-Free Steinberg's Physiological Saline (C.F.S.S.)

Prepared as follows:

A 10 ml

B 5 ml

D 5 ml

Distilled water to 500 ml

TRIZMA pre-set pH crystals pH 7.8 0.28 g

pH adjusted to 7.8 with 0.1 N HCl when necessary

3. Calcium-Magnesium-Free Steinberg's Physiological Saline (C.M.F.S.S.)

Prepared as follows:

A 10 ml

B 5 ml

Distilled water to 500 ml

TRIZMA pre-set pH crystals pH 7.8 0.28 g

pH adjusted to 7.8 with 0.1 N HCl when necessary

4. Modified Calcium-Free Steinberg's Physiological Saline (m.C.F.S.S.)

Prepared as follows:

A 10 ml

B 5 ml

D 5 ml

Distilled water to 500 ml

TRIZMA pre-set pH crystals pH 7.2 1.545 g

pH adjusted to 7.0 when necessary

All salt solutions and salines above were prepared using triple distilled water. They were sterilized by autoclaving in glass bottles,

and stored at room temperature (22° C) until used.

5. Lectins

The following lectins were used in this study.

Wheat germ agglutinin (WGA) from *Triticum vulgaris* was purchased from Miles-Yeda Ltd. (Israel) as a 1.35 mg/ml aqueous solution (lot WGA 15).

Fucose binding protein (FBP) from *Lotus tetragonolobus* was purchased from Miles-Yeda Ltd. as a 3.3 mg/ml solution in phosphate buffer (lot 7).

Concanavalin A (Con A) from *Canavalia ensifórmis* was purchased from Sigma as a highly purified, salt-free, lyophilized powder (Grade IV, lot 123C-5500).

Ricinus communis agglutinin (RCA) from *Ricinus communis* was a gift from Dr. G. M. W. Cook of the Strangeways Laboratory, Cambridge, U.K. The lectin was prepared from castor beans using the technique of Nicolson and Blaustein (1972). The larger molecular weight fraction (RCA 120; molecular weight 120,000) was used.

Soya bean agglutinin (SBA) from *Glycine max*, also a gift from Dr. Cook, was prepared by the method of Lis et al. (1966).

Lectins were dissolved or diluted in C.M.F.S.S. to obtain stock solutions of appropriate concentration, and these stock solutions were stored at -20°C.

6. Sugar Solutions (Haptens)

Haptens were made up as 0.1 M stock solutions, dissolved in C.M.F.S.S. and were stored at -20°C until used. Alpha-methyl-D-glucoside

(lot 82C-1010), alpha-methyl-D-mannoside (lot 52C-3870), and N-acetyl-D-glucosamine (lot 31C-3000) were obtained from Sigma. Beta-lactose was obtained from Nutritional Biochemical Corp. (U.S.A.), and N-acetyl-D-galactosamine (lot X3738) was obtained from Schwarz/Mann (U.S.A.).

7. Drug Solutions

The local anaesthetics procaine hydrochloride (Matheson, Coleman, and Bell, U.S.A.) and xylocaine hydrochloride (Astra Pharmaceuticals Ltd., Canada) were obtained as crystalline powders and were dissolved in either C.M.F.S.S. or m.C.F.S.S. to obtain stock solutions.

The phenothiazine derivatives chlorpromazine hydrochloride and prochlorperazine (methane sulphonate) were obtained in crystalline form from Poulenc Pharmaceuticals Ltd., Canada, and stock solutions were prepared in m.C.F.S.S. The third phenothiazine derivative, trifluoperazine, was donated by Mowatt and Moore, Ltd., Canada (trade name "Clinazine"). Crystalline trifluoperazine was dissolved in m.C.F.S.S. to obtain stock solutions. The local anaesthetics and phenothiazine tranquillizers, in both crystalline form and as stock solutions, were stored at room temperature in the dark, since phenothiazines are photosensitive.

Colchicine was purchased from Sigma (lot 77B-1580). Stock solutions were prepared in C.M.F.S.S. and stored at 4° C.

Cytochalasin B was purchased from Aldrich Chemical Co., U.S.A. (lot PH/2435/60E). Stock solutions were dissolved in dimethyl sulphoxide (Baker Chemical Co., U.S.A.).

8. Glutaraldehyde Fixative

Glutaraldehyde was purchased as a 70% aqueous solution from Ladd Research Industries (U.S.A.). The fixative was prepared by diluting this stock solution to 7% glutaraldehyde with C.F.S.S., adjusted to pH 7.8, and stored at 4° C.

9. Agar

A 2% (w/v) agar solution was prepared by dissolving Special Agar-Noble (Difco Laboratories, U.S.A.) in heated C.M.F.S.S. After cooling the agar in dialysis tubing, it was dialysed for 24 hours at 4° C against C.M.F.S.S. The agar was then melted, divided into 25 ml aliquots, sterilized by autoclaving, and stored at 4° C until used to line agglutination and fixation dishes.

B. EXPERIMENTAL PROTOCOL

1. Obtaining and Staging Embryos

Embryos of *Xenopus laevis* were obtained by hormonal stimulation of sexually mature animals. The frogs were injected subcutaneously in the early afternoon with human chorionic gonadotrophin (Antuitrin "s", Parke-Davis, Canada), females receiving 1,000 I.U., males 500 I.U. In the late afternoon they were paired and left overnight in either dechlorinated tap water or in distilled water. Embryos were collected the following morning and staged as to the degree of development using the developmental tables of Nieuwkoop and Faber (1967). Those approaching the onset of gastrulation, stage 9, were transferred to a 10° C incubator to retard development. Younger embryos were allowed to develop

at room temperature to stage 9, at which time they were also transferred to the 10° C incubator. Embryos incubated at 10° C develop very slowly. However, upon return to room temperature, those held at 10° C for up to 24 hours resume normal development to the hatching stage. Embryos transferred to the 10° C incubator were used in experiments within 4 hours following transfer.

2. The Dissociation of Embryos

Stage 9 embryos were transferred to S.S., where the investing jelly coats and vitelline layers were removed manually using watchmaker's forceps. These embryos were then transferred to fresh S.S. and held on ice until a sufficient number was collected. Dissociation was carried out as follows. Embryos were transferred to C.F.S.S. pre-chilled to the temperature of melting ice; they were incubated at this temperature for approximately five minutes, at which time the saline was replaced by fresh cold C.F.S.S. The embryos were then gently pipetted with a wide-mouth Pasteur pipette to facilitate dissociation. The resulting cell suspension was centrifuged in an International clinical centrifuge for five minutes at 19 x g. The cells were located in a loose pellet while cell debris remained in the supernatant. The pellet was then resuspended in fresh cold C.F.S.S., pipetted further if necessary to achieve complete dissociation into a single cell suspension, re-centrifuged, and resuspended in cold C.F.S.S. This cell suspension was held on ice until the cells were used in subsequent experiments.

3. Assay for Lectin-Induced Agglutinability

Agglutination as determined in this study is defined as the agglomeration of cells via multivalent ligand molecules (for example,

lectins) added exogenously to the cell suspension. In contrast, cell aggregation is an endogenous process dependent upon intrinsic properties of the cells themselves. When cell aggregation and agglutination occur simultaneously, it is difficult to distinguish between the two phenomena. Consequently, cell aggregation interferes with the assessment of agglutination.

Since the presence of calcium in the medium results in rapid aggregation of cells, freshly dissociated cells suspended in ice-chilled C.F.S.S. were used in agglutination tests. Cells were tested for agglutinability mediated by the following lectins: Con A, WGA, FBP, SBA, and RCA. Tests were performed either within porcelain rings mounted on glass slides, or in agar-lined wells of "Linbro multi-dish disposotrays" (Linbro Chemical Co.). Each of these substrates has advantages and disadvantages in the assessment of cell agglutination. When using glass slides, a few dissociated cells tend to adhere to the substrate and cannot be dislodged by the gentle rotation of the slide used to bring the cells into contact with each other in the vortex created. This attachment decreases the number of cells able to participate in the agglutination reaction. However, the glass substrate is useful in that it does not promote cell-to-cell aggregation. On the other hand, dissociated cells do not adhere to the agar substrate; all of the cells being tested can participate in the agglutination reaction. However, the agar substrate promotes cell-to-cell reaggregation. On the average, cells suspended in C.F.S.S. on an agar subs to reaggregate approximately ten minutes after transfer to reas cells transferred to a glass substrate frequently gregation for twenty minutes after transfer. This a aggregation is not of importance

when the agglutination reaction is fast. This is the case for agglutination mediated by all the lectins used except SBA. In this latter case, agglutination is slow enough to be confused with cell reaggregation, and thus glass substrates were always used in tests with this lectin. Additionally, agar seems to interfere with RCA-mediated cell agglutination (decreasing the extent of agglutination observed with a given amount of RCA). Agar is a polysaccharide containing D- and L-galactosyl residues (O'Neill and Stewart, 1956), the latter of which is the sugar hapten for RCA. The agar may be binding lectin which would otherwise bind to the cells. For this reason, all RCA-mediated agglutination tests were performed on glass slides. Agglutination tests with the other three lectins WGA, FBP, and Con A, were carried out on both glass and agar substrates, with comparable results being obtained with either substrate for each lectin.

Agglutination tests were performed in total volumes of 50 or 60 microliters, depending upon the desired lectin concentration and the concentrations of the lectin stock solutions. Final lectin concentrations account for dilution resulting from the addition of C.M.F.S.S., hapten solutions, and/or cell suspension. Hapten specificity of the agglutination reaction was determined by addition of specific sugars to the agglutination mixture to give a final concentration of 0.03 M. Cells were always added to the agglutination mixture after all other components were added. In tests on the inhibition of agglutination by haptens, the sugar and lectin were allowed to mix before the cells were added. Upon the addition of cells, the agglutination plate was gently rotated by hand to bring the cells into contact with each other. Agglutination was then

scored visually under a dissecting microscope.

When agglutination was evaluated, plates were vigorously shaken by reciprocal motion to disperse non-agglutinated cells. Agglutination was scored on a scale of 0, +, ++, +++, ++++, a technique modified from that of Inbar and Sachs (1969). This modified scale for quantifying agglutination represents the following:

0 - the number of "agglutinated" cells in the cell suspension in the absence of lectin at time zero. This base line takes into account those cell clusters sometimes present in cell suspensions.

\+ - up to 30% increase in the number of cells agglutinated over the base line.

++ - 30-60% of the cells agglutinated over the base line.

+++ - 60-90% of the cells agglutinated over the base line, with the cells agglutinated into small clusters of ten cells or less.

++++ - more than 90% of the cells agglutinated over the base line, with the cells agglutinated into clusters containing more than ten cells each.

Although this assay is rather subjective, it does provide a basis for assessing the extent of agglutination induced by lectins.

The dose dependency and hapten specificity for agglutination mediated by each lectin was determined in this manner. Upon completion of agglutination tests, some of the remaining saline-suspended cells were observed under high magnification (X 400) by phase-contrast microscopy for general appearance and surface morphology.

4. Neuraminidase Treatment of Dissociated Cells

Cell suspensions were prepared as described above. Cells were then resuspended in C.M.F.S.S. to which 0.005 M $CaCl_2$ had been added. This exogenous calcium is required for the activity of the neuraminidase used. To the experimental cell suspension neuraminidase from *Vibrio cholerae* was added to a final enzyme concentration of 60 units per milliliter. This enzyme is stated by the suppliers (Hoecht Pharmaceuticals Ltd., Canada) to be free of protease, aldolase, and lecithinase C activities. Controls consisted of cells suspended in the above-mentioned saline plus calcium, but with the enzyme omitted.

Cell suspensions were incubated one hour at room temperature. Cells were then quickly rinsed twice in ice-chilled C.F.S.S. Because both control and experimental cells formed loose aggregates during the incubation period, the cells were redissociated by gentle pipetting and were then tested for lectin-induced agglutinability as outlined above.

5. Treatment of Cells with Local Anaesthetics

Cells dissociated as described above and suspended in C.F.S.S. were treated with either procaine or xylocaine. Drug stock solution was added to experimental cell suspensions to obtain the desired final drug concentration. Both control cells suspended in C.F.S.S. and experimental cells were incubated one hour at room temperature, after which the cells were tested for agglutinability as described above. Experimental cells were added to the agglutination dishes while still in the presence of procaine or xylocaine.

In some experiments, following dissociation with C.F.S.S., cells were centrifuged and resuspended in m.C.F.S.S. In these cases the drugs

were dissolved in m.C.F.S.S. and added as noted above to the experimental cell suspensions. Control and drug-treated cells were incubated one hour at room temperature, after which agglutination tests were performed. Local anaesthetic-treated cells were examined at high magnification by phase-contrast microscopy for general morphological features.

6. Treatment of Cells with Phenothiazine Tranquillizers

The phenothiazine derivatives whose effects were studied are not consistently soluble in saline solutions at pH 7.8. The hydrochlorides of chlorpromazine and trifluoperazine when added to cell suspensions also lowered the pH drastically. For these reasons the drug stocks were made up in m.C.F.S.S. (pH 7.0) and cells were resuspended in m.C.F.S.S. following dissociation with C.F.S.S. The drugs were soluble at pH 7.0, and the increased concentration of TRIZMA buffer in the modified saline helped to maintain the pH within physiological limits.

Drugs were added to experimental cell suspensions to give various final concentrations. Control cells in m.C.F.S.S. and drug-treated cells were incubated at room temperature for 30 to 60 minutes, after which both groups of cells were tested for agglutinability. Cells were added to the agglutination plates while suspended in the incubation medium. Drug dose responses were performed for the effects of phenothiazine tranquillizers upon lectin-mediated cell agglutination. Control and phenothiazine-treated cells were examined for surface morphology at 400X magnification using phase-contrast microscopy.

In some experiments the reversibility of the drug-induced effect was investigated. Control and drug-treated cells were washed up to three times with either m.C.F.S.S. or C.F.S.S. After washing, both

groups of cells were tested again for agglutinability. In some cases these washed cells were incubated in drug-free saline for up to two hours and retested for agglutinability.

7. Treatment of Cells with Colchicine and/or Cytochalasin B

Colchicine and/or cytochalasin B were added to cells suspended in C.F.S.S. to obtain the desired final drug concentrations. Dimethyl sulphoxide was added to the controls for the cytochalasin B experiments, while controls for colchicine experiments were cells suspended in C.F.S.S. Control and drug-treated cells were incubated one hour at room temperature, after which the cells were tested for WGA- and RCA-mediated agglutinability. Control and drug-treated cells were also examined for general morphology under phase-contrast microscopy.

8. Fixation of Cells

Fixation of cells was carried out in agar-lined 15 x 60 mm plastic petri dishes at 4° C. In addition to coating the sides and bottom of the petri dish with agar, a large mound of agar was formed in the center of the dish. The petri dish was fastened to a rotary shaker operating at 80 rpm and a volume of freshly dissociated cells suspended in cold C.F.S.S. was placed in the dish. A volume of 7% glutaraldehyde equal to the volume of the cell suspension was then added drop-wise to the cells in the dish. The agar substrate and rotary motion prevented cells from attaching to the petri dish and kept them in suspension while the agar mound in the center of the dish kept the cells from accumulating and settling out in the vortex created by the rotation. Cells were fixed for 24 hours at 4° C after which they were washed three times with ice-cold

C.F.S.S. These fixed cells were then tested for lectin-mediated agglutinability.

RESULTS

Much of the data presented here represent results pooled from several experiments, each using embryos from different clutches. When using embryos from outbred adults, variability between clutches is commonly observed in such characteristics as the degree of pigmentation and size of the embryos, and the texture of the enveloping jelly coats. Variations in agglutinability of cells derived from different clutches have also been observed. However, in spite of this variability, certain surface properties of cells in this system were consistently demonstrated.

A. AGGLUTINABILITY OF DISSOCIATED CELLS

Lectin dose response experiments were performed for each agglutinin to establish optimal and suboptimal lectin concentrations. The optimal concentration refers to the least amount of lectin which, when added to cells, can elicit a near maximal agglutination response within 2 minutes following treatment with WGA, RCA, and Con A, or within 10 minutes following treatment with SBA. The suboptimal concentration refers to the minimum concentration of lectin which elicits a slight, but clearly observable agglutination response within the time intervals specified above.

The dosage effects of WGA, RCA, Con A, and SBA on cells of *Xenopus* embryos are presented in Tables 1 to 4. The effect of increasing concentrations of WGA on cell agglutination is shown in Table 1. A dose dependency of WGA-mediated agglutination is apparent. Near maximal

TABLE I

DOSAGE EFFECT OF WHEAT GERM AGGLUTININ

WGA (μG/ML)	INCUBATION TIME 2 MIN	5 MIN	10 MIN
0	0	0	0
10 (5)	+	+	++
20 (4)	++	+++	+++
33 (>10)	+++	+++	++++
40 (4)	+++	++++	++++
50 (4)	++++	++++	++++
*HAPTEN + 33 (6)	0	0	0

*0.03 M N - ACETYL - D - GLUCOSAMINE

FIGURES IN PARENTHESES INDICATE THE NUMBER OF EXPERIMENTS SUPPORTING THESE DATA.

agglutination occurs during a 2-minute incubation of cells with 33 µg/ml of WGA (Figure 1a), making this the optimal WGA concentration. The extent of this massive response is made evident by a comparison between Figures 1a and 1b, the latter of which shows cells not agglutinated. The suboptimal concentration for this lectin is approximately 10 µg/ml. The agglutination response to WGA is also time-dependent at optimal or lower than optimal lectin concentrations, with agglutination increasing with incubation time (Table 1). When compared with other cells, WGA-induced agglutination of embryonic *Xenopus* cells is very rapid at optimal lectin concentration. The response is near maximal within 2 minutes; incubation for an additional 8 minutes does not appreciably increase the extent of agglutination. In addition, Table 1 shows the complete inhibition by the hapten N-acetyl-D-glucosamine of agglutination induced by optimal concentrations of WGA (Figure 1b).

Xenopus blastula cells also agglutinate in the presence of RCA (Table 2). The results obtained with this lectin are very similar to those reported for WGA. The extent of agglutination at any given incubation time is directly proportional to lectin concentration up to the optimal RCA concentration of 33 µg/ml. The response at optimal concentrations is, as with WGA, relatively rapid, the majority of cells being agglutinated within 2 minutes (Figure 2a). Since at 20 µg/ml, RCA produces submaximal agglutination, and at 10 µg/ml it fails to elicit a detectable response after 2 minutes of incubation, 16 µg/ml was chosen as the suboptimal concentration of this lectin. Beta-lactose, added to a final concentration of 0.03 M, completely inhibits cell agglutination induced by optimal (Figure 2b) or higher RCA concentrations up to 50 µg/ml (Table 2).

TABLE II

DOSAGE EFFECT OF RICINUS COMMUNIS AGGLUTININ

RCA (μG/ML)	INCUBATION TIME 2 MIN	5 MIN	10 MIN
0	0	0	0
10 (2)	0	+	+
20 (5)	++	++	+++
33 (10)	+++	+++	++++
40 (1)	+++	++++	++++
50 (4)	++++	++++	++++
*HAPTEN + 33 (5)	0	0	0
*HAPTEN + 50 (2)	0	0	0

*0.03 M β - LACTOSE

FIGURES IN PARENTHESES INDICATE THE NUMBER OF EXPERIMENTS SUPPORTING THESE DATA.

Figure 1a. Dissociated cells in the presence of WGA (33 µg/ml), 2 minutes after the addition of cells. (Magnification X 40)

Figure 1b. Dissociated cells in the presence of WGA (33 µg/ml) and 0.03 M N-acetyl-D-glucosamine, 5 minutes after the addition of cells. (X 40)

Figure 2a. Dissociated cells in the presence of RCA (33 µg/ml), 2 minutes after the addition of cells. (X 40)

Figure 2b. Dissociated cells in the presence of RCA (33 µg/ml) and 0.03 M beta-lactose, 5 minutes after the addition of cells. (X 40)


1a
2a

The Con A-induced agglutinability of embryonic *Xenopus* cells is shown in Table 3. A lectin dose dependence is observed with Con A similar to that reported for the two previously discussed lectins, except that the optimal concentration, approximately 100 µg/ml, is considerably higher. Agglutination is rapid and near maximal after 2 minutes of incubation (Figure 3a). Either of two sugars, alpha-methyl-D-glucose or alpha-methyl-D-mannose, at a concentration of 0.03 M completely inhibits the agglutination induced by up to 166 µg/ml of Con A (Figure 3b). The suboptimal concentration of Con A is approximately 33 µg/ml (Table 3).

In contrast to the results reported above for WGA, RCA, and Con A, agglutinability of these cells with SBA appears, by comparison, to be much lower. Higher doses of this lectin are necessary to induce near maximal agglutination, the optimal concentration being approximately 166 µg/ml (Figure 4a). Nevertheless, the response is dose-dependent (Table 4). Furthermore, SBA-mediated agglutination is time-dependent and appears to be a slower process when compared to that mediated by WGA, RCA, or Con A. Only at the highest SBA concentrations is agglutination detectable prior to 5 minutes of incubation. Agglutination by 166 µg/ml of SBA is completely inhibited by N-acetyl-D-galactosamine (Figure 4b).

The agglutinability of dissociated embryonic *Xenopus* cells with FBP was tested. No FBP-induced agglutination is observed at lectin concentrations up to 167 µg/ml.

The preceding data indicate that WGA, RCA, Con A, and to a lesser extent SBA readily agglutinate dissociated cells from stage 9 embryos of *Xenopus laevis*, and that this lectin-induced agglutination is effectively inhibited by the appropriate hapten sugars. To ensure that this

TABLE III

DOSAGE EFFECT OF CONCANAVALIN A

CON A (μG/ML)	INCUBATION TIME 2 MIN	5 MIN	10 MIN
0	0	0	0
33 (6)	+	++	++
66 (3)	++	+++	+++
100 (3)	+++	+++	++++
132 (2)	+++	++++	++++
166 (6)	++++	++++	++++
250 (2)	++++	++++	++++
*HAPTEN + 166 (3)	0	0	0

*.03 M α - METHYL - D - GLUCOPYRANOSIDE OR
.03 M α - METHYL - D - MANNOPYRANOSIDE

FIGURES IN PARENTHESES INDICATE THE NUMBER OF EXPERIMENTS SUPPORTING THESE DATA.

TABLE IV

DOSAGE EFFECT OF SOYA BEAN AGGLUTININ

SBA (μG/ML)	INCUBATION TIME 2 MIN	5 MIN	10 MIN	20 MIN
0	0	0	0	0
10 (2)	0	0	0	+
20 (2)	0	0	0	+
33 (1)	0	0	0	
40 (4)	0	0	+	++
50 (5)	0	+	+	++
66 (7)	0	+	+	++
80 (1)	0		++	+++
100 (2)	0	++	++	
132 (3)	+	++	++	+++
166 (3)	++	++	+++	
*HAPTEN + 166 (1)	0	0	0	

*0.03 M N - ACETYL - D - GALACTOSAMINE

FIGURES IN PARENTHESES INDICATE THE NUMBER OF EXPERIMENTS SUPPORTING THESE DATA.

Figure 3a. Dissociated cells in the presence of Con A (100 µg/ml), 2 minutes after the addition of cells. (Magnification X 40)

Figure 3b. Dissociated cells in the presence of Con A (100 µg/ml) and 0.03 M alpha-methyl-D-glucose, 5 minutes after the addition of cells. (X 40)

Figure 4a. Dissociated cells in the presence of SBA (166 µg/ml), 8 minutes after the addition of cells. (X 40)

Figure 4b. Dissociated cells in the presence of SBA (166 µg/ml) and N-acetyl-D-galactosamine, 8 minutes after the addition of cells. (X 40)


3a

inhibition is sugar-specific, an experiment was performed in order to test the hapten specificity of agglutination induced by each lectin. The results of this experiment are presented in Table 5. All sugars were used at a final concentration of 0.03 M; lectins were used at optimal or near optimal concentrations. WGA-mediated agglutination is inhibited completely by N-acetyl-D-glucosamine, and a slight inhibition is obtained in the presence of N-acetyl-D-galactosamine. RCA-mediated agglutination is completely inhibited only by beta-lactose, while N-acetyl-D-galactosamine is slightly inhibitory. None of the other sugars tested inhibit RCA-mediated agglutination. In this particular experiment agglutination by Con A is strongly inhibited by either alpha-methyl-D-glucoside or alpha-methyl-D-mannoside. N-acetyl-D-glucosamine is moderately effective in inhibiting Con A-induced agglutination, while beta-lactose and N-acetyl-D-galactosamine have no effect. Complete inhibition of SBA-induced agglutination is observed only in the presence of either N-acetyl-D-galactosamine or beta-lactose.

Amphibian embryos at the late blastula stage are composed of presumptive ectodermal cells located at the animal pole, cells of the marginal zone which will give rise to the mesoderm, and presumptive endodermal cells located at the vegetal pole of the embryo. The degree of pigmentation decreases from the animal pole toward the vegetal pole, while the number and size of yolk granules, as well as cell size, increases in the same direction. The criteria of cell size and degree of pigmentation were used in order to determine if any of the lectins studied preferentially agglutinated a particular cell type. Upon close microscopical examination of the agglutinated and non-agglutinated cells, no

TABLE V

HAPTEN SPECIFICITY OF LECTIN-MEDIATED CELL AGGLUTINATION

HAPTEN ADDED 0.03 M	LECTIN USED WGA* (33 UG/ML)	RCA* (33 UG/ML)	CON A * (166 UG/ML)	SBA** (166 UG/ML)	NO** LECTIN
NO HAPTEN	+++	+++	++++	+++	0
N-ACETYL-D-GLUCOSAMINE	0	+++	++	+++	
N-ACETYL-D-GALACTOSAMINE	++	++	++++	0	
B-LACTOSE	+++	0	++++	0	
α-METHYL-D-GLUCOSIDE	+++	+++	+	+++	
α-METHYL-D-MANNOSIDE	+++	+++	+	+++	

*Agglutination scored at 2 minutes.

**Agglutination scored at 10 minutes.

selective agglutination is observed for any of the lectins at the concentrations used in these experiments. In all cases, agglutinated cell masses appear to be composed of indiscriminate mixtures of different cell types. The relative proportion of cells of any one type present in agglutination masses reflects the relative proportion of cells of that type present in the cell suspension.

B. THE EFFECT OF NEURAMINIDASE TREATMENT OF CELLS ON AGGLUTINATION

Neuraminidase is a glycosidase which specifically cleaves off exposed terminal sialic acid residues from glycoproteins, glycolipids, and oligosaccharides. The reaction involves the hydrolysis of the alpha-ketosidic linkage between the keto group of the terminal N-acetyl-neuraminic acid and a subterminal sugar residue (Rafelson, Schneir, and Wilson, 1966).

To investigate the possible effects of neuraminidase treatment, the agglutinability of enzyme-treated and control cells with optimal and suboptimal concentrations of each lectin was studied. Using any of the four lectins at optimal concentrations, no reduction in cell agglutinability is observed following neuraminidase treatment. The results of similar experiments using suboptimal lectin concentrations are summarized in Table 6. Neuraminidase treatment has no effect on Con A- or WGA-mediated agglutination when control and enzyme-treated cells are compared. However, neuraminidase treatment greatly enhances the agglutinability of cells in response to RCA. This enhanced response at low RCA concentration is rapid, comparable with respect to time-dependence to the response

TABLE VI

EFFECT OF NEURAMINIDASE TREATMENT ON LECTIN-INDUCED AGGLUTINATION

	CON A (30 μG/ML)		WGA (10 μG/ML)		RCA (16 μG/ML)		SBA (66 μG/ML)		FBP (165 μG/ML)	
	2 MIN	8 MIN	2 MIN	5 MIN	2 MIN	8 MIN	4 MIN	10 MIN	2 MIN	10 MIN
	(1)*		(2)*		(4)*		(5)*		(2)*	
CONTROL	+	++	+	++	0	+	0	+	0	0
NANASE	+	++	+		++	+++	+	++	0	0

* NUMBER OF EXPERIMENTS.

of control cells to higher lectin concentrations. Figure 5 illustrates control cells in the presence of 16 µg/ml of RCA after 2 minutes of incubation. A comparison with Figure 6, which shows neuraminidase-treated cells under the same conditions, illustrates the enhanced agglutination due to enzyme treatment. A similar although less dramatic enhancement of agglutination by SBA occurs after neuraminidase treatment. The enhanced agglutinability by 66 µg/ml of this lectin shows the same temporal characteristics mentioned previously for SBA; it is a slower response than that induced by Con A, RCA, or WGA. A comparison between Figures 7 and 8 illustrates this enhancement. The former shows control cells incubated with 66 µg/ml of SBA after 8 minutes incubation while the latter represents neuraminidase-treated cells under the same conditions; increased agglutination is evident in enzyme-treated cells. Neuraminidase treatment does not induce agglutination in the presence of FBP (166 µg/ml).

C. EFFECT OF LOCAL ANAESTHETICS ON AGGLUTINATION

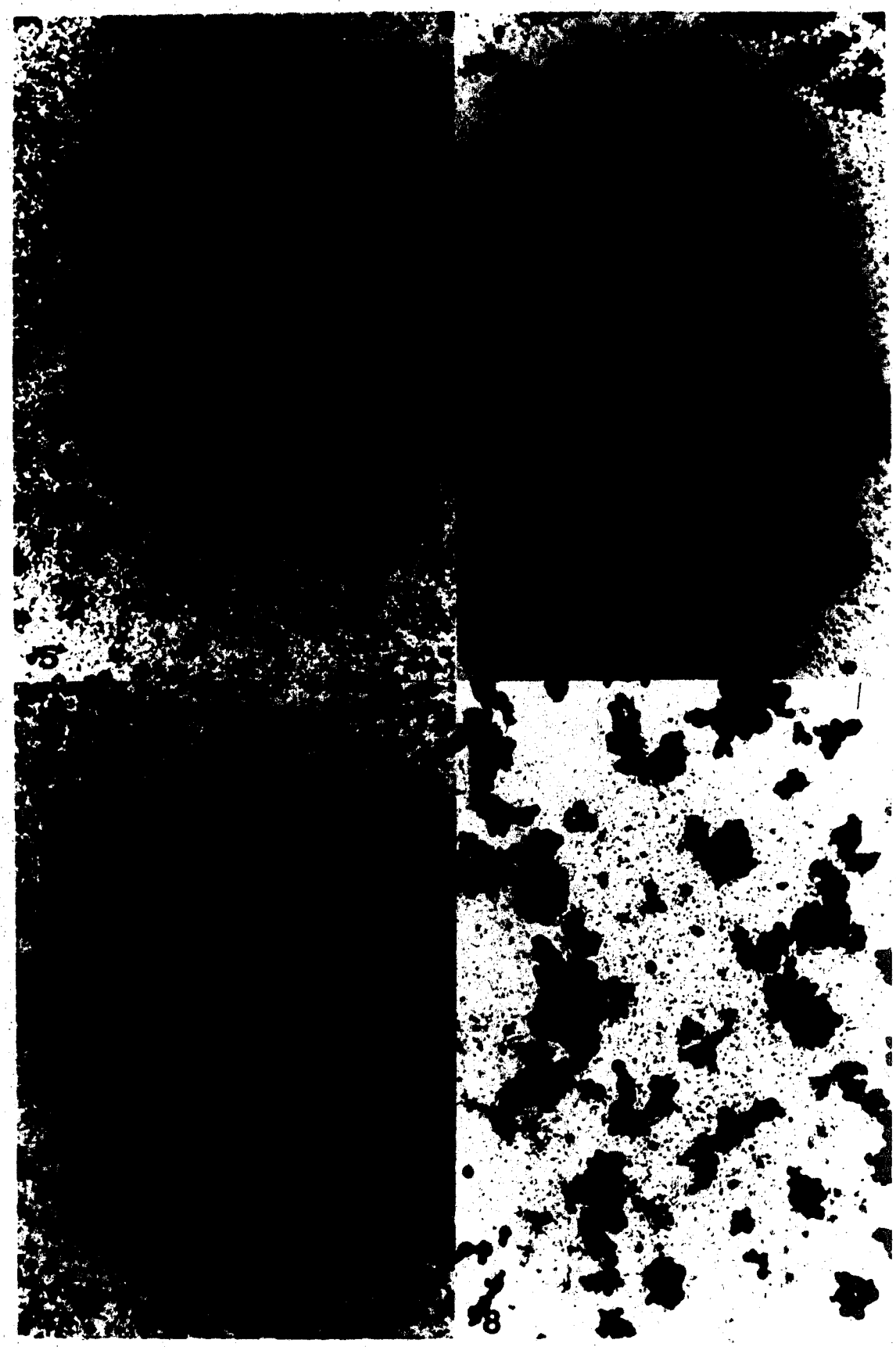
In other cell systems, local anaesthetics and phenothiazine tranquillizers have been found to influence some cell surface mediated phenomena such as lectin-induced agglutination and cell capping (Poste *et al.*, 1975a; Ryan *et al.*, 1974). In order to further investigate the mechanisms involved in the lectin-mediated agglutination of embryonic *Xenopus* cells, the effects of these drugs upon this process were studied. Cells were treated with either of two local anaesthetics, procaine or xylocaine. The drug concentrations used ranged from 7.5×10^{-3} M to 1.4×10^{-1} M for procaine, and from 1.1×10^{-2} M to 1.1×10^{-1} M for xylocaine. These concentrations have been reported to elicit the cell

Figure 5. Saline-treated, control cells in the presence of RCA (16 µg/ml), 5 minutes after the addition of cells. (Magnification X 40)

Figure 6. Neuraminidase-treated cells in the presence of RCA (16 µg/ml), 5 minutes after the addition of cells. (X 40)

Figure 7. Saline-treated, control cells in the presence of SBA (66 µg/ml), 8 minutes after the addition of cells. (X 40)

Figure 8. Neuraminidase-treated cells in the presence of SBA (66 µg/ml), 8 minutes after the addition of cells. (X 40)


8
8

surface alterations mentioned previously (Ryan *et al.*, 1974; Poste *et al.*, 1975b). In these experiments the possible effects of local anaesthetics on agglutinability at optimal or suboptimal concentrations of RCA or WGA were investigated.

When cells are incubated in the presence of low concentrations of anaesthetics, no difference in agglutinability between experimental and control cells is observed with either lectin. Agglutinability is neither enhanced nor diminished. However, as the concentration of either drug increases, cells appear to become more labile, as is evidenced by increased cell lysis during incubation. In spite of this, even at the highest drug concentrations there remains a sufficient number of non-lysed cells to perform agglutination tests. A problem arises, however, in the test itself. Upon addition of suboptimal concentrations of either RCA or WGA to anaesthetic-treated cells, further cell lysis occurs. Such concentrations of either lectin rarely cause cytolysis of cells not treated with local anaesthetics. Lysed cells clump together in large, slimy masses of cell debris. Superficially, these masses have a similar appearance to clumps of agglutinated cells, making it difficult to evaluate the extent of the agglutination of cells treated with high concentrations of either procaine or xylocaine. Since the presence of the appropriate hapten inhibits this lectin-dependent cytotoxicity as well as cell agglutination, other criteria are required to distinguish between clumping of cells resulting from cell lysis and true lectin-mediated agglutination. In this investigation, such a distinction was based upon the speed of the agglutination reaction and the gross morphology of agglutinated cells. Agglutination induced by WGA or RCA is a rapid

process; it is nearly complete 1 minute after the addition of optimal concentrations of lectin. Also, cells agglutinated exclusively by lectin are tightly bound into cohesive masses, with the boundaries of individual cells still being distinguishable. On the other hand, cell clumping due to cytolysis is a slightly slower process, requiring approximately 2 to 3 minutes or more. Masses of lysed cells have a different texture, appearing viscous and easily deformable. Also, the peripheries of lysed cells are not distinct and individual cell boundaries cannot be distinguished within the mass. When these criteria are considered in the evaluation of the lectin-induced agglutination of cells treated with high concentrations of either procaine or xylocaine, there does not appear to be a drug-related change in the agglutinability of cells in this system. However, the results of these experiments are not conclusive due to the above-mentioned complicating factors.

D. THE EFFECT OF PHENOTHIAZINE TRANQUILLIZERS ON AGGLUTINATION

Preliminary experiments with chlorpromazine dissolved in C.M.F.S.S. showed that, at a concentration of 2×10^{-3} M, this drug can inhibit lectin-mediated agglutination. Similar results were obtained when prochlorperazine or trifluoperazine was used at the same concentration. However, a problem arose in that this inhibitory effect was not consistently reproducible, probably due to the variable solubility of these drugs in physiological salines at pH 7.8. In those experiments where the drugs failed to inhibit agglutination, they did not appear to be dissolved. Insolubility was characterized by the presence of a fine,

white, opalescent precipitate suspended in the saline, and also by a lack of the drop in pH that accompanies dissolution of the hydrochloride derivatives of these phenothiazines. It seems reasonable to assume that the drugs must be dissolved in the suspending medium in order to affect the cells. For this reason subsequent experiments entailed dissolution of the drugs and resuspension of the cells in m.C.F.S.S. prior to addition of drugs to experimental cell suspensions. Following this change, drug-induced inhibition of cell agglutination was consistently observed with all three tranquillizers.

Using m.C.F.S.S., drug dose-response tests were performed for each tranquillizer to establish minimum concentrations which can inhibit lectin-mediated cell agglutination. Agglutinability of drug-treated and control cells with WGA, and in some cases, with RCA at optimal lectin concentrations was tested. The results of these experiments are summarized in Table 7. These data indicate a similar dose-dependency for all three drugs. At 2×10^{-4} M, chlorpromazine, prochlorperazine and trifluoperazine each fail to inhibit agglutination. At 4×10^{-4} M, agglutination is frequently inhibited, while at 8×10^{-4} M or higher, this inhibition is consistently observed. It appears that the minimum concentration of any one of these three phenothiazine derivatives which is effective in inhibiting the agglutination response lies between 4×10^{-4} M and 8×10^{-4} M. This phenothiazine-mediated inhibition is illustrated by a comparison between Figure 9, showing control cells agglutinated by WGA, and Figures 10, 11, and 12, which show chlorpromazine-treated, prochlorperazine-treated, and trifluoperazine-treated cells in the presence of optimal concentrations of lectin.

TABLE VII

DOSAGE EFFECT OF PHENOTHIAZINE DERIVATIVES UPON CELL AGGLUTINATION

DRUG CONCENTRATION**	CHLORPROMAZINE		PROCHLORPERAZINE		TRIFLUOPERAZINE	
	WGA*	RCA*	WGA*	RCA*	WGA*	RCA*
2×10^{-4} M	□ □ □	□	□ □ □	□	□ □ □	□
4×10^{-4} M	□ ▪	□	□ ▪	□	□ □	□
8×10^{-4} M	▪ ▪ ▪ ▪ ▪ ▪	▪ ▪ ▪	□ ▪	□ ▪	□ ▪	□
1.0×10^{-3} M					▪	▪
1.2×10^{-3} M	▪ ,		▪ ▪	▪	▪	
1.5×10^{-3} M	▪ ▪	▪				
1.6×10^{-3} M	▪		▪		▪	
2.0×10^{-3} M	▪ ▪	▪	▪ ▪ ▪ ▪	▪ ▪	▪	

* 33 UG/ML LECTIN

□ ONE EXPERIMENT. CELLS AGGLUTINATE.

▪ ONE EXPERIMENT. INHIBITION OF AGGLUTINATION.

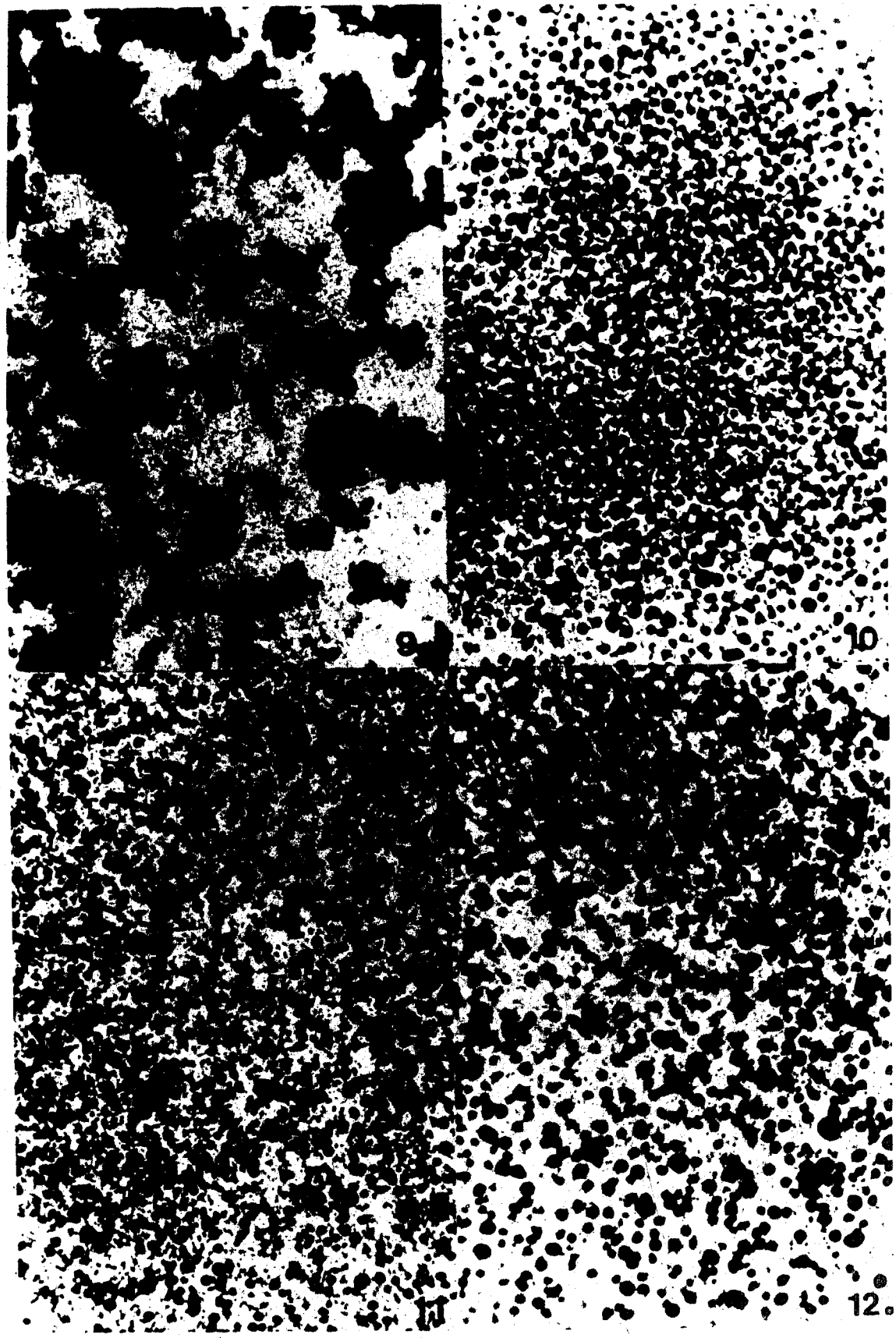
** CELLS INCUBATED 1 HOUR AT ROOM TEMPERATURE.

Figure 9. Control cells from the phenothiazine experiments in the presence of WGA (33 µg/ml), 2 minutes after the addition of cells. (Magnification X 40)

Figure 10. Cells pre-incubated in 8×10^{-4} M chlorpromazine, in the presence of WGA (33 µg/ml), 5 minutes after the addition of cells. (X 40)

Figure 11. Cells pre-incubated in 8×10^{-4} M prochlorperazine, in the presence of RCA (33 µg/ml), 5 minutes after the addition of cells. (X 40)

Figure 12. Cells pre-incubated in 8×10^{-4} M trifluoperazine, in the presence of WGA (33 µg/ml), 5 minutes after the addition of cells. (X 40)


9
10
11
12

The results presented in Table 7 suggest that agglutination by either WGA or RCA is similarly affected by phenothiazine tranquillizers. Experiments were performed to investigate whether these drugs differentially affect the agglutination mediated by the lectins WGA, RCA, Con A, and SBA. Cells were incubated in 2×10^{-3} M chlorpromazine or prochlorperazine, or in 1×10^{-3} M trifluoperazine after which they were tested for agglutinability with each of the four lectins at optimal concentrations. The results of these experiments are reported in Table 8. These data indicate that each of the phenothiazine derivatives tested inhibits the agglutination mediated by any of the four lectins.

Cells were tested for lectin-induced agglutinability at 30, 45, and 60 minute periods of drug incubation. At 30 minutes of incubation, lectin-induced agglutination is still evident and is comparable to that of untreated controls. Some inhibition of agglutination is evident in drug-treated cells after 45 minutes, while a 60 minute drug incubation period is necessary for complete inhibition of the agglutination response. The reversibility of the inhibition of agglutination brought about by phenothiazine tranquillizers was investigated. After incubation of cells in the presence of any of these three drugs, standard agglutination tests were performed to confirm the drug's inhibitory effect upon that process. Following this, drug-treated cells were rinsed up to three times with fresh drug-free saline and, in some cases, reincubated in the absence of drug for two hours. Cells were tested again for lectin-induced agglutinability. Under these experimental conditions the inhibition of lectin-induced agglutination is not reversible.

TABLE VIII

EFFECT OF PHENOTHIAZINE TRANQUILLIZERS ON LECTIN-INDUCED AGGLUTINATION

	WGA* (33 μG/ML)	RCA* (33 μG/ML)	CON A* (166 μG/ML)	SBA* (166 μG/ML)
CONTROL	+++	+++	++++	++
CHLORPROMAZINE - HCL**	0	0	0	0
PROCHLORPERAZINE**	0	0	0	0
TRIFLUOPERAZINE - HCL***	0	0	0	0

* AGGLUTINATION SCORED AT 4 MIN.

** 2×10^{-3} M

*** 1×10^{-3} M

E. EFFECTS OF COLCHICINE AND CYTOCHALASIN B ON AGGLUTINATION

Colchicine is an alkaloid which interferes with microtubule polymerization (Wilson, 1975) while cytochalasin B has been reported to disturb many cell processes that are mediated by microfilaments (Wessels *et al.*, 1971; Pollard and Weihing, 1974). Microtubules and microfilaments have been reported to mediate cell surface associated functions in other cell systems (Edelman, 1976). In order to determine whether these cellular organelles are involved in lectin-induced agglutination of embryonic *Xenopus* cells, dissociated cells were treated with either colchicine or cytochalasin B, or with both drugs simultaneously.

Colchicine was used at concentrations ranging from 30 to 120 μM while cytochalasin B was used at a concentration of 20 μg/ml. In experiments where cells were incubated in the presence of both colchicine and cytochalasin B simultaneously, the drugs were used at concentrations of 60 μM and 20 μg/ml respectively. After incubation with the drugs, cells were tested for agglutinability with RCA and WGA at optimal and suboptimal lectin concentrations. Agglutination appears to be neither enhanced nor inhibited by either colchicine or cytochalasin B, or by both drugs in combination. However, as was the case with cells treated with local anaesthetics, colchicine and cytochalasin B appear to make the cells more labile and thus enhance the cytotoxicity of the lectins. Cells incubated in the presence of dimethyl sulphoxide also exhibit this increased lability and elevated susceptibility to the cytotoxic effects of lectins. These effects complicated the evaluation of cell agglutinability in the same manner as described previously (see Results, section C).

F. THE EFFECT OF FIXATION ON AGGLUTINATION

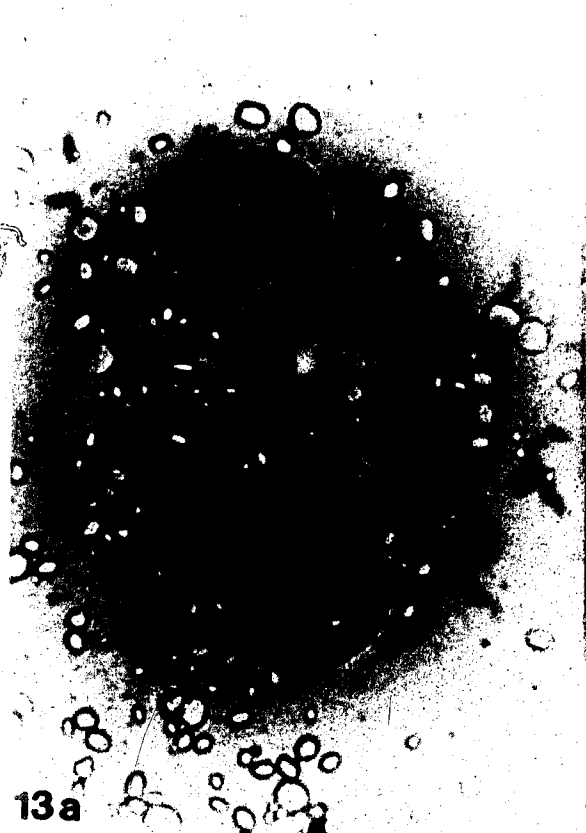
Glutaraldehyde fixation has been reported to inhibit lectin mediated agglutination, presumably by interfering with the mobility of lectin receptors (Inbar *et al.*, 1973). Experiments were performed to determine its effect in this system. Glutaraldehyde fixation of embryonic *Xenopus* cells for 24 hours renders them non-agglutinable with either RCA or WGA (33 µg/ml). Fixed cells agglutinate to a certain extent in the presence of Con A (166 µg/ml); however, this agglutination response is drastically reduced from that observed with unfixed cells. Maximal agglutination of fixed cells with Con A (166 µg/ml) corresponds to a score of approximately ++ on the test scale, as opposed to a score of ++++ for unfixed cells. Closer examination under the dissecting microscope of fixed cells agglutinated with Con A makes it apparent that cell debris is also being agglutinated. Indeed, with phase-contrast microscopy the small cell clusters appear to be composed of cells enmeshed in a mass of agglutinated yolk platelets. Yolk platelets released from ruptured cells are found in all cell suspensions, fixed and unfixed; however, only in experiments with Con A and fixed cells has agglutination of yolk been clearly observed.

G. CELL MORPHOLOGY

Cells from control or drug-containing suspensions as well as fixed cells were examined by phase-contrast microscopy. The cells are spherical, varying in diameter from approximately 20 µ to over 110 µ. The majority of pigmented cells are less than 70 µ in diameter, the pigment being localized in the cortex of the cell at one of its poles. All cells

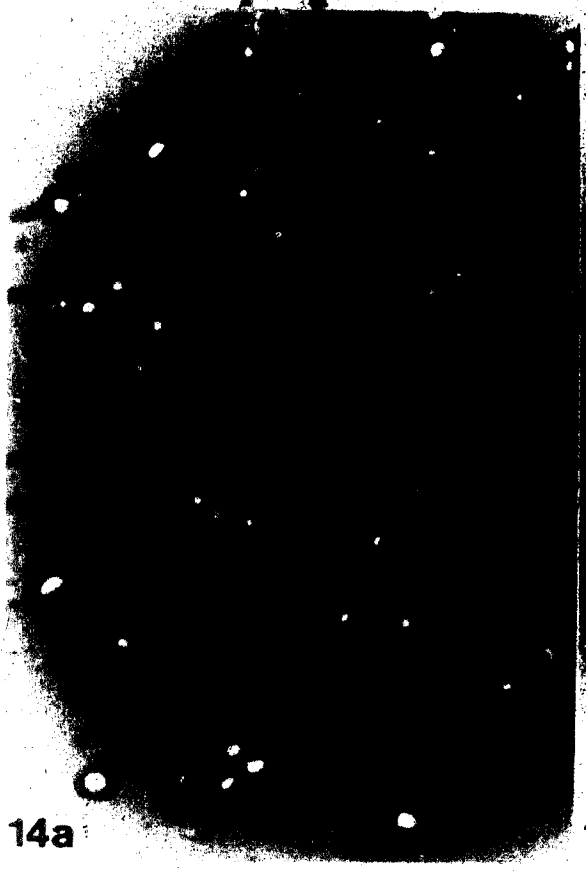
Figures 13a and 13b. Dissociated cells under phase-contrast microscopy. (Magnification X 400). Note the lobopodia (arrows).

Figures 14a and 14b. Dissociated cells incubated one hour with 10 nanograms (8×10^{-9} M) under phase-contrast microscopy. (X 400)

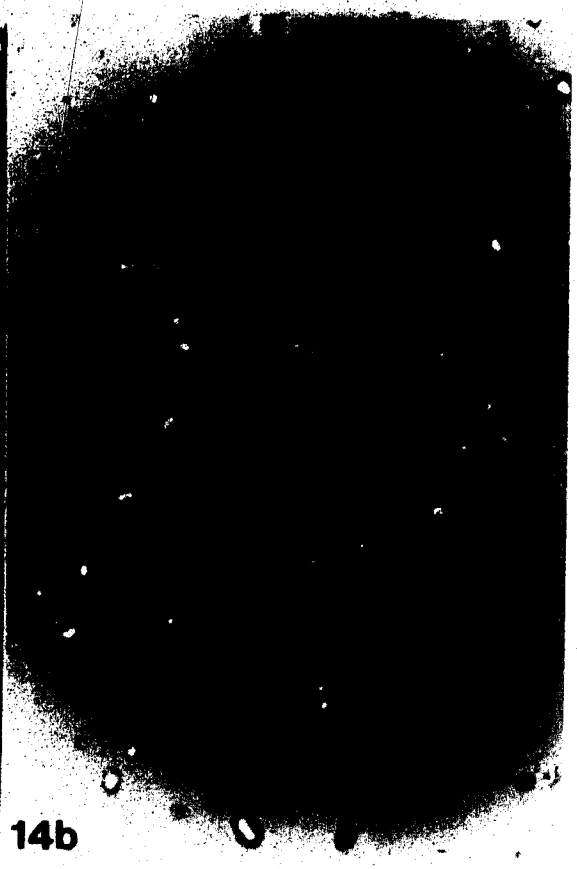


13a

13b



14a



14b

contain large numbers of cortical yolk platelets, the latter being more numerous in the large endodermal cells. Freshly dissociated cells and those used as controls in the drug experiments are characterized by a smooth surface, with many presumptive ectodermal and mesodermal cells extending large, rounded lobopodia (Figures 13a and 13b). These lobopodia are very active, changing position by flowing around the cell's periphery. In most cases, cytoplasmic organelles or granules appear to be excluded from the cytoplasm of the lobopodia, making the protuberances transparent. Occasionally, in a small number of cells, granular cytoplasmic contents are seen flowing within the lobopodia. Cell processes such as these were first described in dissociated amphibian cells (*Ambystoma*) by Holtfreter (1946). More recently, Satoh *et al.* (1976) have investigated this phenomenon in embryonic *Xenopus* cells in detail, distinguishing between several types of processes extended by these cells. In particular, the latter authors describe "hyaline blebs or lobopodia" which appear to correspond to the lobopodia described above. Treatment of cells with either local anaesthetic does not prevent the extrusion of lobopodia or alter the contour of the cells' peripheries.

Those concentrations of phenothiazine derivatives that fail to inhibit lectin-mediated agglutination do not appear to affect the surface morphology of cells or the formation of these processes. However, either chlorpromazine, prochlorperazine, or trifluoperazine at concentrations which effectively inhibit agglutination also inhibit the formation of lobopodia. In these cases, the morphology of the peripheral regions of the cells is altered. The cell cortex, and/or presumably the plasma membrane, appear to be contracted, the cortical yolk platelets protruding at the periphery of the cell. This contracted appearance and the absence of lobopodia are illustrated in Figures 14a and 14b, which

show cells incubated with 8×10^{-4} M trifluoperazine for one hour.

Colchicine-treated cells maintain a smooth surface morphology and extrude many typical lobopodial extensions. On the other hand, cytochalasin B prevents the extrusion of lobopodia and cells treated with this drug present a rough surface morphology, similar to that exhibited by cells treated with concentrations of phenothiazine tranquillizers that inhibit lectin-induced agglutination. Cells treated simultaneously with both colchicine and cytochalasin B exhibit the same surface characteristics as cells treated with cytochalasin B alone.

Glutaraldehyde-fixed cells appear spherical and opaque under phase-contrast microscopy. The uneven distribution of pigment in the cell cortex is maintained in pigmented cells; however, no lobopodia are observed. Fixed cell peripheries appear rougher than those of unfixed control cells, but are not nearly as rough as those of cells treated with cytochalasin B or 8×10^{-4} M phenothiazine tranquillizers.

DISCUSSION

The work of Moscona (1971), Kleinschuster and Moscona (1972), and Zalik and Cook (1976) indicates that early embryonic chick cells possess exposed cell surface receptors for certain lectins, and that these receptors are capable of participating in a lectin-mediated agglutination response. Early embryonic cells appear to resemble many types of transformed cells with respect to their agglutinability. These studies also suggest that as tissue differentiation proceeds in the embryo, the differentiating cells acquire agglutinability characteristics more closely resembling those of nontransformed cells of differentiated adult tissues; that is, cells still possess the exposed lectin receptors which, although capable of binding lectin, cannot participate in the agglutination of the cells bearing them.

The results presented here show that cells from late blastulae of *X. laevis* also display cell surface properties similar to transformed cells with respect to interactions between surface carbohydrate moieties and a variety of lectins. Embryonic *Xenopus* cells are agglutinable with WGA, RCA, Con A, and SBA, indicating that these cells possess surface receptors which bind these lectins, and that these receptors are in such a state as to render the cells bearing them agglutinable. Cells from *Xenopus* blastulae do not appear to be agglutinated by FBP.

Interpretation of the results of agglutination tests is at best speculative. As to the actual biochemical nature of the lectin receptors present at the surfaces of dissociated cells of *Xenopus* embryos, very little can be said based on the results of agglutination tests alone.

The specificity of the sugar-binding properties of lectins can be studied via comparative affinity/precipitation tests using a range of purified oligosaccharides and monosaccharides as hapten substrates for the lectin. However, it is not possible to directly apply the results of such sugar-binding studies which are conducted under highly controlled conditions to the interpretation of observations made on the interaction of lectins with cell surface carbohydrates. Little is known about the chemical nature of oligosaccharides present on the surfaces of cells, making it difficult to determine which sugars in the oligosaccharides a lectin is interacting with. It is known that subterminal sugars influence the interaction between some lectins and terminal sugar residues of a polysaccharide. Nagata and Burger (1974), studying the binding affinities of a number of sugars to WGA, found that this lectin binds the disaccharide and trisaccharide polymers of N-acetyl-D-glucosamine with higher affinity than it does the monomeric sugar. Goldstein *et al.* (1975) extended these observations, showing that WGA binds more strongly to the trisaccharides of N-acetyl-D-glucosamine than to the disaccharides, for both of which the lectin shows greater affinity than for the monosaccharide. By comparing the affinities of WGA for a wide variety of oligosaccharides, these same investigators showed that this lectin can distinguish between monomeric N-acetyl-D-glucosamine, the disaccharides, trisaccharides, tetrasaccharides and the pentasaccharides of this sugar. The lectin distinguishes between these haptens not only on the basis of the number of N-acetyl-D-glucosaminyl residues contained by these molecules, but also on the basis of the type(s) of linkage(s) joining the monosaccharide residues. Nicolson and Blaustein (1972), comparing the relative abilities of a number of monosaccharides, disaccharides, and trisaccharides to

inhibit RCA-mediated cell agglutination, found that disaccharides carrying D-galactosyl residues are more inhibitory than D-galactose itself. They also showed that the nature of the linkage between the sugar residues influences the inhibitory capacity of haptens composed of two or more sugars. On the basis of studies such as these, it has been proposed that the sugar-binding sites of WGA and RCA may accommodate more than a single sugar residue. Goldstein *et al.* (1975) suggest that each WGA binding site may associate with up to three covalently linked N-acetyl-D-glucosamine residues, making the trisaccharide of this sugar most complementary to the WGA sugar-binding site, followed by the disaccharide, and then the monosaccharide. Such a decreasing complementarity along with an influence as of yet undefined resulting from the nature of the inter-sugar linkages, could perhaps account for the decreased affinity of the lectin for haptens with fewer N-acetyl-D-glucosaminyl residues. Similarly, Lis and Sharon (1973) point out that the data of Nicolson and Blaustein (1972) may indicate that the sugar-binding site of RCA possibly accommodates two or more sugar residues, and that as a consequence of this, the binding affinity of this lectin for a given terminal residue of an oligosaccharide is greatly influenced by the nature of subterminal sugars.

If the specificity of lectins has been underestimated and is expressed not in terms of single sugar residues, but rather in terms of two or more residues joined via specific linkages, lectin molecules may prove to be far more useful as investigative probes in cell surface research once such specificities are elucidated. Indeed, that the biological specificity of lectins has been underestimated is suggested by the studies of Allan and Johnson (1976) on the binding to cells of four

lectins, all of which are stated to bind mannosyl residues "specifically." The results indicate that Con A and pea lectin bind to two separate populations of receptors on the surface of a cell, even though the binding of both is inhibited by alpha-methyl-D-mannose. On the other hand, lectins from fava beans and lentils each compete to a different degree with pea lectin for the same receptors, although again the binding of all three is inhibited by the same hapten. These observations may be indicative of an as yet unappreciated binding specificity inherent in the interactions of these four lectins with cell surface carbohydrates.

Other factors influence the interpretation of cell agglutination data. For example, although the monosaccharide N-acetyl-D-glucosamine inhibits WGA-induced cell agglutination, Burger and Goldberg (1967) noted that the sugar nucleotide uridine diphosphate-N-acetyl-D-glucosamine does not. These authors hypothesized that steric hindrance resulting from the bulky nucleotide group attached to the sugar perhaps prevents the binding of the associated sugar residue by the lectin molecule. In a similar manner, neighbouring cell surface components may sterically interfere with the binding of a lectin to a particular surface moiety, thereby influencing the detectable agglutinability of the cells concerned. Such steric hindrance may be especially significant in the light of evidence that at least two lectins, Con A and WGA, can recognize and bind specifically to nonterminal residues in an oligosaccharide chain. Goldstein *et al.* (1973) noted that Con A binds to nonterminal alpha-D-mannopyranosyl residues in oligosaccharides and bacterial glycoproteins. Similarly, WGA has been shown to interact with nonterminal N-acetyl-D-glucosaminyl residues present in glycoproteins such as carcinoembryonic antigen (Goldstein *et al.*, 1975). One might expect that in the interaction

between a lectin molecule and a specific non-terminal sugar component of a large, highly branched cell surface carbohydrate chain, steric hindrance from nearby side chains and from neighbouring molecules could be considerable.

Another characteristic of lectins that ought to be kept in mind when interpreting cell agglutination data is that the sugar-binding specificities and affinities of lectins are not "all or nothing" phenomena. Lectins bind sugars specifically and nonspecifically, depending on the concentrations of the hapten relative to that of the lectin (Greenaway and LeVine, 1973). Furthermore, in studies investigating the binding affinity of lectins for a variety of different sugar residues, sugars are found that are bound with less than maximum but with more than minimum affinity (Goldstein *et al.*, 1975). This particular characteristic of lectins is reflected in the results obtained in the present study on the hapten specificity of agglutination. Although N-acetyl-D-glucosamine completely inhibits WGA-induced cell agglutination, N-acetyl-D-galactosamine also shows slight inhibitory activity; beta-lactose completely abolishes RCA-mediated agglutination while N-acetyl-D-galactosamine also appears to inhibit the agglutination induced by this lectin, but to a lesser degree. The most commonly used hapten inhibitors for Con A alpha-methyl-D-glucose and alpha-methyl-D-mannose, have great inhibitory activity on Con A-induced agglutination, but N-acetyl-D-glucosamine as well appears to have some activity in this respect. These data illustrate that the sugar-binding property of lectins is not strictly sugar-specific.

The evidence cited above illustrates the present lack of understanding of the nature of lectin-sugar interactions. Thus the

interpretation of hapten "specific," lectin-induced cell agglutination tests must, to a certain extent, be inconclusive with respect to the biochemical nature of the lectin receptors present at the cells' surfaces. Bearing in mind the complicating factors noted above, a tentative interpretation of the results of agglutination tests performed in this study will be offered, if only to provide a testable hypothesis upon which to base further investigations. The experimental evidence suggests that cells of late blastulae of *X. laevis* may possess surface carbohydrates bearing the following sugar residues such that they are available for interactions with lectin molecules: N-acetyl-D-glucosamine-like residues (WGA), D-galactose-like residues (RCA and SBA), N-acetyl-D-galactosamine-like residues (SBA), and alpha-methyl-D-glucose- or alpha-methyl-D-mannose-like residues (Con A). The cell surface components bearing these specific residues, at terminal or subterminal positions, are capable of participating in an agglutination response. To verify this hypothesis and further characterize these cell surface lectin receptors, isolation and biochemical analysis of the receptor molecules is necessary.

With respect to the lack of FBP-mediated agglutinability exhibited by embryonic *X. laevis* cells either before or after treatment with neuraminidase, little can be said based on the results of these experiments. That cells may bind lectin and yet remain nonagglutinable is a well documented observation (Sharon and Lis, 1975). Thus, the experiments reported here suggest only that cells from *Xenopus* blastulae do not possess FBP receptors capable of participating in an agglutination response. These cells may however possess FBP receptors. To distinguish between these two possibilities, an investigation into the binding of labelled FBP to the surface of these cells is required.

Neuraminidase treatment of embryonic *Xenopus* cells enhances their agglutinability with RCA and SBA, but not with Con A or WGA. This suggests a change at the cell surface resulting from removal of exposed terminal sialic acid residues, a change which preferentially affects receptors for RCA and SBA. Since both of these lectins bind D-galactosyl and N-acetyl-D-galactosyl residues with high affinity, and since the most common sugars subterminal to sialic acid residues in membrane glycoproteins are D-galactose and N-acetyl-D-galactosamine (Spiro, 1973), one of the most likely explanations for the enhanced agglutinability is that neuraminidase, by removing terminal sialic acid residues, is exposing previously inaccessible receptors to the lectins. Indeed such an explanation has been proposed by many investigators to account for the appearance of cell agglutinability with SBA and/or RCA after neuraminidase treatment of the cells (Lloyd and Cook, 1974; Zalik and Cook, 1976). That neuraminidase treatment of cells exposes SBA and RCA receptors that are otherwise not accessible to the lectin molecules has been shown in other systems by lectin binding studies (Vlodovsky and Sachs, 1975; Chan and Oliver, 1976). However, the exposure of previously inaccessible lectin receptors cannot adequately account for the enhanced RCA- and SBA-mediated agglutinability of embryonic *Xenopus* cells subsequent to neuraminidase treatment.

In the present study, the enhanced agglutinability of cells is detected using suboptimal lectin concentrations. the cells are known to possess an adequate number of RCA and SBA receptors to initiate a maximal agglutination response at optimal lectin concentrations, the factor limiting the response under the conditions used to test for enhanced agglutinability would appear to be the decreased lectin concentration and

not the number of receptors available to the lectin. This is presumably the case for both control and neuraminidase-treated cells, since both are extensively agglutinated with optimal concentrations of RCA or SBA. Because the number of accessible lectin receptors is not limiting at sub-optimal lectin concentrations, the enhanced agglutinability of neuraminidase-treated cells must be due to some factor other than a mere increase in the number of receptors available to RCA and SBA. One possibility is that on untreated cells, SBA and RCA molecules bind to galactosyl and N-acetyl-D-galactosaminyl residues which are subterminal to sialic acid residues on cell surface oligosaccharides. At physiological pH, sialic acid is ionized. Presumably, molecules at the surface of a single cell bearing such anionic residues would repel each other. These repulsive forces between sialic acid-bearing lectin receptor molecules could interfere with the clustering of receptors into patches. The redistribution of lectin-receptor complexes at the cell surface from a random array into patches may contribute to the agglutination of some types of cells (Nicolson, 1974; Sachs, 1974). Also, sialic acid-derived electrochemical forces of repulsion may prevent the interaction of lectin and lectin-bound receptors (for RCA and SBA) on adjacent cells, thus hindering formation of the intercellular lectin-bridged linkages between cells which are believed to be the basis for cell agglutination. By enzymatic removal of sialic acid residues adjacent to prospective RCA and SBA binding sites, the electrochemical forces of repulsion hindering the formation of patches and of intercellular lectin bridges may be more easily overcome, thus enhancing cell agglutinability with these two lectins.

In addition to generating electrochemical forces, terminal sialic acid residues close to RCA and SBA binding sites could impede lectin binding, patching, and intercellular cross-bridging by steric interference with these processes, as well as by decreasing the affinity of these receptors for RCA and SBA. Neuraminidase treatment of cells could reduce such steric hindrance, or perhaps increase the affinity of the receptors for these lectins, thereby enhancing the cells' agglutinability with RCA and SBA. Which, if any, of these proposed mechanisms are responsible for the effect of sialidase in this system remains a problem for future investigation. It is of interest that Harris (1970), studying the electrophoretic mobility of cells from late blastulae of *Xenopus*, found that neuraminidase treatment had no effect on the electrophoretic mobility of these cells and did not release detectable amounts of sialic acid from the cell surface. Harris used a concentration of neuraminidase one-third that used in the present study, and incubated the cells at 37° C for 30 minutes. Preliminary tests in the present investigation showed that *Xenopus* cells treated with neuraminidase according to the method of Harris did not exhibit enhanced RCA- or SBA-mediated agglutinability, suggesting that under such conditions neuraminidase does not remove exposed sialic acid residues from oligosaccharide chains at the cell surface.

A strictly objective and efficient method for the quantification of the extent of cell agglutination has yet to be developed, especially for use with cells as large as those from amphibian blastulae. As noted earlier, although the technique used in the present study for assessing cell agglutinability is rather objective and has its shortcomings, it is useful and it is practical. However, one negative aspect of the protocol

used in these exp[illegible]ught to be discussed briefly, this aspect being the lack of control over the density of cell suspensions used in the agglutination tests. Variation in the number of cells used per agglutination test obviously influences the number of lectin receptors present per test. Such variability would, under conditions of constant lectin concentration, result in a variability in the ratio of the number of lectin receptors to the number of lectin molecules available for binding to the receptors. Changes in this ratio ought to affect the extent of the agglutination response (Nicolson, 1974). This is a significant point with respect to the attempt in the present investigation to establish lectin dose responses. However, in spite of such variability between tests, the results reported here are valid as being indicative of general characteristics of agglutinability. For each dose response experiment, aliquots of cells from the same cell suspension were used in each agglutination test. Thus the number of cells used per test was approximately the same at all the lectin concentrations used in each experiment. The results of separate dose response experiments in most cases compared favorably with each other, and where variability between experiments was observed, the same general lectin-dose and time dependencies characteristic for that particular lectin were observed.

Preferential agglutinability of distinct populations of cells was not observed among the cells derived from *Xenopus* blastulae. However, it ought to be pointed out that these investigations have not proven that such differences in agglutinability do not exist. For example, distinction between different cell types in this study was based only on general characteristics of presumptive endodermal, ectodermal and mesodermal cells,

characteristics such as cell size, the degree of p[illegible]on, and the yolk content of the cells. Such features, although useful, are not definitive cell markers among embryonic amphibian cells. A more important factor that must be considered in evaluating the present results stems from the possibility that cells in close proximity may be able to influence surface-related properties of one another such as agglutinability. Evidence for such a capacity comes from studies on cell adhesion to artificial substrates. Chipowsky *et al.* (1973) not only noted that dissociated cells adhere to Sephadex beads derivatized with galactose, but also that suspended cells are more adherent to cells directly attached to the beads, and that yet more cells adhere to those not in direct contact with the beads. Thus, the majority of cells in the aggregates are not adhering to the beads, but to other cells. Chipowsky *et al.* described this process as ". . . autocatalytic, in that the cells that are bound to the beads seem to become more sticky, facilitating the binding of new cells to their surface. . . ." Whitehead and Marcus (1975) reported a similar phenomenon in the adhesion of cells to Sepharose beads derivatized with Con A. Cells attached to the beads [illegible] layers, the cells [illegible] outer layers [illegible] being in contact with [illegible]

[illegible]

[illegible]

[illegible]

bead which initially induced the changes in the surface properties of a limited number of cells.

These observations of Chipowsky *et al.* and Whitehead and Marcus suggest that cells can alter the surface properties of neighbouring cells and perhaps thereby effect a self-propagating transfer of certain surface-related properties between cells. The possible implications of such induced cooperativity, although of obvious importance in cellular interactions, have been largely overlooked in the investigations on intercellular adhesion and lectin-mediated agglutinability. In the present study, such a cooperative effect may have masked subtle differences in the agglutinability of cells from different presumptive germ layers. In agglutination tests using mixed cell populations, the response of a more agglutinable cell type may enhance or induce the response of cells which would otherwise not be as agglutinable. To specifically investigate the possible existence of differences in their agglutinability, cells from each of the three presumptive germ layers ought to be separately tested.

Differences in cell surface properties between cells from different presumptive germ layers of the amphibian embryo have been demonstrated using the technique of cell electrophoresis. MacMurdo-Harris and Zalik (1971) have found differences in the electrophoretic mobilities between cells derived from the presumptive ectoderm, mesoderm and endoderm of [illegible] *Xenopus*. Also, the electrophoretic mobilities of cells derived from each germ [illegible] change as development progresses. Schaeffer *et al.* (1973) recorded the electrophoretic mobilities of cells from different presumptive germ layers of blastulae, and early, mid, and late gastrulae of *Rana pipiens*. These investigators also noted differences in cell electrophoretic mobilities of cells from different

germ layers, and between cells from the same germ layer at different stages of development. Whether or not such differences in cell electrophoretic mobility reflect cell surface changes which are detectable also by the use of lectins remains to be seen.

Fixation of cells from *Xenopus* embryos inhibits their agglutination with RCA and WGA and severely restricts their agglutinability with Con A when lectins are used at optimal concentrations. These results are consistent with the majority of those obtained from studies in other systems into the effects of aldehyde fixation on cell agglutinability (Inbar *et al.*, 1973; Sachs *et al.*, 1974). The inhibitory effect of fixation is believed to stem from either or both of two related cell changes. The fluidity of the plasma membrane and the mobility of membrane proteins within the plane of the membrane are thought to be greatly reduced by fixation. As noted earlier, the redistribution of cell surface lectin-bound receptors into clusters or patches has been correlated with the agglutinability of cells. It has been proposed that fixation prevents this clustering and thus reduces the agglutinability of cells (Inbar *et al.*, 1973). Fixation is also believed to reduce membrane deformability, making the plasma membrane more rigid. This too is believed to reduce the agglutinability of cells by decreasing the intercellular contact upon which the agglutination process depends (Gibson *et al.*, 1975).

In general, even fixed cells can be rendered agglutinable if the lectin concentration is increased above that required to agglutinate unfixed cells (Nicolson, 1974). This may be relevant to the present observation of agglutination of fixed *Xenopus* cells with Con A. It was noted in these experiments that the yolk platelets from ruptured cells in

fixed cell suspensions agglutinate. The agglutination of yolk platelets is not an entirely unexpected observation in light of the suggestion of Singal and Sanders (1974) that these organelles may be a source of sugar residues that become associated with newly formed endoplasmic reticular membranes. Such sugars at the peripheries of yolk platelets may bind Con A either specifically or nonspecifically. Unfixed yolk platelets may also bind Con A, although they have not been observed to agglutinate. There is much more cell debris and yolk in unfixed cell suspensions than in fixed suspensions, since the latter are washed several times after fixation and since fixed cells are not prone to lysis. Thus in fresh cell suspensions the binding of Con A by yolk platelets may effectively lower the amount of lectin available for binding to the cell surface. In fixed cell suspensions, fewer yolk platelets would bind less lectin, resulting in a higher concentration of Con A available for binding to the cells. Such an increased binding of lectin to fixed cells could account for the slight agglutinability of these cells with Con A. The binding of lectin by yolk in unfixed cell suspensions could also account for the relatively high concentrations of Con A required to maximally agglutinate embryonic *Xenopus* cells.

Recently, much attention has been drawn to the existence of cytoplasmic contractile and structural elements in nonmuscle cells. It now seems likely that most types of cells possess these elements (Pollard and Weihing, 1974). Evidence supporting the possible existence of such a structure associated with the cytoplasmic face of the plasma membrane is of particular relevance to the present study. The concept of a peripheral cytoskeleton has evolved rapidly in the wake of a great many investigations into the mechanisms by which cells exert control over

membrane and cell surface properties. Currently, the most dynamic and popular model of plasma membrane structure and organization includes a phospholipid bilayer spanned by many protein molecules (Singer and Nicolson, 1972), the cytoplasmic ends of which may be associated with elements of a peripheral cytoskeleton (Edelman, 1976). The nature of this association may be in part responsible for the mobility of trans-membrane proteins within the plane of the membrane.

The cytoskeleton itself is envisaged as having two functionally distinct components, one of which is sensitive to microtubule-disrupting drugs, the other to microfilament-disrupting drugs. In general, the microtubular element is thought to function as a structural support and perhaps to selectively "anchor" certain plasma membrane proteins and glycoproteins so as to restrict their mobility within the membrane (Poste *et al.*, 1975c). The microfilamentous element may be responsible for the controlled movements of membrane proteins and glycoproteins within the plane of the membrane as occurs in cell capping. In such a model, it is evident that these two elements of the peripheral cytoskeleton would be functionally opposed to one another; the microfilamentous component effects the directed movement of specific membrane proteins and glycoproteins, while the microtubular component restricts this movement. By coordinating the interaction of these two cytoskeletal elements, a cell could regulate the distribution and mobility of transmembrane proteins and glycoproteins, including those lectin receptors which span the membrane (Edelman, 1976).

As discussed earlier, the mobility of lectin receptors, as manifested in the redistribution of lectin-receptor complexes at the cell surface, has been implicated in the agglutination of some types of cells.

It is now believed that the peripheral cytoskeleton may modulate receptor mobility, and thereby regulate the agglutinability of these cells. For this reason, the possible role of colchicine-sensitive and cytochalasin B-sensitive structures in the lectin-mediated agglutination of embryonic *Xenopus* cells was investigated. It appears that neither drug, used either separately or together, alters the agglutinability of these cells with RCA and WGA at optimal or suboptimal lectin concentrations. It has been reported that SV40-transformed 3T3 cells are rendered non-agglutinable by pre-treatment of the cells with colchicine (Poste *et al.*, 1975c). This is not the case for embryonic *Xenopus* cells. This difference in response to colchicine treatment may be indicative of a more fundamental difference in surface properties between embryonic cells and those of transformed cells. The agglutinability of embryonic cells does not appear to be regulated by colchicine-sensitive or cytochalasin B-sensitive cytoplasmic elements.

It is also of interest to note that cytochalasin B affects the surface morphology of dissociated cells of *Xenopus* blastulae. The extrusion of cytoplasmic lobopodia is inhibited and the cell periphery appears somewhat contracted. This suggests that the maintenance of a smooth surface morphology and the formation of lobopodia may involve contractile cytoplasmic elements which are sensitive to cytochalasin B. That colchicine does not inhibit the extension of lobopodia suggests that microtubular functions are not essential to this process.

Circumstantial evidence indicates that local anaesthetics may affect surface-related phenomena such as cell capping and agglutination via a mechanism involving the disruption of both cytochalasin B-sensitive and colchicine-sensitive cytoskeletal elements. It has been suggested that the effect of local anaesthetics on cells mimics the combined effects

of cytochalasin B and colchicine (Poste *et al.*, 1975a; Poste *et al.*, 1975b, 1975c). Treatment of embryonic *Xenopus* cells with either of the anaesthetics procaine or xylocaine does not appear to alter their agglutinability with RCA or WGA. This is consistent with the above hypothesis in so far as agglutinability is concerned, since incubation of these cells with cytochalasin B plus colchicine also does not change the cells' agglutinability. However, local anaesthetics do not change the morphology of dissociated *Xenopus* cells, nor limit the extrusion of lobopodia, whereas cells treated with cytochalasin B lack lobopodia and appear slightly contracted. This illustrates that the local anaesthetics and cytochalasin B differ in their mechanisms of action in interfering with cell surface properties.

Poste and his associates (1975a, b, and c) have shown that the local anaesthetics enhance the agglutinability of nontransformed cells, but do not affect the agglutinability of their transformed counterparts. These investigators have also found that incubation of cells with both cytochalasin B and colchicine renders nontransformed cells agglutinable, while similar treatment has no effect on the agglutinability of transformed cells. These results indicate that treatment of cells with local anaesthetics, or with colchicine plus cytochalasin B increases the agglutinability of cells not otherwise agglutinable, but has no discernible effect on the agglutinability of cells which are readily agglutinated without drug treatment. That the agglutinability of embryonic *Xenopus* cells is not affected by any of these drug treatments is not surprising, since these cells, like transformed cells, are already highly agglutinable.

The phenothiazine tranquillizers are a group of pharmacologically active drugs which share many membrane-modifying properties with the local

anaesthetics. These properties, reviewed by Seeman (1972), include inhibition of the action potential in excitable membranes, lateral expansion of cell and artificial membranes, increasing the fluidity and disorder within membranes, displacement of membrane-bound calcium, and alteration of the activity of certain soluble and membrane-associated enzymes. At low concentrations (10^{-6} M to 10^{-5} M) the cationic phenothiazine derivatives protect cells from hypotonic lysis, whereas at higher concentrations (5×10^{-3} M) they induce haemolysis (Ahtee, 1966; Seeman, 1972). Like the local anaesthetics, the phenothiazine tranquillizers inhibit virus-induced cell fusion (Poste and Reeve, 1972). The mechanism by which these drugs exert these influences upon biological membranes is largely unknown.

Phenothiazine tranquillizers have also been reported to affect two lectin-mediated phenomena. Ferguson *et al.* (1975) have shown that chlorpromazine, at a concentration of 5×10^{-6} M, inhibits the Con A-induced blastogenic response of mouse lymphocytes. These investigators suggest that chlorpromazine may inhibit blastogenesis by decreasing the Con A-induced cell aggregation which they believe to be necessary for blast transformation. Ryan *et al.* (1974) demonstrated that chlorpromazine (2×10^{-5} M), trifluoperazine (10^{-4} M) or either of two local anaesthetics can inhibit the capping of surface-bound Con A by human polymorphonuclear leukocytes and of anti-Ig-Ig complexes on the surfaces of mouse lymphocytes. The results of these two studies, as well as the similar membrane activities of the cationic phenothiazine derivatives and the local anaesthetics, suggested that the tranquillizers may influence the agglutinability of cells. Consequently, this possibility was investigated in the present study.

The three phenothiazines, chlorpromazine, prochlorperazine, and trifluoperazine, appear to inhibit lectin-mediated agglutination of embryonic *Xenopus* cells at optimal lectin concentrations. This inhibition is drug-dose dependent and, under these experimental conditions requires an incubation time of approximately one hour to completely abolish cell agglutinability. With respect to the drug dose required to effect this inhibition, the minimum concentration appears to lie between 4×10^{-4} M and 8×10^{-4} M. Variability in the density of cell suspensions treated with drugs could perhaps account for those cases wherein 8×10^{-4} M drug did not abolish agglutinability and where 4×10^{-4} M did (see Table 7). A particularly dense cell suspension may require a drug concentration higher than 8×10^{-4} M to completely inhibit the agglutinability of all the cells, if inhibition were to require a minimal uptake of drug per cell. Conversely, 4×10^{-4} M may provide enough drug to abolish the agglutinability of more sparsely suspended cells.

On the basis of agglutinability tests alone, it is not possible to account for this phenothiazine-induced inhibition of the agglutination of cells. There are many possible mechanisms which could account for abolition of cell agglutinability. Chlorpromazine is known to affect membrane properties in several ways. Seeman *et al.* (1972) have reported an influence of phenothiazine tranquillizers upon the distribution of intramembranous particles in the plasma membranes of sheep erythrocytes. It has also been suggested that chlorpromazine preferentially binds to specific cell surface moieties such as the human HLA antigen, A1, and beta-adrenergic receptors (Smeraldi and Scorza-Smeraldi, 1976). These results indicate that the phenothiazines could be inhibiting the

agglutination of embryonic *Xenopus* cells by interfering with interactions between lectins and cell surface lectin receptors. Chlorpromazine has also been reported to preferentially solubilize specific proteins of human erythrocyte ghosts, in particular, cell surface glycoproteins (Boivin and Galand, 1975). Solubilization occurs at drug concentrations of 2×10^{-3} M and higher, which is within the range of concentrations used in the present study. Thus, the phenothiazines could be inhibiting cell agglutination by removing lectin receptors from the plasma membrane or by altering their distribution at the cell surface.

As mentioned earlier, phenothiazine tranquillizers can induce hypotonic hemolysis at concentrations of 5×10^{-3} M or higher. They have also been shown to induce the massive release of serotonin from blood platelets (Ahtee, 1966). Telkka *et al.* (1964), using electron microscopy, found that incubation of rabbit platelets in 10^{-3} M chlorpromazine for one hour results in the rupture of the plasma membrane with some cytoplasmic leakage. However, the organelles inside these ruptured platelets are largely intact. These electron microscopical observations are particularly interesting in light of the changes observed in embryonic cell surface morphology accompanying the inhibition of agglutination. Cells so inhibited appear contracted, with the cortical yolk platelets delineating an irregular cell periphery. No plasma membrane is discernible under phase-contrast microscopy at 400 X magnification. These observations suggest another possible mechanism for the phenothiazine-induced inhibition of cell agglutination. The drugs could be severely disrupting the plasma membrane, perhaps even rupturing it, thereby preventing the lectin-cell interactions required for cell agglutination.

The above are offered as possible mechanisms by which phenothiazine

tranquillizers decrease the agglutinability of embryonic *Xenopus* cells. With regard to the findings of the present study, these explanations remain purely speculative and are not intended to represent the entire spectrum of possibilities. They do, however, provide a basis for future investigations. In particular, the lectin-binding properties as well as the ultrastructural morphology of the peripheries of cells treated with cationic phenothiazines ought to be examined; the former to test whether or not lectin receptors are being removed by the drugs, the latter to investigate whether or not the integrity of the plasma membrane is being affected by the tranquillizers.

SUMMARY

1. *Xenopus lac* embryos at the late blastula stage were dissociated by treatme ith cold calcium-free saline and gentle pipetting. Cells so dissociated are active in the extrusion of lar lobopodial projections from their peripheries.

2. Dissociated cells are agglutinable with the lectins WGA, RCA, and Con A, and to a lesser extent with SBA. The extent of the agglutination is dependent upon the concentration of lectin. Lectin-mediated agglutination can be inhibited by the addition of the appropriate sugar hapten. Cells are not agglutinated by FBP.

3. Neuraminidase treatment of cells enhances their agglutinability with RCA and SBA, but has no effect on their Con A-, WGA-, or FBP-mediated agglu inability.

4. Treatment of cells with xylocaine or procaine alters neither their agglutinability nor their surface morphology. Cells so treated still extrude lobopodia.

5. Treatment of cells with phenothiazine tranquillizers irreversibly reduces their agglutinability. This inhibition is dependent upon drug concentration. Cells treated with phenothiazine tranquillizers appear to be co acted at their peripheries and do not extrud lobopodia.

6. Treatment of cells with colch ther their agglutinability nor their surface morp ith cin B does not alter cell agglutinability; however, cell e with this drug appear to be slightly contracted at their peripheries and lack lobopodia. Cells treated with both colchicine and cytochalasin B simultaneously resemble

cells treated with cytochalasin B alone with respect to agglutinability and surface morphology.

7. Cells fixed with glutaraldehyde are no longer agglutinable with RCA or WGA, and their agglutinability with Con A is drastically reduced. Both platelets present in fixed cell suspensions appear to be agglutinated by Con A. Fixed cells lack lobopodia and appear slightly contracted.

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