AN ANALYSIS OF CELLULAR ADHESION AND SURFACE MEMBRANES IN A SMALL AMOEBA

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ABSTRACT

AN ANALYSIS OF CELLULAR ADHESION AND SURFACE MEMBRANES IN A SMALL AMOEBA

By

Richard Lee Hoover

An investigation of the surfaces of two Acanthamoeba strains was carried out. During exponential growth one, A. (=Mayorella) palestinensis (MP), clumped while the other, A. (=Hartmanella) rhysodes (HR), did not. Microelectrophoretic data indicated that the net surface charge at pH 7.2, where MP clumped and HR did not, was the same for the two cells. Different pI values were also found, indicating differences in the exposed chemical moieties. Cells fixed in 2% glutaraldehyde, which had similar pI's to live cells, maintained a certain adhesive specificity i.e. live HR and dead MP clumped but live HR and dead HR did not. When these fixed cells were then treated with chemicals that affected carbohydrate moieties, the dead HR and live HR clumped, implicating a role for carbohydrates in adhesion.

The phytohemagglutinins, concanavalin A, wheat germ agglutinin and soybean agglutinin, caused HR to aggregate but only soybean agglutinin had any effect on MP. This not only indicated a difference in carbohydrate structure but also a

predominance of glucose-like sites on the surface of HR and a lack of them on MP.

Proteins from the two isolated surfaces were compared by disc acrylamide electrophoresis. The results showed that the proteins of HR (the non-clumper) were smaller than those of MP. Also, all proteins stained with periodic acid Schiff reagent, indicating glycoproteins.

Analyses of glycosyltransferases indicated activity in both surface membranes. Glucosyltransferase and galactosyltransferase were present in HR but only galactosyltransferase was present in MP and at a specific activity twice that of HR. This data correlated with the results from the phytohemagglutinins which also indicated glucose sites on the surface of HR and not MP.

Two unique lipids were isolated from the surface membranes of MP which apparently were not present in HR. Analysis of these by thin layer and gas chromatography indicated one to be a triglyceride and the other a glycolipid.

All of the data implicated carbohydrates as a very important factor in the adhesion phenomenon. The exact mechanism is not known but it is felt that it relates to the lyophobic colloid model outlined by many investigators. Any substance which can reduce the repulsive energy barriers may enhance the probability and strength of adhesion between cells. In those cases, it may be the type of carbohydrate exposed on the surface that influences this process.

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By

Richard Lee Hoover

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INTRODUCTION

Two prolific areas of research in recent years have been the study of cell membrane structure and function and the study of cellular adhesion. From the time of the early isolations and characterizations of erythrocyte ghosts (Gorter and Grendel, 1925; Ponder, 1949) and of myelin sheaths (Robertson, 1958), journals have been filled with material concerning function, content and morphology of various membranes. There is also an abundance of literature on cell adhesion and its importance in cell motility, morphogenesis, parasitology and cancerous growth. However, with this superfluous amount of research it is still not known what makes cells stick together and how the surface membrane is actually integrated into this process.

In this work, I will be looking at two different strains of amoebae with different adhesive properties and trying to analyze what factors on or in the surface membrane cause them to clump. The investigation will be carried out using the whole cells and the isolated membranes. From this I hope to be able to say something about what factors influence adhesion and thereby many other biological phenomena.

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Cell Contacts

When speaking or writing about cell adhesion, it should be realized that there are essentially four different types of cell contacts; therefore, it must be clear as to which is being discussed. (1) There are the maculae adherens, also called desmosomes and terminal bars (Porter, 1956; Fawcett, 1961). They appear as a series of parallel layers lying between the two opposing surface membranes and extending into the cytoplasm of the cell, when observed with the electron microscope. This type of contact does not extend over much of the surface of the cell, but there may be more than one per surface. These have been considered the more stable of the cell contacts and may be responsible for holding adult tissues together. Very little evidence is available concerning the presence of maculae adherens in embryonic tissues. As with all the other contacts to be discussed, it is possible that the contacts are merely fixation artifacts made when preparing the tissues for electron microscopy.

(2) The zonula occludens or the close opposition of plasma membranes appears as a membranous structure composed of five layers, three dark lines separated by two lighter bands supposedly protein (dark) and lipid (light) (Farquhar and Palade, 1963). Apparently the two outermost layers of the cell membrane, the protein layers, have fused to form one layer approximately 20-30 Å in thickness. The standard electron

microscope cannot detect the separate entities. Besides being considered responsible for adhesions, they have also been shown to be areas where intercellular communication might occur very easily. These may be related to an area of low electrical resistance (Lowenstein, 1966).

(3) The septate desmosomes which are in close opposition to the surface membranes have been described to a much lesser extent than the others (Overton, 1963). The surface of the two membranes are separated by approximately 75 Å; however, they are joined by cross-structures (25 Å in diameter) which connect them together. Likewise, these may be more important in adult cell contacts since they apparently do not occur in embryonic tissues.

(4) Finally, the 100-200 Å gap or the zonula adherens, the most prevalent contact in nature, is observed as two complete trilaminar membranes with a separation of 100-200 Å (Farquhar and Palade, 1963). This distance and its significance in adhesion could be caused by at least three different means. (1) The space is filled molecular components which cannot be detected by the electron microscope. (2) The space is filled with a fluid containing no structure and very little macromolecular material. (3) The gap is an artifact produced by dehydration during fixation of materials; however, its prevalence and its coexistence with other types of contacts in the same tissue make this seem highly unlikely. Curtis (1964), using live cells and interference reflection

microscopy, has shown that cells moving across a glass surface do not come in contact with the glass any closer than approximately 100 Å. By changing the conditions of the supporting media, he affected gap distance and ultimately cell adhesion. Permeation experiments have also shown that hemoglobin molecules can perfuse between cells before fixation but not afterwards. This implies the existence of a gap and the presence of a low viscosity material in living tissues (Farquhar and Palade, 1963). There is more evidence for the existence of the gap in real life, but it will not be presented here. The mere ubiquity of this form of contact is the best argument against its being antifactual. It is this type of cellular contact most scientists are concerned with and likewise what I am concerned with in this work.

Theories of Adhesion

There are many theories both physical and chemical to explain adhesion and how it occurs between cells and between various substrates and cells. In order to understand what I have done and what its significance, it is necessary to review briefly the prominent theories.

When considering cellular adhesion, it is necessary to realize that there are repulsive forces as well as adhesive forces and that it is the net force of these two that causes adhesion. Biophysically speaking, there are several types

of attractive forces that could be involved (Curtis, 1967): covalent bonding between surfaces; hydrogen bonding between surfaces; ionic bonding e.g. $NH_3^+ - {}^-OOC$ or $COO^- - {}^+Ca^+ - {}^-OOC$; ionization and deionization of individual groups which are at a pH where partially ionized; charge mosaics arranged so that opposite patches of charge face one another; electrostatic attraction between charged surfaces of differing surface potential; long and short range Van der Waals forces, and image forces due to the tendency for ions to move away from regions of low dielectric constant, lowering osmotic pressure between two cells. Electrostatic forces arise from the charge field set up by various chemical groups on the surface. Since almost all cells under physiological conditions have a net negative charge and since like charges repel one another, electrostatic forces of similar cells are usually repulsive. However, if the electrostatic forces are of different sign or of different strength but of the same charge, they will be attractive. With respect to this it has been proven that Ca^{++} can have an effect on adhesion. Calcium binds to a large number of negative charges, neutralizing the charges and thereby reducing the repulsive forces which would then permit the cells to approach more closely. One major objection to this theory is that as the distance between cells increases the strength of the repulsive forces decreases and at a distance over 15 Å the forces have no effect; therefore, in the 100-200 Å type contact, electrostatic forces probably will not be all that important (Pethica, 1961).

Covalent linkages between surfaces are hard to rationalize because of the close contact needed for this to occur, at least 10 Å. But, if some type of binding material is introduced as an intermediary between the two surfaces that form a covalent bond, then it may be possible. Nevertheless, most cells that form clumps or aggregates can be dissociated much easier than expected if covalent bonds were present.

Van der Waals forces may play a role in adhesion but how to measure and quantify this is almost an impossible task. The theory is based on the fact that there is an attractive force between two surfaces if the dielectric constant between the surface and suspending medium are different--the larger the difference, the greater the force. Since there is a difference in the dielectric constants of the cells and the aqueous medium, the conditions for attraction do exist and make this a possible factor.

It has also been found that energies of attraction and energies of repulsion involved with London Van der Waals and electrostatic forces equal one another at two distinct distances from two particles of similar properties (Schenkel and Kitchener, 1961). At all other distances either the forces of repulsion or attraction prevail. At approximately 10 Å from the surfaces and again at the biologically significent 100-200 Å, the forces of attraction are prevalent. In this respect it can be visualized how the interaction of these forces may effect adhesion and that the 100-200 Å is real and significant.

Curtis (1967), through considerations of surface potentials, London dispersion forces, gap distances, and strength of adhesion proposed a lyophobic colloid model to explain adhesion. This is concerned primarily with the initial adhesion and does not exclude other explanations such as molecular contact or bridging by Ca^{++} and other molecules. The theory must take into account the deformability of cells and that more rigid cells are less adhesive. In order for adhesion to be initiated between two surfaces there must be a point of low surface potential, but if the cell is in the shape of a sphere, the potential would presumably be equal over the entire surface. The formation of pseudopodia which occur initially in most cell adhesion processes, such as motility, reduces the surface potential at that point on the surface. Charges are spread out allowing parts of the cell surface to come in closer contact to another cell. As the cells form new pseudopodia new contacts are made and eventually two cells clump. This is repeated many times before a large aggregate of cells is formed. Once this initial type contact is complete then other forms of contacts might predominate such as Ca^{++} bridging or direct molecular contact between the two surfaces. The theory also takes into account the differences in strength of adhesive bonds. Once two cells have come in very close contact (2-10 Å) then their separation is more difficult; however, if the adhesion is maintained at a distance of 100-200 Å, disaggregation is much easier.

Thus, embryonic tissues which have very few close contacts are much easier to dissociate than adult tissues.

An additional comment on the calcium bridging concept should be made because there are at least two ways for this to occur. The Ca^{++} binds the two surfaces together either directly, or indirectly by binding to one surface and then to an intermediate substance which in turn binds to another Ca^{++} and the other cell surface. This supposedly accounts for the larger 100-200 Å gap. However, in many systems it has been found that the addition of chelating agents such as EDTA has no effect on adhesion (Band and Mohrlok, 1969) even though the Ca^{++} is removed. Also this theory cannot be substantiated when cells are subjected to changes in pH. With a drop in pH there would be an ionization of certain groups which in turn would produce a net positive charge; therefore, not allowing the binding of calcium (Steinberg, 1962). Yet, at the lower pH's, cells will adhere and form aggregates (Curtis, 1963). The role of calcium in initial contacts may be to reduce surface potentials and then later to strengthen the adhesive bond once it is formed.

One of the more recent studies on cell adhesion has based its theory on molecular contact. Roseman (1970) proposes that on the surface of like cells there are multiglycosyltransferase complexes which are capable of transferring specific carbohydrates from a donor molecule to an acceptor molecule, which in this case is also on the surface. When the reaction between

the enzyme, the donor and the acceptor occurs, a certain degree of adhesion results--interlocking the substrate with the enzyme. This is very similar to the theory of Tyler (1947) who proposed that adhesion occurred by a reaction much like that of antigens-antibodies; however, the theory suffers on two points. (1) The surfaces must come in very close approximation for this to occur. (2) The cells must line up in a particular manner so that the substrate lines up with the enzyme. Nevertheless, an investigation of transferases can be useful in demonstrating the types of carbohydrates on the surface which may or may not be responsible for adhesive properties.

The most often suggested chemical contributing to adhesion is the ubiquitous sialic acid. The carboxyl group (COO^-) exposed on the surface would be responsible for the negativity and may even enhance the binding of Ca^{++}, setting up conditions for adhesion. Much has been done to show that sialic acid is the major moiety exposed on the surface and that it actively contributes to the negativity and adhesion of the cell. For example, Weiss (1965) and Cook et al. (1961) have shown that the charge on the surface determined by microelectrophoresis is reduced after the cells are treated with neuraminidase (sialidase). The more recent work of McQuiddy and Lilien (1971), however, has shown that the removal of sialic acid did not interfere with the reaggregation of neural retina cells from 7-, 10- or 14-day chick

embryos. Moscona (1962) has also demonstrated that 7-day chick embryo retina cells aggregate better than the 10-day cells even though the 7-day cells have less sialic acid. All of this points to the fact that sialic acid is not important in attraction and initial aggregation; instead, its role may be one of repulsion. But still not to be overlooked is the fact that in close contacts such as desmosomes, Ca^{++} bridging may be important.

Many investigations have been carried out looking for other specific molecular components on the surface which could contribute to adhesion. One approach to this has been to make antibodies to the surface to see if they could either enhance or hinder adhesion. Spiegel (1954a,b) did this using both embryonic amphibian cells (Rana pipiens and Triton alpestus) and sponge cells (Microciona prolifera and Cliona celata). Antibodies made from the whole cells were used to carry out the adhesion studies. Because of the heterogeneity and the number of antibodies, it is very difficult to discern the actual chemical causing any effect. Furthermore, in order for adhesion of this kind to occur, the cells would need to line up exactly (antibody to antigen) and come in very close contact. Boyse et al. (1968) later showed that inhibition of adhesion may merely be the non-specific coating of the surfaces and not the binding of the specific adhesive sites.

Curtis (1964 and 1966) and Orr and Roseman (1969) have shown that a substance from horse serum can enhance the

aggregation of cells at low temperatures. The material is thought to be a macroglobulin similar to immunoglobulin. Oppenheimer and Humphreys (1971) isolated specific macromolecules from mouse tumor cells required for adhesion. They found it to be very large macromolecule, negatively charged with a molecular weight of about 10^6. Daday and Creaser (1970) isolated a protein extractable with ethylenediamine tetracetic acid that was necessary for reaggregation of avian embryonic cells. They concluded that this material is located in the space between the two surfaces and is bridged to each surface by divalent cations. Taylor (1964) and Taylor and Orton (1967) have also found in the yeast, Hansenula wingei, specific factors that are responsible for agglutination. The material was isolated by digesting the cell walls with enzymes and passage over a Sephadex G-75 column. The chemical had a molecular weight of 570,000 and was sensitive to mercaptoethanol. Later Crandall and Brock (1968) found similar materials on yeast of two opposite mating types and proposed an interaction analogous to the antibody-antigen reaction. It was also shown the substances are specific because they can enhance or block adhesion depending on which type of yeast material are mixed. Supposedly, this factor is a glycoprotein of lower molecular weight than that of Taylor's; therefore, it may be actually the part of the molecule containing the active site.

Kondo and Sakai (1971) have isolated and characterized a reaggregating-promoting substance from sea urchin eggs which they call 'Ovacquenin'. The substance is very similar chemically to hyalin, an insoluble protein of the hyaline layer which surrounds the fertilized egg. They treat the sea urchins in early blastula or morula with isotonic urea and EDTA to disaggregate the cells and extract the material. Like that of hyalin (stored in the cortical granules) and its mode of formation and effectiveness, ovacquenin is secreted in monomeric form and binds immediately to the surface. From here the molecule, in the presence of divalent cations, is built up until it comes in contact with other cells--thus adhesion.

Aggregation of chick embryo and mammalian cell cultures have been shown to be influenced by the action of mucopolysaccharides. All of the substances which enhanced aggregation could also be destroyed by pretreating them with hyaluronidase (Pessac and Defendi, 1972).

The most often mentioned chemical involved in adhesion is either a protein or a glycoprotein. Margoliash et al. (1965) and Humphreys (1963) have characterized substances isolated from dissociated sponge cells which influence reaggregation and which contain carbohydrate and protein. The size of the two glycoproteins varies, Humphreys', $5X10^6$ and Margoliash's, 20,000; however, this may be due to the fact that the latter's is a monomeric form. How these substances

bring about their effect on cells is still in question and unanswered by these authors.

Other evidence, derived from experiments in which protein synthesis was inhibited, has been used to implicate the important role of proteins in adhesion. Moscona and Moscona (1966) could inhibit reaggregation of neural retina cells with puromycin and actinomycin (here there was a slight lag before an effect). Kemp et al. (1967) has also shown puromycin effects adhesion but not to the extent nor for the same reasons as Moscona reported. Kemp pointed out that puromycin causes other effects--depression of carbohydrate metabolism and uptake and utilization of glucose on fat cells and a reduction in uptake of O_2 in sea urchins. The effect on adhesion may be on the surface directly by acting on actomyosin-like proteins and changing the shape of the cells. Previously, Jones (1965) had shown that p-benzoquinone reduced agglutination of embryo, chick fibroblast cells, because it increased the rigidity of the surface. The work of Dunn et al. (1970) supports this idea that the effect of puromycin is more than the inhibition of protein synthesis.

As for the actomyosin-like protein, Gröschel-Stewart et al. (1970) have detected a similar protein on the surface of dissociated embryonic chick cells which may be involved in aggregation. They treated cells with a fluorescent antiserum made against the smooth muscle actomyosin of chick gizzard and were able to show a reduction in the aggregative

properties of the cells. They regarded this to mean that contractible proteins located on the surface are involved in cell adhesion.

Kolodny (1972) has found that thymidime, actinomycin, emetine, salicylate, neuraminidase, X-irradiation, and serum deprivation have no effect on the readhesion and spreading of trypsinized 3T3 cells or SV-40 transformed cells; however, colchicine and reduced temperatures decreased adhesiveness. Since both of the latter inhibitors effect microtubule synthesis and assembly, they believed that contractile proteins play a significant role in adhesion. Finally, Oppenheimer et al. (1969) have shown that teratoma cells deprived of L-glutamine do not adhere normally. One effect of this is that the known pathway for synthesis of glycoproteins, glycolipids or polysaccharides had been interrupted. If L-glutamine was then added back with the addition of an antagonist of L-glutamine (azaserine or 6-diazo-5-oxonorleucine) non-adhesive teratoma cells still could not adhere normally.

The discussion thus far has shown that there are many theories for cell adhesion and that they must be considered just theories since they do not fit many other systems than the one used in the initial experiments. The problem becomes even more complicated when the phenomenon of sorting out is considered. Ever since Wilson (1907 and 1911) and Galtsoff (1925) showed that after mixing two species of sponges

together they will sort themselves out into similar cells, many other theories have been proposed in addition to those mentioned before. Moscona (1965) proposed that each surface has a particular substance on it which makes it unique and specific for cells of the same type. Therefore, like cells are more adhesive to themselves than to unlike cells and because of this all cells will initially clump together, but as soon as they find a surface more satisfactory e.g. a like cell, they will detach and adhere to it. Steinberg's theory (1970) on differential adhesiveness is very similar to this idea except that there is no mention of particular chemicals on the surface. Cells can adhere to any substance but will do so with a preference to those surfaces which are more ideal for stronger adhesions. Curtis (1970) in addition has proposed a timing hypothesis to explain this phenomenon. During development or even after cells are mechanically or enzymatically dissociated, they acquire their adhesive properties at different times during reformation. He carried out such an experiment and showed that in fact different type cells do recover adhesive properties at different times. Armstrong's (1971) results on the one hand contradict this hypothesis because he found that early in the experiment, cells form hetergeneous clumps. If Curtis' hypothesis were correct, then one would first expect to see homogeneous clumps of cells as one type became adhesive together. As the other cells slowly acquire adhesive properties they would adhere to the preformed sphere. Kiremidjian and Keper (1972)

on the other hand support the timing hypothesis for they found changes in adhesiveness of Rana pipiens pronephric cells during development. Recently, Curtis and Van de Vyver (1971) have shown that there are soluble factors in two strains of the sponge Ephydatia fluviatilis that effect adhesion. If the factor is mixed with homologous cells, adhesiveness increases but if added to heterologous cells, it decreases. This helps to explain the conflict between the timing hypothesis and what Armstrong has observed. One would expect to see initially random adhesion between the two different cell types; but, as the cells begin to produce this factor, they would make the local area unfavorable and expel the unlike cell. In this way you can have initial heterogeneous clumps and evoke the timing hypothesis because the cells have not yet gained their complete adhesive properties. And so the argument goes on.

It has also been proposed that sorting out may even occur by some form of chemotactic mechanism (Townes and Holtfreter, 1955). In the hypothesis a gradient is set up which causes cells to segregate to a certain region. In the study of slime mold aggregation, it has been found that the cells depend on secretion of chemicals to find their way (acrasin or c-AMP) (Keller and Segel, 1970; Robertson et al., 1972). Whether this mechanism could apply to embryonic systems is still unknown because of insufficient data demonstrating the secretion of any material which might direct movement.

Roth et al. (1971) have hypothesized that the glycosyltransferase-acceptor complexes could play a role in cell recognition and sorting out. The theory allows for initial adhesions and eventual separation and segregation into clumps of like cells. The cells are clumped by the substrate-enzyme reaction but as soon as the carbohydrate chain is complete or as soon as the donor molecules are depleted, the cells detach leaving the carbohydrate moiety and find a new substrate, thereby sorting out.

The contact phenomenon plays an important role in many other aspects of biology. For example, two reports, one by Morris and Moscona (1970) and one by Abe et al. (1972) have shown that the induction of glutamine synthetase and alkaline phosphatase, respectively, depend on cell interactions. As the cells make more contacts with one another, there is either an increase or an initial expression of the enzyme activity.

There is the additional incentive to study cellular adhesion for medical purposes. Many relationships in medicine, such as that of the host-parasite, are partially expressed as the ability or inability to make contacts. A better understanding of this process can lead to advances in preventing or curing these problems. Recently, pathologists have found that certain amoebae penetrate the nasal mucosa and other tissues of man and other mammals and enter the central nervous system where they ultimately cause death. In order for parasite invasion to occur, certain conditions must be met which favor this. One of these is how easily the amoebae can

penetrate the host. This not only depends on the substrate but also on the adhesiveness of the cells to the substrate.

Furthermore and maybe even more important, is the relationship of cell adhesion to cancerous growth. The host-parasite relationship also would apply here i.e. nature of substrate and adhesiveness of cells. Much work has been done to compare the surfaces of the normal and malignant cells. Sheppard et al. (1971) showed that the 3T3 cells after infection with SV-40 virus agglutinated in the presence of wheat germ agglutinin (WGA). Kapeller and Doljanski (1972) found that normal chick embryo cells transformed by DNA and RNA oncogenic viruses exhibited more binding of WGA and Concancvalin A (Con A) than the untransformed cells. Sela et al. (1970) showed that soybean agglutinin (SBA) agglutinates mouse, rat and human cell lines transformed by viral carbinogens but has no effect on transformed hamster cells. Also because normal cells can be aggregated after treatment with trypsin, it is felt that the carbohydrate sites are in a cryptic form and that transformation exposes them. Similarly, Inbar and Sachs (1969) found that the sites for Con A exposed on the transformed cells can be expressed in normal cells after proteolytic digestion. Cline and Livingston (1971) and Ozanne and Sambrook (1971) using radioactive labelled Con A and WGA found that the number of carbohydrate sites as judged by bound radioactive agglutinin on the surfaces of normal and transformed cells, are not significantly different

and that the agglutinability differences must arise from other surface factors.

Chemical analyses of the surfaces of normal and transformed cells have resulted in conflicting reports. Buck et al. (1970) found glycopeptides from transformed cells contained an enrichment of higher molecular weight peptides as compared to the normal control cells. Later, Buck et al. (1971) confirmed these findings by showing a consistent tendency of material from transformed cells to elute on Sephadex G-50 ahead of that of normal cells, indicating larger glycopeptides. Converse to this, Hakomori et al. (1968) found that the hematosides in normal cells were larger than those in their transformed counterparts. They believed this to be due to an incomplete formation of the carbohydrate chains attached to the lipid. This coincides with the earlier work of Hakomori and Murakami (1968) who found smaller molecular weight glycolipids in transformed cells. Kijimoto and Hakomori (1971) in conjunction with these observations found that the activity of UDP-gal: latosylceramide α-galactosyltransferase was reduced (10-50%) in polyoma transformed cells from that of normal cells. It was also of interest to note that the activity of this enzyme was higher in contact-inhibited cells than in low density populations.

Wu et al. (1969) and Meezan (1969) have also shown that the neutral and amino sugars, especially sialic acid and N-acetyl galactosamine, were reduced in SV-40 transformed

mouse fibroblast cells. This would relate to the size of glycoproteins and glycolipids and indicate that they are smaller in the transformed cells. Chiarugi and Urbano (1972) found with acrylamide electrophoresis that the membrane glycoproteins of polyoma virus transformed cells decrease in glycosylation as compared to the normal cells.

Ultrastructurally, Ambrose et al. (1970) has shown that normal cells have a regular array of fibrils near or associated with the surface and that malignant cells, which have irregular shapes, do not contain these oriented structures. The implication here was that these may be actin-like fibers which form a cytoskeleton or are involved in contractility of the cell--all of which may play a role in cellular adhesion.

To reiterate, cell adhesion can be correlated to malignancy by the fact that transformation of normal cells and malignant cells reduce adhesiveness (Edwards et al., 1971). It is for this reason that many experiments studying cell adhesion have been carried out with virally transformed cells. It has been fortuitous that the adhesion studies have aided in the study of cancer--its prevention and its cure.

I have tried to demonstrate two points in this introduction: (1) although research on cell surfaces and cell adhesion is plentiful, there are still arbitrary questions, and (2) the importance and relevance of a study on cell adhesion and its role in biology and medicine. In this work to be

presented, I have encompassed many of the techniques discussed above and used them to explore one particular system hoping to confirm or establish new facts concerning cell adhesion and the cell surface. The work is based on the initial observations of Band and Mohrlok (1969) who found that two strains of Acanthamoeba--A. (Hartmanella) rhysodes (HR) and A. (Mayorella) palestinensis (MP)--have different adhesive properties. During exponential growth MP clumps while HR does not. Neither form clumps at low temperatures or if dead. However, after fixation in glutaraldehyde and osmium tetroxide followed by dehydration and lipid extraction, the cells still maintain a certain specificity i.e. dead MP will clump with either live amoeba while dead HR will clump only with the live MP. From this, two primary points can be concluded: (1) there must be a living metabolic component in order for clumping to occur since dead cells alone do not clump, and (2) the surface of the two amoebae must be different. It is with the second question that I am primarily concerned within this work. Not only have I looked at the isolated surface membranes and their various chemical moieties, but also I have tried to approach the problem using the whole cell. The amoebae offer an ideal system in that they contain the perfect control for adhesion (clumping and non-clumping) plus the fact that the fixed cells act as ideal models. They can be subjected to many tests without affecting other cell processes. In the present study as well as the

former one, it must be realized that the adhesion of these cells resembles that of initial contacts and that the cells do not form tissues or any type of tight junctions. Nevertheless, the study is applicable to cellular adhesion and hopefully in turn to the many processes controlled by this phenomenon.

MATERIALS AND METHODS

Culture Methods

Acanthamoeba (=Mayorella) palestinensis (MP) and Acanthamoeba (=Hartmanella) rhysodes (HR) were grown axenically on a peptone-glucose nutrient medium (PPG) (0.12g NaCl; 0.003g $MgCl_2$; 0.003g $CaCl_2$; 0.003g $FeSO_4$; 0.142g Na_2HPO_4; 0.136g KH_2PO_4; 10g proteose peptone (Difco); 18.0g glucose; water to 1 liter (Band, 1959). Cells were inoculated into 2 ℓ flasks which had been silicone-coated and incubated at 27±1°C on a rotary shaker until the density of cells was reached.

Whole Cell Electrophoresis

The measurements for this were done on a Northrop-Kunitz rectangular cataphoresis cell (Arthur H. Thomas Co., Philadelphia). The chamber had been modified by replacing the platinum electrodes with Ag-$AgCl_2$ electrodes (Nadell and Creger, 1962) and equipping it with a water jacket. All measurements were made at 25°C. The cells were observed through a microscope with a 10X objective and a 25X focusable eyepiece containing a micrometer. The amoebae were washed

several times in the suspending medium before actual measurements. After each measurement the current was reversed and a new measurement made in the other direction. Rate of migration was determined over various distances and at different milliamps. Viscosity, conductivity, and pH of the solutions were measured at 25°C just prior to the experiments. Mobilities were corrected to the viscosity of water at 25°C. Electrophoretic mobility ($\bar{v}$) was expressed as velocity (μ/sec) per unit field strength (volt/cm). Therefore,

$$\bar{v} = \frac{duAKc}{tiRm} \ (\mu sec^{-1} volt^{-1} cm^{-1})$$

where A = cross sectional area of observation chamber (cm^2); Kc = cell constant of conductivity cell employed, calculated from the equation Kc = Rm/Rs where Rs is the specific resistance and Rm is the measured resistance, in this case of 0.1N KCl at 25°C; d/t = distance (μ) cell moved in a certain time (sec); i = current (amps) flowing through the chamber; and RM = the measured resistance of the suspending medium (ohms/cm^3). Measurements were made at the two stationary levels of the chamber (0.211 and 0.789 X the thickness of the chamber). Cells fixed in 2% glutaraldehyde and 1% osmium tetroxide were also used for these measurements.

Modifications of the Cell Surface and the Effect on Clumping

Cells fixed in glutaraldehyde (2%) and osmium tetroxide (1%) and washed through a series of alcohols up through

propylene oxide were treated with enzymes and poly-lysine to see if adhesive properties could be changed. The marker for these experiments is based on the observation that dead HR (non-clumping amoeba) and live HR will not clump but that dead MP (clumping amoeba) and live HR will (Band and Mohrlok, 1969). After treatment, the reactions were stopped and the cells were washed several times with PPG. They were then mixed with equal proportions of live HR or MP. Observations were made to see if the above mentioned specificity was changed. The concentrations and experimental conditions for the enzymes and poly-lysine were:

Substance	Experimental Conditions
(1) Trypsin, 2X cryst. from Bovine pancreas, Sigma Chem. Co.	3.0mg/20ml, pH 7.0, 37°C, 1 hr.
(2) α-amylase (bacterial) B grade, Calbiochem	0.01mg/10ml, pH 4.8, 37°C, 1 hr.
(3) β-amylase (α-amylase free) B grade, Calbiochem.	2.0mg/1000ml, pH 6.9, 37°C, 1 hr.
(4) Pronase, B grade, Calbiochem.	7.0mg/10ml, pH 7.4, 37°C, 1 hr.
(5) Elastase, from pancreas, Sigma Chem. Co.	1.0mg/1ml, pH 8.8, 37°C, 1 hr.
(6) β-glucoronidase, Worthington Biochem. Corp.	1.0mg/1ml, pH 4.5, 37°C, 1 hr.
(7) Neuraminidase from Cl. perfringens, Type V, Sigma Chem. Co.	1.0mg/50ml, pH 5.0, 37°C, 1 hr.
(8) DNAase, deoxyribonuclease I from bovine pancreas, 1X cryst. and lyoph., Sigma Chem. Co.	1.0mg/50ml, pH 7.15, 37°C, 24 hr.

Substance	Experimental Conditions
(9) RNAase, ribonuclease A, Type 1-A from bovine pancreas, 5X cryst. and ribonuclease T_1 from Aspergillus oryzae, Grade III, Sigma Chem. Co.	1.9mg/ml, (20 mg/ml and 18 mg/ml, respectively), pH 6.0, 37°C, 1 hr.
(10) Collagenase, Worthington Biochemical Corp.	1.0mg-1ml, pH 7.4, 37°C, 18 hr.
(11) Cellulase from Aspergillus niger, Type I, Sigma Chemical Co.	10.0mg/1ml, pH 4.0, 37°C, 24 hours.
(12) β-glucosidase, B grade Calbiochem	1.0mg/ml, pH 5.0, 37°C, 4 hr.
(13) Poly-d-lysine, hydromide, mol. wgt. 75,000-150,000, Sigma Chemical Co.	1.0mg/10ml distilled H_2O, 25°C, 1 hr.
(14) Poly-L-lysine, hydrobromide, Type I, II, I-B, Sigma Chemical Co.	1.0mg/10ml distilled H_2O, 25°C, 1 hr.

Several snake venoms (all from Sigma Chemical Co., St. Louis) were also used; Bothrops atrox (DNAase, hyaluronidase, ATPase, protease and L-amino acid oxidase), 0.2mg/10ml, pH 8.1 at 37°C for 1 hour; Crotalus viridis (L-amino acid oxidase) 0.2mg/10ml, pH 7.6 at 37°C for 1 hr; Pseudechis porphyriacus (lecithinase and L-amino acid oxidase) 0.2mg/10ml, pH 7.8 at 37°C for 1 hr; and Vipera ammodytes (L-amino acid oxidase, hyaluronidase, lethinase and protease) 0.2mg/10ml, pH 7.8 and 37°C for 1 hr.

Preparation and Treatment of Phytohemagglutinins

Concanavalin A (Sigma Chemical Co., St. Louis) (Con A) was added to live and dead cells at concentrations of

100μg/ml, 500μg/ml and 1000μg/ml in a low salt solution (0.5M NaCl. 5.4 X 10^{-4}M $MgSO_4$ and 3.6 X 10^{-4}M $CaCl_2$). Cell aggregation was used to determine extent of effect.

Soybean agglutinin was prepared according to Liener (1952). Two hundred fifty grams of soybean flour were suspended in 3 ℓ of distilled H_2O at room temperature and the pH adjusted to 6.7 with constant stirring. This was continued for 1 hr. The solution was then acidified with HCl to pH 4.6 and allowed to settle overnight at 4°C. The supernatant was siphoned off and the remaining liquid removed by filtration. To each liter of the water extract, 300g of ammonium sulfate was gradually added. The precipitate was removed by filtration and 100g of ammonium sulfate added to the clear yellow filtrate and allowed to settle overnight at 4°C. The supernatant was discarded and the precipitate collected by centrifugation. A slurry was made of the precipitate with H_2O and then dialyzed against distilled H_2O for 36 hr. at 4°C. Any precipitate still remaining was removed by centrifugation. The pH of the solution was adjusted to 4.6 and the volume brought up to 200ml. To this 70g of ammonium sulfate was added followed by centrifugation at 10,000g for 10 min. The pellet was redissolved in 70ml of 0.05M phosphate buffer pH 6.1. This solution was dialyzed against 60% ethanol at -5° to -10°C for 24 hr. The precipitate formed was removed by centrifugation, dissolved in a small amount of H_2O and dialyzed against distilled H_2O overnight at 4°C. After removal of any remaining precipitate, the solution was

lyophilized and stored frozen until further use. This crude fraction was then dissolved in 5ml of 0.001M PO_4^- buffer, pH 6.8, and applied to a 2.8X15 cm column of hydroxylapatite (calcium phosphate) (Lis et al., 1966) and eluted with PO_4^- buffer in a stepwise manner--50ml each of 0.001M, 0.05M, 0.1M, 0.2M and 0.5M. The fractions were monitored by optical density readings at 280mμ and the 0.2M PO_4^- eluate was dialyzed extensively against H_2O and lyophilized. This was redissolved in water, passed over a hydroxylapatite column again and eluted with 0.2M PO_4^- buffer, pH 6.8. The optically dense fractions at 280mμ were combined and dialyzed against H_2O and finally lyophilized. This was termed SBA and used in the same concentrations and tested in the same manner as Con A.

Wheat germ agglutinin (WGA) was prepared according to the procedure of Burger and Goldberg (1967). One gram of wheat germ lipase (Calbiochem) was suspended in 50ml H_2O and ground with a mortar and pestle, then homogenized in a Teflon homogenizer at room temperature. This suspension was put in a water bath at 63°C for 15 min. and afterwards centrifuged to remove any precipitate. Finally, it was passed through glass wool to clear completely the supernatant. To the supernatant (approx. 42.5ml) was added 11.5g of ammonium sulfate and stirred for 20 min. The precipitate was collected by centrifugation, redissolved in 5ml H_2O and dialyzed against H_2O overnight at 0°C. Any resulting precipitate was removed by centrifugation. The dialyzed fraction was condensed to 7 ml, applied

to a Sephadex G-75 column (2.25X65 cm) and eluted with water. The fractions were monitored with OD at 280mμ and collected in 5ml samples (Figure 1). Electrophoresis was carried out on the final fraction according to the methods of Clarke (1964). A pattern was found similar to that found by Burger so it was assumed the sample was pure WGA. The samples were lyophilized and stored below 0°C. Concentrations like those of Con A and SBA were used on the cells. In all three cases, the experiments were carried out for 1 hr. with observations being made at 15 min. intervals.

Membrane Isolation

Surface membranes, Golgi membranes and endoplasmic reticulum were isolated according to Chlapowski and Band (1971). Surface membranes were also isolated by a modification of the same method. In the original method Mg^{++} was present in all solutions but in the modification, it was absent in order to eliminate the sucrose gradient centrifugation. Cells were collected by centrifugation at 500g for 5 min and washed once in 0.0005M Tris-HCl, pH 7.5 and 0.25M sucrose (T). All subsequent steps were carried out at 0-4°C. The final pellet was suspended in an equal volume of T and homogenized in a Potter-Elvehjem glass Teflon grinder at 2000rpm by 8 up and down strokes. The homogenate was spun down at 2000g in a Sorvall HB-4 swinging bucket rotor for 15 min. The supernatant was poured off and the pellet

Figure 1. Optical density readings at 280 mμ of fractions of wheat germ agglutinin eluted from a Sephadex G-75 column.

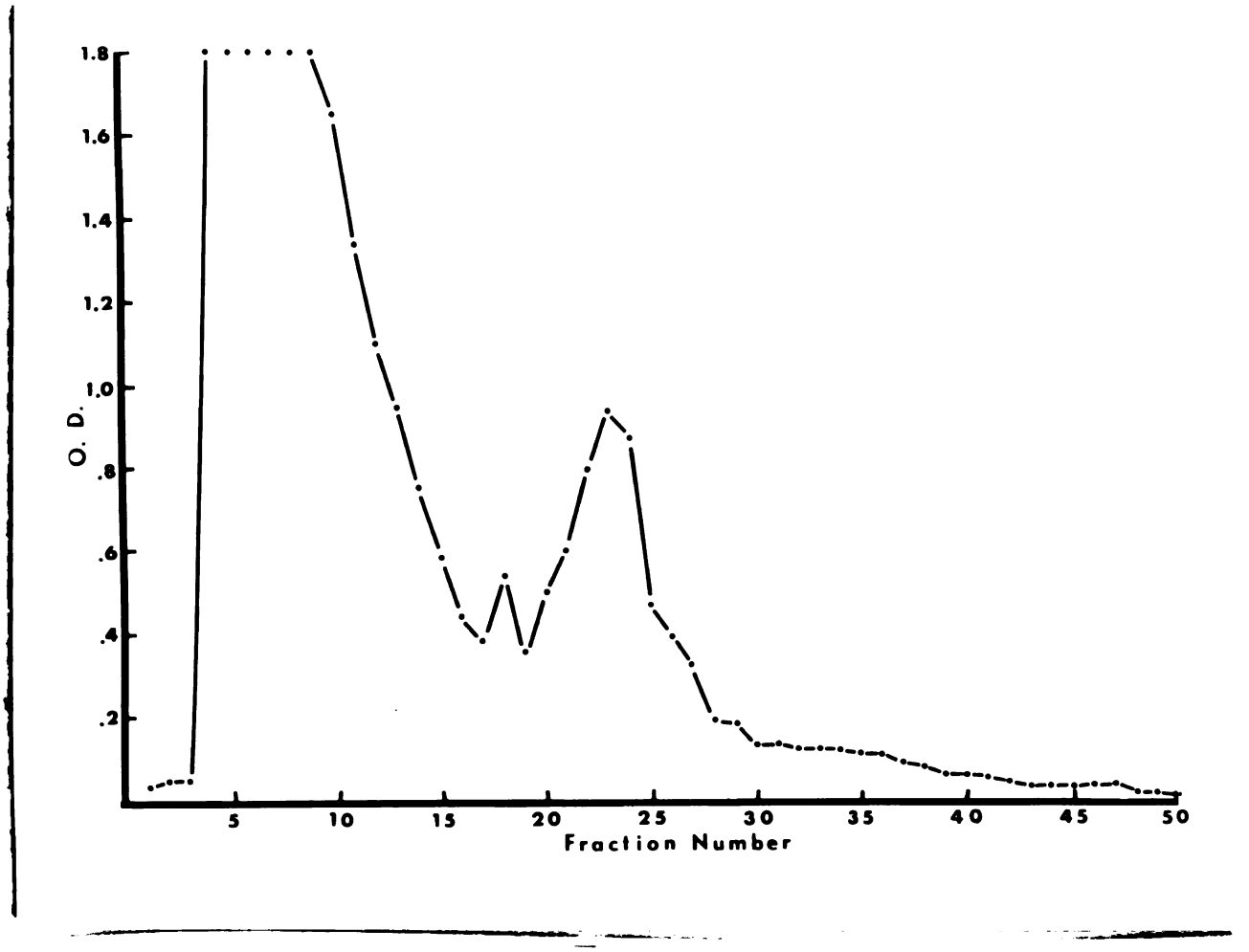

Figure 1

resuspended in T and recentrifuged as before. This was repeated 4X. After the final centrifugation, 0.005M Tris-HCl, pH 7.5, 0.025M KCl and 0.25M sucrose (TK) was used to wash the pellet twice. Finally, the precipitate (primarily surface membranes, mitochondria and a few unbroken nuclei) was suspended in 0.25M sucrose and 0.001M ethylenediamine tetracetic acid (EDTA), pH 7.5, and centrifuged at 2000g for 15 min. This step was repeated at 1100g for 10 min. until the supernatant became clear as judged by phase microscopy. The fluffy white pellet was pure surface membranes.

In some instances where there were a large number of cysts, sucrose gradient centrifugation had to be inserted before the washes with sucrose-EDTA. This consisted of a 4ml continuous gradient of TK (1.3M-2.0M sucrose) centrifuged at 100,000g for 30 min. The membrane layered at a density of 1.18g/cc and were removed with a J-shaped needle. This was followed by the sucrose-EDTA washes.

Analysis for Purity of Membranes

Water washed membranes were assayed for ATPase activity (Mg^{++}-Ca^{++} and Na^{+}-K^{+} dependent) by the methods of Hays and Barland (1966) and of Kleinig (1970). In both instances Mg^{++}-Ca^{++} dependent ATPase was separated from Na^{+}-K^{+} dependent ATPase by the addition of ouabain which inhibits the Na^{+}-K^{+} dependent enzyme. The 5'-nucleotidase assays were carried out according to Kleinig (1970) and Schultz Thompson (1969).

The free phosphate released in the experiments was measured by the Fiske-Sabbarow test (Linberg and Ernster, 1956). Phase microscopy was also used as a minitor for purity because nuclei and mitochondria, the most prevalent contaminants, can be seen easily. The presence or absence of RNA and DNA was detected as a measurement of OD at 260mμ.

Membrane Solubilization

The surface membranes were solubilized by several methods but predominantly by that of Kiehn and Holland (1968) and that of Zahler et al. (1970). In the former method the isolated membranes were dialyzed extensively against 0.1% sodium dodecyl sulfate (SDS), 0.1% β-mercaptoethanol, 0.5M urea, 0.001% EDTA and 0.01M Tris-HCl, pH 9.0. In the latter method the membranes were dissolved in phenol:acetic acid:8M urea (2:1:1) (PAU). In both of these methods, solubilization, as judged by clarity of solution, was complete for HR but not MP.

Acrylamide Electrophoresis

Gel electrophoresis was carried out according to Clarke (1964) on 5% acrylamide with all samples except those dissolved with PAU. Stock solutions of acrylamide, N_4-tetramethylethylenediamine (TEMED), ammonium persulfate and gel buffer were made up prior to running the experiments. The acylamide solution (A) consisted of 30.0g acrylamide, 1.0g N,N'-methylenebisacrylamide and 123ml H_2O: TEMED (B) was a 0.28% solution;

ammonium persulfate (C) was a 0.14% solution; and the buffer (D) consisted of 29.0g glycine, 6.0g Tris and 980ml H_2O. To make the 5% gels 2 volumes of A, 1 volume of B, 4 volumes of C and 1 volume of D were mixed together. In some experiments 1% SDS and/or 8M urea was incorporated into the gels. The upper and lower electrolytes consisted of a 1:10 dilution of the stock composed of 29.0g glycine, 6.0g Tris, 5ml 1N HCl and 975ml H_2O, pH 8.1. Electrophoresis was carried out at 1ma/gel until the tracking dye (0.5% bromophenol blue in 1% acetic acid) reached the bottom.

The procedure for molecular weight determination using acrylamide electrophoresis was that of Weber and Osborn (1969). Stock solutions were made as follows: (A) 22.2g acrylamide, 0.6g bis and H_2O up to 100 ml; (B) 7.8g $NaH_2PO_4 \cdot H_2O$, 38.6g $Na_2HPO_4 \cdot 7H_2O$, 2g SDS and H_2O up to 1 ℓ, pH 7.0; and (C) ammonium persulfate, 15mg/ml. The tracking dye consisted of 0.05% bromophenol blue in H_2O. Gels were prepared by mixing 15ml of B, 13.5ml of A, 1.5ml of C and 0.045ml TEMED. Membrane samples as well as the standards were prepared by incubating them in 0.01M PO_4^- buffer, pH 7.0, containing 0.1% SDS and 0.1% β-mercaptoethanol at 37°C for 3 hr. Three microliters of tracking dye, 1 drop of glycerol, 5 μl β-mercaptoethanol, 50μl dialysis buffer and 10-50μl protein were added on top the gels. Upper and lower chambers of the electorphoretic unit contained a 1:1 dilution of solution B. Electrophoresis was carried out at 8 ma per gel for

4 hours. Measurements were made of the length of the gel before and after staining and of the distances the tracking dye and protein migrated during the run. Mobility was calculated as

$$M = \frac{\text{dist. of protein migration}}{\text{length of gel after destaining}} \times \frac{\text{length before staining}}{\text{dist. of dye migration}} -$$

Standards included: serum albumin (Bovine, Fraction V, Sigma), catalase (lyophilized, Worthington Biochem.), ovalbumin (Grade V, salt free, Sigma), pepsin (2X cryst and lyoph., Sigma), trypsin (2X cryst. Bovine pancreas, Sigma), lysozyme (3X cryst. from egg white, B grade, CaIbiochem), RNAase A (5X cryst. from bovine pancreas, Sigma), cytochrome C (Type III from horse heart, Sigma) and insulin (Bovine pancreas, Sigma).

Gels for the PAU samples were made as follows: 3g acrylamide, 0.08g bis, 6g urea and 14ml glacial acetic acid were dissolved in water to a volume of 30ml; 0.15g ammonium persulfate was dissolved in 10ml of IOM urea and mixed with the acrylamide solution plus 0.1ml TEMED. This was allowed to polymerize in the tubes for 3 hr. Pre-electrophoresis was carried out for 3 hr. at 5ma/gel with 35% acetic acid as the upper and lower buffers. Samples (0.1ml membrane dissolved in 0.9ml PAU) were layered on the gels and run at 2.5 ma/gel for 3-5 hr. The electrolyte was 10% acetic acid.

Several methods were employed to stain the gels. (1) They were stained with 0.1% naphthol blue black in 7% acetic acid for 1 hour and destained by diffusion in 7% acetic

acid overnight. (2) They were stained with Coomassie blue according to Fairbanks et al. (1971): (a) 25% isopropanol, 10% acetic acid and 0.035% Coomassie blue, overnight; (b) 10% isopropanol, 10% acetic acid and 0.003% Coomassie blue, 6-9 hr.; (c) 10% acetic acid and 0.003% Coomassie blue, overnight; and (d) 10% acetic acid for several hr. (3) Or, they were first soaked overnight in 40% methanol and 7% acetic acid at 37°C and then stained with 0.25% Coomassie blue in 7% acetic acid. Then they were destrained by diffusion in 7% acetic acid-40% methanol.

Gels were stained for carbohydrate according to Zacharius et al. (1969). They were fixed for 20 min in 12.5% trichloroacetic acid; rinsed in H_2O 2minl; immersed in 1% periodic acid in 3% acetic acid for 50 min; rinsed in H_2O overnight; soaked in Fuchsin-sulfited stain in the dark for 50 min; washed 3X for 10 min each in freshly prepared 0.5% metabisulfite; and washed overnight in H_2O.

Composition of the Membranes

Protein was measured by the method of Lowry (1951) with albumin as a standard.

Carbohydrate content was determined by the anthrone test according to Dische (1955) with glucose as a standard.

Sialic acid determinations were carried out following the procedure of Jourdian et al. (1971) with N-acetylneuraminic acid as the standard.

Determination of nitrogen was by the micro-Kjeldahl method of Steyermark (1961) with glycine as a standard.

Isolation and Analysis of Specific Lipids from MP Surfaces

Lipids from the MP surface membrane were isolated from a colloidal solution remaining after solubilization by any of the above methods. The solubilized membrane was spun down at 10,000g for 10 min. and the supernatant recentrifuged at 200,000g for 30 min. in a SPINCO SW-50 swinging bucket rotor. The material floated on the surface and was removed with a J-shaped needle attached to a syringe. After suspension in H_2O, the lipids were again centrifuged at 200,000g for 30 min. and removed as before. The floating material was extracted with chloroform:methanol (2:1), washed 2X with H_2O and dried under N_2. The dried substance was redissolved in chloroform:methanol (2:1) and run on silica gel coated thin layer plates. The plates were developed in several different solvents--chloroform:methanol:water (100:42:6), chloroform:methanol:acetone (70:30:1) and chloroform:methanol (8:1). Spots were detected with I_2 (1% in methanol), concentrated H_2SO_4 and α-naphthol in H_2SO_4. The spots from the I_2 treated plates were scrapped off and eluted from the silica gel with the running solvent. The material was then analyzed with gas chromatography and mass spectrometry.

Glycosyltransferase Activity in Surface Membranes

The methods outlined by Basu et al. (1968) for glucosyltransferase analysis were used on surface membranes, Golgi and endoplasmic reticulum fractions of each amoebae. Assay mixtures (final volume, 0.10ml) contained 0.4mg Cutscum detergent; 0.2mg Triton X-100; 20μM Bicine buffer (Matheson Scientific), pH 7.8; 0.5μM $MnCl_2$; 0.2μM ceramide (lipid acceptor); 87μM (10μl) UDP-^{14}C-glucose (227mCi/mM, New England Nuclear); and 0.6mg protein. The mixture was incubated at 32°C for 1 hr. Controls without protein and lipid were also set up to determine enzymatic and hydrolytic breakdown of UDP-^{14}C-glucose. Other controls were run using 7-day embryonic chick brain as described previously (Basu et al., 1968). Incubation mixtures were prepared by dissolving the lipid acceptor and detergent in (2:1 chloroform:methanol, drying and then adding the rest of the assay mixture. The reaction was stopped with addition of 20μl of 5.0μM KCl and 2.5μM EDTA followed by 0.5ml chloroform:methanol (1:1). The lower layer was removed and washed once with 0.2ml of chloroform:methanol: 0.1M KCl (3:47:48). The chloroform layer was dried under nitrogen and redissolved in chloroform:methanol (2:1). This was applied to Whatman SG-81 silica gel impregnated paper which had been saturated with 1% sodium tetraborate and dried at 100-110°C. The system was developed in chloroform:methanol: water (60:17:2). The chromatogram was dried and cut into strips and placed in scintillation vials with the following

cocktail: 6.0g 2,5-diphenyloxazole (PPO), 0.01g 1,4-bis-2-(5-phehyloxazolyl)-benzene (POPOP) and 100ml toluene. The samples were counted on a Packard Tri-carb Liquid Scintillation Spectrometer Model 3320. Background and quenching were calculated into each sample.

Galactosyltransferase activity was measured on only surface membranes by a slight modification of the method of Fleischer et al. (1969). The assay mixture (80μl) consisted of 6μM sodium cacodylate, pH 6.75; 3μM $MnCl_2$; 3μM mercaptoethanol; 78μM (5μl) UDP-^{14}C-galactose (254.5mCi/mM, New England Nuclear); 3μM N-acetylglucosamine (acceptor); and approximately 50μg protein. Controls without protein and without N-acetylglucosamine were also run. The mixtures were incubated at 37°C for 1 hr. Reaction was stopped with the addition of 17μl of 0.3M EDTA, pH 7.4 with NaOH, and cooling. The mixtures were then passed over an ion exchange column (0.5X2.0 cm) of AG-2 X 8, 200-400 mesh Cl^- form (BioRad Laboratories, Richmond, Calif.) which had been washed with distilled H_2O. Two 0.5ml aliquots of H_2O were used to elute the material. The only substances passing through the column were the N-acetylglucosamine+galactose-C^{14} (N-acetyllactosamine-^{14}C) and the free galactose-C^{14}. The unreacted UDP-galactose remained bound to the column. The samples were mixed with the scintillation cocktail (5.5g PPO, 0.1g POPOP, 667ml toluene and 333ml Triton X-100) and counted as before.

RESULTS

Microelectrophoresis

Table 1 presents the electrophoretic mobilities of living HR (lHR) and living MP (lMP) at different pH's. Each value represents an average of two experiments of 50 measurements each. From this data there are two significant observations to be made. First, at the physiological pH (7.2) where adhesion occurs in Mp and not HR, the electrophoretic mobilities and thereby the surface charge are essentially the same. Secondly, the pI or isolectric point of the two cells is different. This is measured as the pH at which there is zero electrophoretic mobility when a current is applied across the chamber. This was extrapolated from the graph of the pH vs. electrophoretic mobility (Figure 2). The data indicate the pI's are different and that MP has the higher, thus, a difference in the chemicals on the two surfaces.

Table 2 is the data showing the electrophoretic mobilities of glutaraldehyde fixed HR at the different pH's. Again this is the average of two experiments of 50 measurements each. This is to demonstrate what groups on the surface may be effected by fixation. There is a reduction in the

Table 1. Electrophoretic mobilities of live HR and MP* ($\mu m/sec/volt/cm^2$).

pH	HR	MP
7.2	-.8059	-.8086
6.5	-.9042	-.8751
5.2	-1.0273	-.4752
4.1	-1.1630	-.4799
3.2	-.4298	+.5643
2.2	+1.0491	+1.3980

*HR = Hartmanells rhysodes

MP = Mayorella paleslinosis

Figure 2. Electrophoretic mobilities of live HR and MP. The pI's for HR and MP are 2.9 and 3.4, respectively. ·=MP, X=HR.

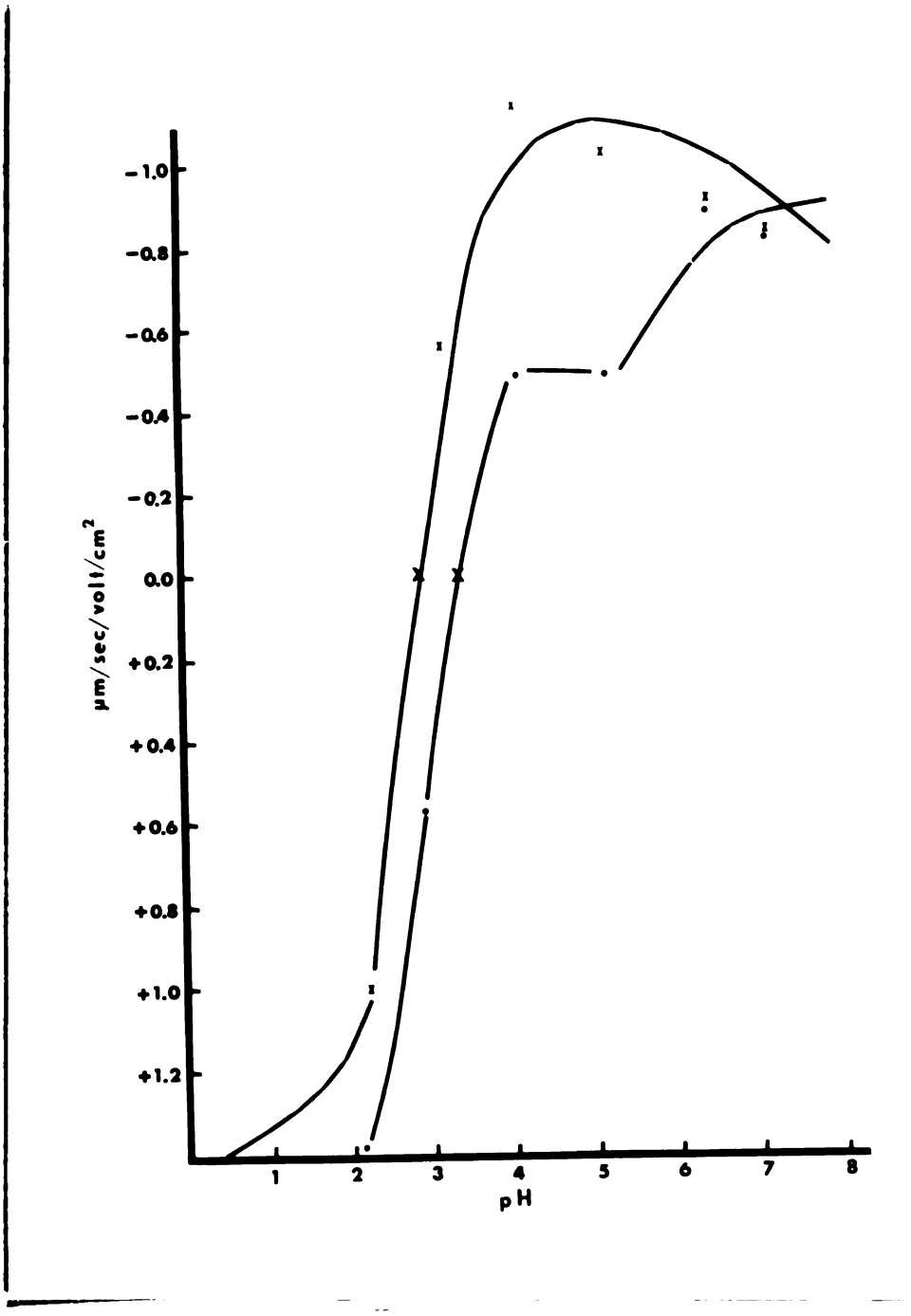

Figure 2

Table 2. Electrophoretic mobilities of glutaraldehyde (2%) fixed HR and MP ($\mu M/sec/volt/cm^2$).

pH	HR	MP
7.2	-.7412	-.6789
6.5	-.6073	
5.2	-1.640	
4.1	-1.3503	
3.2	-	
2.2	+.5636	

charges or the electrophoretic mobilities of the fixed cells but the pI (2.9) is the same as with the live cells (Figure 3).

Table 3 represents the data from electrophoretic measurements of fixed cells treated with various chemicals, including four snake venoms (2 experiments, 50 measurements each). It is only with the latter substances that a drastic decrease in charge at pH 7.3 is seen. It should also be noted that these substances cause dead HR (dHR) and lHR to clump. The other values compare to the controls that were fixed and lipid extracted.

Effects of Various Substances on Adhesive Specificity

Table 4 gives the results of the two separate experiments in which the glutaraldehyde fixed cells were treated with various enzymes, poly-lysine and membrane solubilizers. In these experiments two test groups were observed in particular--the lHR and dMP, to see if clumping could be blocked and the lHR and dHR to see if clumping could be induced. In no instance could the clumping of dMP and lHR be blocked. However, the enzymes that effect carbohydrate moieties such as β-amylase, elastase, β-glucoronidase, sialidase, collagenase, cellulase, β-glucosidase, etc. destroyed the specificity of HR i.e. lHR and dHR clumped. Only one from this group, α-amylase, had no effect on the clumping properties of HR. None of the proteolytic enzymes except protease (subtilisin)

Table 3. Electrophoretic mobilities of chemically treated glutaraldehyde (2%) fixed HR and MP (um/sec/volt/cm^2).

pH	Treatment	HR	MP
7.3	Lipid extracted	-.7882	-.6789
7.3	Cold 5% trichloro-acetic acid (TCA)	-.6604	-.6985
7.3	Cold TCA and lipid extracted	-.6768	-.7489
7.3	Lipid extracted then cold TCA	-.7596	-.7896
7.3	Lipid extracted and venom of *Bothrops atrox*	-.5189	-.5874
7.3	Lipid extracted and venom of *Pseudechis porphyriacus*	-.5277	-.5368
7.3	Lipid extracted and venom of *Vipera ammodytes*	-.5462	-.5660
7.3	Lipid extracted and venom of *Crotalus viridis*	-.5277	-.5660
6.5	Lipid extracted and poly-L-lysine	-.6035	-.6593
6.5	Lipid extracted, 8M urea, 1% SDS and 0.1% β-mercapto-ethanol	-.8092	-.8280

Figure 3. Electrophoretic mobilities of glutaraldehyde fixed HR. The pI (2.9) is comparable to that of the live cells.

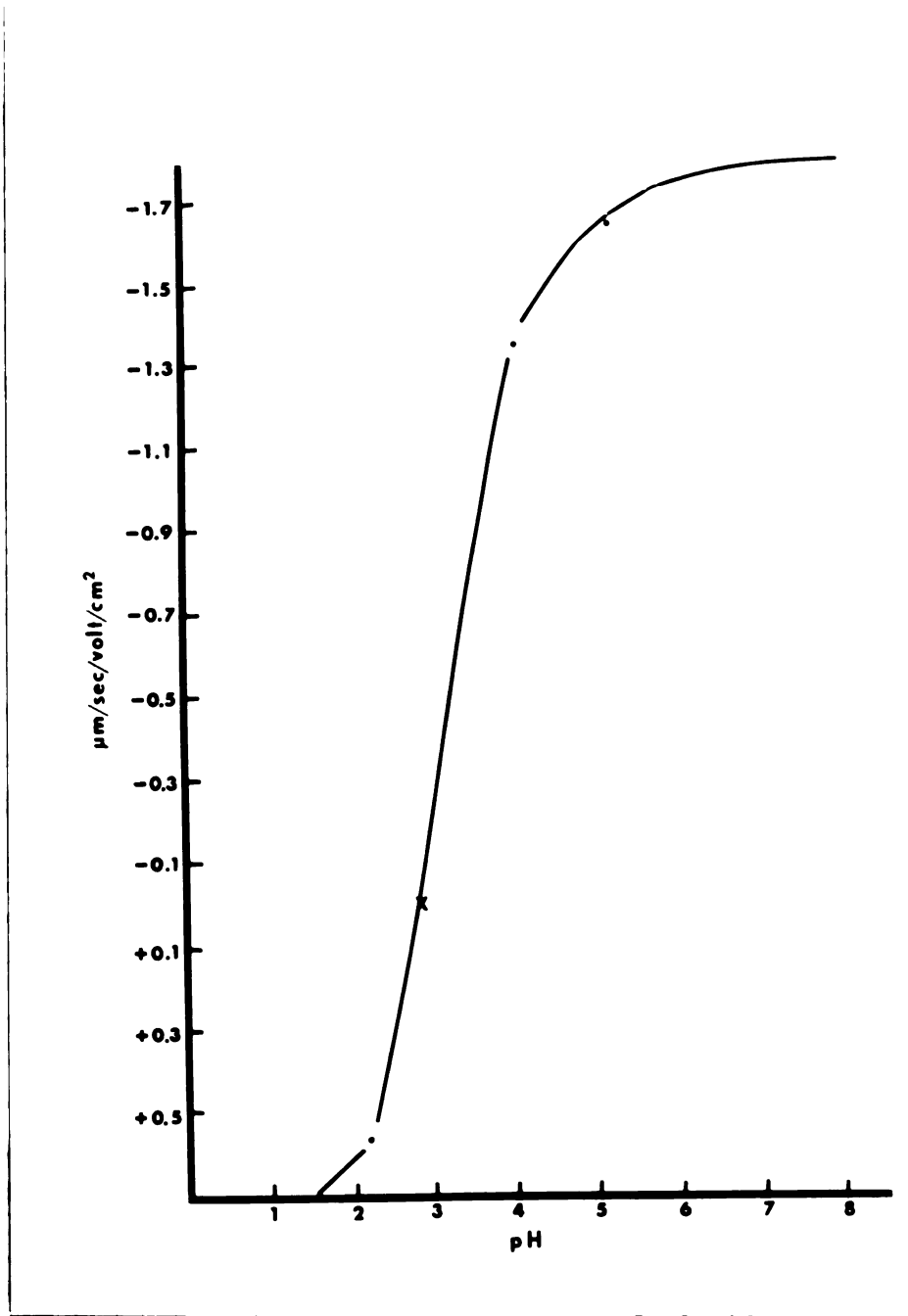

Figure 3

Table 4. Effects of various chemicals on the adhesive properties of fixed cells (d) when mixed with live cells (l).

Test substance (conc.)	lMP dMP	lMP dHR	lHR dMP	lHR dHR
Control-nothing added	+	+	+	-
Trypsin (3.0 mg/20 ml)	+	+	+	-
β-amylase (0.01 mg/10 ml)	+	+	+	+
α-amylase (0.002 mg-1000 ml)	+	+	+	-
Pronase (7.0 mg/10 ml)	+	+	+	-
Elastase (10 mg/10 ml)	+	+	+	+
β-glucoronidase (1.0 mg/1 ml)	+	+	+	+
Neuraminidase (1.0 mg/50 ml)	+	+	+	+
Collagenase (10.0 mg/10 ml)	+	+	+	+
Cellulase (10.0 mg/1 ml)	+	+	+	+
RNAase (0.1 mg/1 ml)	+	+	+	-
DNAase (1.0 mg/1 ml)	+	+	+	-
RNAase, DNAase, and pronase (as above)	+	+	+	-
α-glucosidase (1.0 mg/1 ml)	+	+	+	+
2M NaCl	+	+	+	+
1M NaCl	+	+	+	+
8M urea, 1% SDS and 0.1% mercaptoethanol	+	+	+	+
Dimethylformamide-HCl	+	+	+	+
Protease (subtilisin)	+	+	+	+
Poly-d-lysine (10 mg/100 ml)	+	+	+	-
Poly-L-lysine (10 mg/100 ml)	+	+	+	-
1% β-mercaptoethanol overnight, room temp.	+	+	+	+
Brthrops atrox (0.2 mg/10 ml)	+	+	+	+
Crotalus viridis (0.2 ml/10 ml)	+	+	+	+
Pseudechis porphyriacus (0.2 mg/10 ml)	+	+	+	+
Vipera ammodytes (0.2 mg/10 ml)	+	+	+	+

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had any effect; and neither RNAase nor DNAase nor a combination of both had any effect on this group. High concentrations of sodium chloride (1M and 2M), several solutions used to solubilize the surface membranes (dimethylformamide; 8M urea, 1% SDS and 1% β-mercaptoethanol; and 1% β-mercaptoethanol) and all the snake venoms which contain at least two different enzymes caused the aggregation of live and dead HR. Coating the cells with poly-L-lysine or poly-D-lysine also caused all the cells to clump.

Binding of Phytohemagglutinins to the Surfaces of HR and MP

Table 5 shows the results of three different experiments when the agglutinins were added to both live and glutaraldehyde fixed HR and MP. The non-clumper, HR, aggregated in the presence of all the agglutinins; however, there was a definite degree to the extent of clumping based upon size of clumps and number of single cells. Con A was by far the best agglutinating agent because all HR cells clumped into large aggregates as soon as the agglutinin was added. Time did not seem to be important for adhesion to occur. In the cases of WGA and SBA, the size of the clumps decreased and the numger of single cells increased. Furthermore, time did play a role in these adhesions in that it took longer for the cells to agglutinate in the presence of these substances than it did with Con A. Even though it was slight, SBA was the only agglutinin to have any effect on MP.

Table

| Phytoh (

Concan

Wheat g

Soybean

+ =
++ =
+++ =

Table 5. Effect of phytohemagglutinins on the clumping of HR and MP.

Phytohemagglytinin (conc μg/ml)	HR	MP
Concanavalin A		
100	+++	-
500	+++	-
1000	+++	-
Wheat germ agglutinin		
100	++	-
500	++	-
1000	++	-
Soybean agglutinin		
100	+	-
500	+	+
1000	+	+

+ = less than 30% of cells in clumps

++ = 30-60% of cells in clumps

+++ = 100% of cells in clumps

Isolation, Composition, Solubilization and Electrophoresis of Surface Membranes

Table 6 presents the percentage composition of the isolated membranes based on dry weight. The results are an average of four experiments. There are several significant figures that should be pointed out. First, a comparison of the protein and lipid content show that the MP membrane contains a higher percentage of protein but a lower percentage of lipid than HR. Second, the carbohydrate content is the same for both (10%). Third, the sialic acid content of MP is much higher (almost 2X) than that of HR. Fourth, nitrogen determinations indicate a similarity between the two membranes. And finally, the nucleic acid content of both membranes appears to be practically zero.

The presence of Mg^{++} strengthens the surface and makes the membranes practically insoluble in any of the solvents used. However, the exclusion of Mg^{++} permitted total solubilization of HR and almost complete solubilization of MP. Several different methods were used that gave similar patterns on electrophoresis (Clarke, 1964). They were:

(1) 2% SDS

(2) 5mM EDTA, 5mM β-mercaptoethanol and 2.5% SDS (Lenard, 1970)

(3) 8M urea, 1% SDS and 1% β-mercaptoethanol

(4) 20% SDS

(5) 1% SDS (Nachman and Ferris, 1970)

Table 6. Composition of surface membranes expressed as percentage dry weight.

Material	MP	HR
Protein	39.1-42.0	34.1
Lipid	50	58
Carbohydrate	10-11	10-11
Sialic acid	6.8	3.5
Nitrogen	28.8	31.0

(6) 1% SDS and 5mM EDTA

(7) 1% SDS, and 1% β-mercaptoethanol, pH 7.0 (Weber and Osborn, 1969)

(8) 1% sodium deoxycholate (Emmelot and Dias, 1970)

(9) 1% Triton X-100 (Miller, 1970)

(10) 1% Lubrol WX (Fitzpatrick et al., 1969)

(11) Membranes in dimethylformamide-HCl dialyzed against 8M urea in 35% acetic acid, then 8M urea in 2% sodium acetate, next in 8M urea, 1% SDS, pH 7.2, and finally 8M urea, 1% SDS, 0.1% β-mercaptoethanol, pH 7.2 (Schnaitman, 1969)

(12) Phenol:acetic acid:8M urea, 2:1:1 (Zahler et al., 1970)

(13) 5mM EDTA, 0.1% SDS, 0.1% β-mercaptoethanol, 0.01M Tris, pH 9.0 (Kiehn and Holland, 1968)

(14) 5% SDS, 5% mercaptoethanol, 0.01M γ-aminobutyric acid and 0.05M Tris pH 8.5 (Laico et al., 1970).

When the membranes were then run on acrylamide gels, it was evident the means of solubilization did play a role in the pattern observed. Figure 4 presents the protein patterns for all the membranes solubilized by any method except the phenol/acetic acid/urea method (PAU). This figure, as well as those for other gel patterns, is representative of two or more separate experiments. On these gels as well as all others the areas where dissimilarity occurs between the gels is designated by lines on the outside and where similarity occurs with lines between the gels. The gels are 5% acrylamide made according to Clarke (1966) with nothing else added; however, in Figure 5, where 8M urea had been added the number of bands increases by at least one. Yet, as in Figure 4, the patterns of HR and MP membranes are quite similar with a slight

Figure 4. Electrophoretic patterns of HR and MP surface membranes on 5% acrylamide following solubilization by the methods of Kiehn and Holland (1969). The stain is naphthol-blue black.

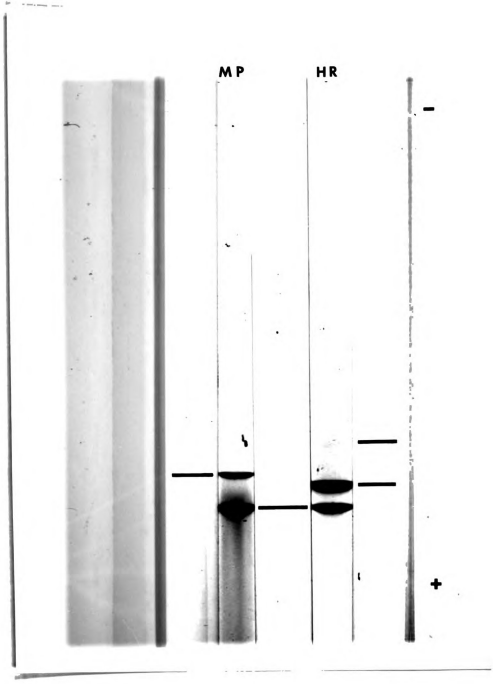

Figure 4

Figure 5. Electrophoretic patterns on HR and MP surface membranes on 5% acrylamide with 8M urea added to the gels. Solubilization was by the method of Kiehn and Holland (1969). The gels were stained with naphthol-blue black.

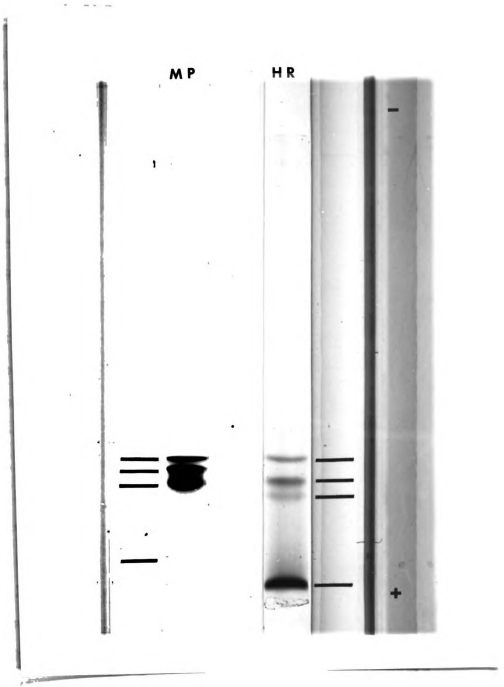

Figure 5

indication of larger proteins in MP. Periodic acid Schiff reagent was also used to stain these gels as well as those used for molecular weight determinations. In both cases, the protein bands subsequently stained for carbohydrate, indicating glycoproteins.

The molecular weight of these proteins was determined by SDS electrophoresis (Weber and Osborn, 1969) after solubilization according to Kiehn and Holland (1968) or Weber and Osborn (1969). Figure 6 is a graph of relative mobility vs. molecular weight of the standards. The position of the membrane proteins are designated by arrows. The molecular weights of the proteins range between 50,000-70,000 with the proteins of MP being a little larger than those of HR.

Figure 7 shows a comparison of MP and HR surface membrane proteins after electrophoresis in an acid buffer and solubilization in PAU. Not only does the number of bands increase (from 2-3 to approx. 20) but also the number of bands showing no similarity between the two. The HR membrane has a larger amount of lower molecular weight proteins and smaller amount of higher molecular weight proteins than does MP. This is based on the correlation between distance of migration and size of proteins.

One other interesting aspect to this is presented in Figure 8. When the PAU solubilized membranes are dialyzed back into a urea-SDS system and run on gels in a basic buffer, in this case according to Clarke (1964), the number of bands

Figure 6. Relative mobility of membrane proteins and standards with SDS acrylamide electrophoresis.

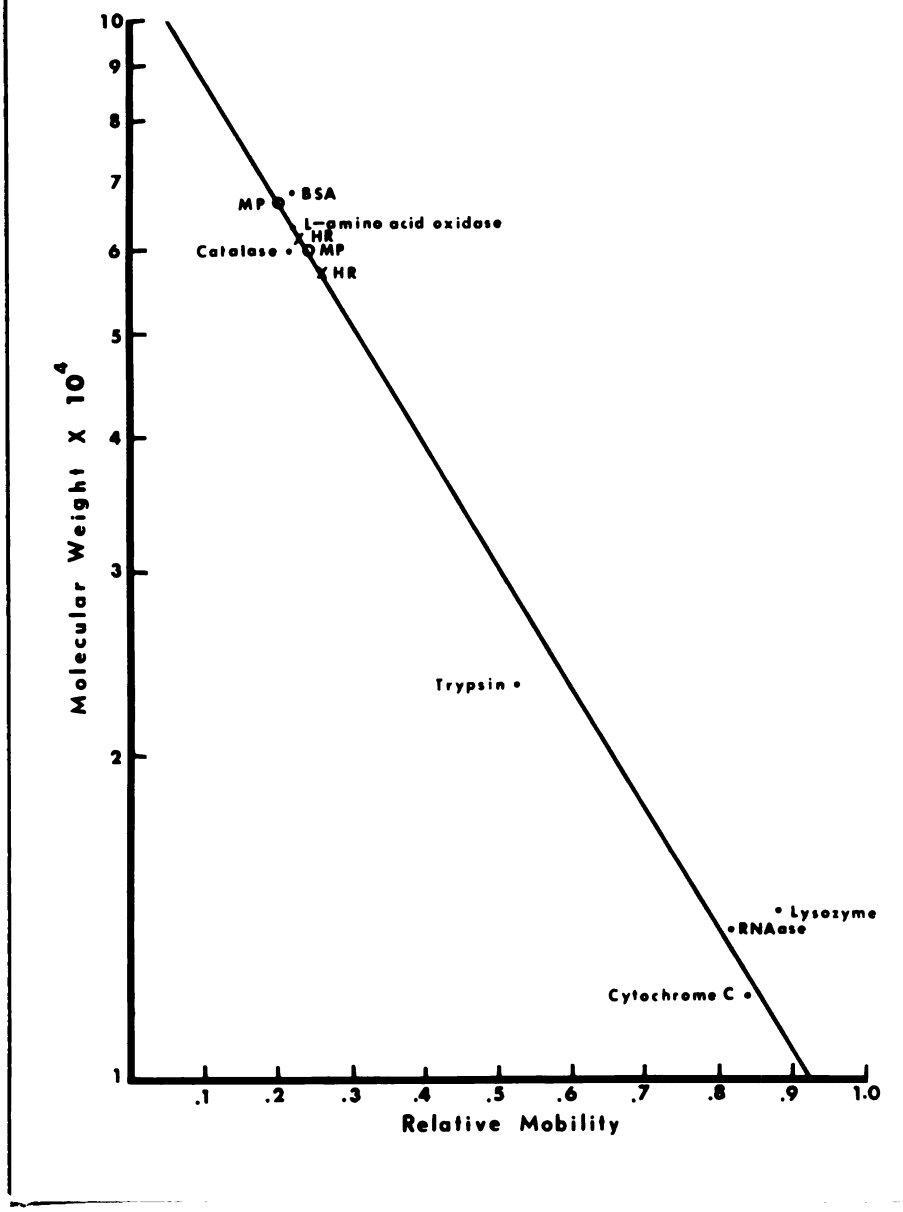
Molecular Weight X 10^4
10
9
8
7
6
5
4
3
2
1
BSA
MP
L-amino acid oxidase
HR
Catalase
MP
HR
Trypsin
Lysozyme
RNAase
Cytochrome C
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Relative Mobility

Figure 6

Figure 7. Electrophoretic patterns of HR and MP surface membranes following solubilization in PAU and electrophoresis in acid gels. The stain is Coomassie blue.

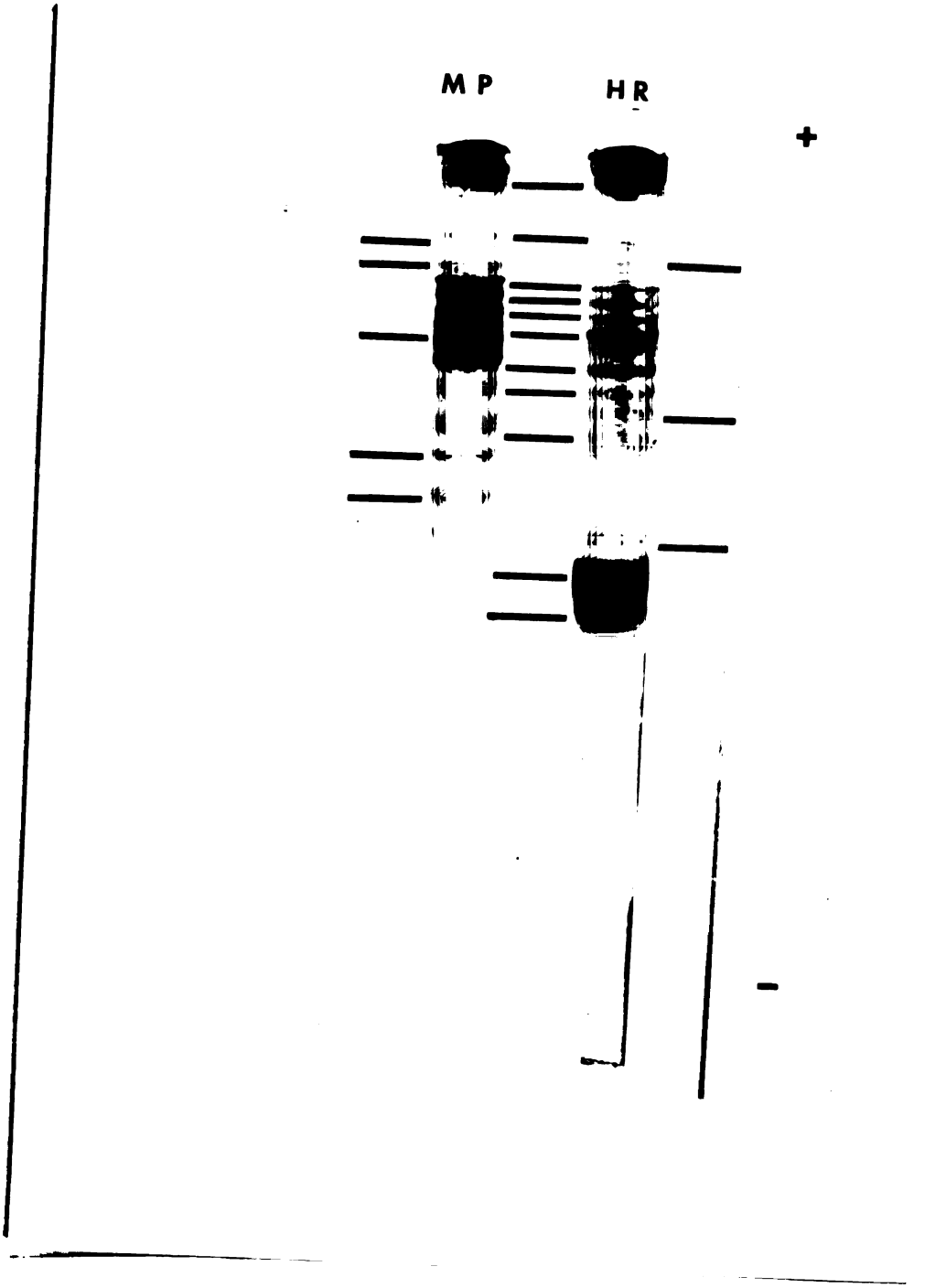

Figure 7

Figure 8. Electrophoretic patterns of HR and MP surface membranes of 5% acrylamide following dialysis from an acid solvent into a SDS-urea system. The stain is naphthol blue black.

Figure 8

is again two. The reverse of this is also true i.e. if this SDS-urea solution is then dialyzed back into PAU and run on the acetic acid gels, the number of bands increases and the similarity decreases.

Glycosyltransferase Activity

The distribution of the glucosyltransferase activity is seen in Table 7. The data is an average of two experiments. The surface membrane of HR exhibits radioactivity on the paper where ceramide-^{14}C-glucose runs (Basu et al., 1968). The microsome fraction of HR and the Golgi fraction of MP also display this activity; however, the surface membranes of MP do not appear to have any activity. The other areas of radioactivity found on the paper can be explained by the lipid nature of the substrate and the type of experiment. Any activity at the origin is due to contamination picked up from one of the non-lipid layers when transferring to the paper. Activity at the top is due to the hydrolysis or breakdown of the UDP-C^{14}-glucose into free C^{14}-glucose. Both of these factors are indicated by the data from the two controls in which neither membrane nor lipid acceptor were added. In comparison, it was also found the radioactive spots correspond to those found with 7 day embryonic chick brain which were shown by Basu et al. (1968) to be the products of glucosyltransferase activity.

Table 7. The distribution of radioactivity (glucosyltransferase activity) in various membrane fractions of HR and MP. Values are expressed as dpm/μg protein.

Fraction	Relative position on chromatogram		
	Origin (dpm)	Middle (dpm)	Top (dpm)
HR surface membrane	69	42	63
HR Golgi	242	-	-
HR Microsome	163	36	-
MP surface membrane	276	-	40
MP Golgi	105	60	54
MP Microsome	200	-	26
No proteins	196	-	-
No proteins or acceptor	54	-	153

Galactosyltransferase activity was present in both surface membranes (HR and MP) but the specific activity in MP was twice that found in HR (Table 8). All the data (average of two experiments) are expressed as dpm/μg membrane protein added and the values corrected for both enzymatic and hydrolytic breakdown. Paper chromatography of the reaction products revealed two spots--one corresponding to galactose and the other corresponding to N-acetyllactosamine. The donor, UDP-^{14}C-galactose, does not appear to be passing through the column since the amount of radioactivity put on is at least 10X that which is coming through (Table 8)--the unused is bound to the column.

Isolation and Characterization of Two Lipids from the MP Surface Membrane

After solubilization of the membranes, it was observed that the MP solution was cloudy. Spinning at 10,000g caused the insolubilized membrane to settle to the bottom but the supernatant still remained cloudy. After centrifugation at 200,000g for 30 min. this material floated to the top of the tube where it was collected for analysis. None of this substance was observed in the HR membrane preparations.

Figure 9 is a representation of this material after chromatography on silica gel coated-thin layer plates with three different solvent systems. Figure 9A reveals one spot at the origin with the solvent petroleum ether/ethyl ether/

Table 8. Galactosyltransferase activity of the surface membranes of HR and MP expressed in dpm/μg protein passing through an ion exchange column.

Fraction	10 min. counts	Specific activity dpm/μg protein
HR surface membrane (1X conc.)	20,892	31
HR surface membrane (2X conc.)	48,508	37
HR surface membrane No acetylglucosamine (2X conc.)	9,917	7
MP surface membrane (1X conc.)	16,844	36
MP surface membrane (2X conc.)	69,814	77
MP surface membrane No acetylglucosimine	13,196	14
No protein	558	
No protein or acceptor	608	

Radioactivity of 5 μl of UDP-^{14}C-galactose--332,198 cpm

Figure 9. Thin layer (silica gel) chromatograms of the insoluble lipids of MP surface membrane. All spots were visualized with iodine except C where concentrated sulfuric acid was used. The solvents were:

A chloroform/methanol/acetone--70:30:1

B and C chloroform/methanol/water--100:42:6

D chloroform/methanol--8:1

acetone (70:30:1) but with chloroform/methanol/water (100:42:6) there are two spots, one of which travels with the solvent front (9B). Both spots also stain with concentrated sulfuric acid but only the top one with anthrone (9C). Neither stain for sialic acid. Chloroform/methanol (8:1) retards both spots on the plate so that now the top spot travels approximately 2/3 the distance of the solvent front (9D).

The spots were then scraped off and eluted with chloroform-methanol and dried under nitrogen. Following methanolic hydrolysis, the materials were run on a mass spectrometer and gas chromatography. The lower spot appears to be a triglyceride. The upper spot is still under investigation.

DISCUSSION

Microelectrophoresis

The main objective of this work was to investigate the two amoebae surfaces. One method used extensively, particularly on red blood cells, has been microelectrophoresis or whole cell electrophoresis. In this procedure, the cells are suspended in a medium inside a closed system across which a current is applied. Based on the conductivity, the viscosity and pH of the solution, the charge on the surface can be calculated. There are four assumptions that must be taken into account when determining these measurements. (1) The usual hydrodynamical equation for the motion of a viscous fluid may be assumed to hold both in the bulk of the liquid and within the electrical double layer (layer of molecules surrounding the charged sphere that represents ions attracted to the surface because of the charges). (2) The presence of the charged sphere produces a distortion of the electrical field in such a way that the electrical current passes tangentially along the surface of the particle. (3) The electrical double layer is so thin that the electrical field can be considered parallel to the double layer at all points. (4) The electrical field does not deform the

double layer (Bull, 1943). According to Henry's law if the particle is small enough and the thickness of the double layer is large enough, the particle approaches the condition of an isolated charged sphere; therefore,

$$QE = 6\pi rnu$$

where Q = charge on particle; E = potential gradient; r = radius of sphere; n = viscosity of solution; and u = velocity of sphere. The right side of the equation represents resistance based on Stoke's law. If the size of the non-conducting particle is large in comparison to the thickness of the double layer, electrophoretic velocity is independent of size and shape of the particle; however, if the thickness of the double layer is comparable to the radius of the particle, the electrophoretic velocity becomes a function of both the size and shape of the particle. In these measurements the conditions were met for the former situation; therefore the amoebae act as charged spheres.

Furthermore, measurements must be done at the two stationary levels within the cataphoresis cell, calculated to be 0.211 and 0.789 of the total thickness of the glass cell, because of electrosmosis. In a closed system like this, there are four forces that may exist and cause a particle to move: (1) electrosmosis or the movement of a liquid relative to a solid under the influence of an external field applied tangentially to the interface; (2) streaming potential or

the potential resulting from the movement of a liquid relative to a solid in response to a mechanical force applied tangentially to the interface; (3) the Dorn effect or movement of a solid phase in respect to a liquid under the influence of a mechanical force e.g. gravity and the sedimentation potential; and (4) electrophoresis or the movement of a solid phase in respect to a liquid under the influence of an external electrical field. Thus in this system and under the conditions of these experiments, two of these forces were applicable--electrophoresis and electrosmosis. It is necessary, then, to recognize both and to separate them. The easiest means of doing this is to take measurements at those levels in the cell where electrophoresis is the only force and in this case at those stationary levels mentioned above.

The data from these experiments indicated quite clearly that net surface charge does not play a direct role in the clumping of the amoebae. At pH 7.2 where MP clumps and HR does not, the electrophoretic mobilities were essentially the same. Therefore, net surface charges must be the same. If one were to accept the theory that electrostatic forces keep the cells separated, then it would seem probable that either MP should not clump or HR should since they have the same charge at this pH. Considering the cells as charged spheres, Weiss (1964) also points out that the strength of the electrostatis forces of repulsion may not be relevant in

acting as an inhibitor to adhesion. The cells may be able to break through this barrier e.g. by pseudopodia formation, but once through they may not be able to remain adherent; therefore, they will not clump.

From these experiments it was also possible to determine the isolectric point of the cell or that pH at which the number of positive charges equals the number of negative charges, thus a net charge of zero. One can then assume something about the nature of the chemical moieties on the surfaces. It was observed that the pI of HR (2.9) was lower than that of MP (3.4) which means there are different types of groups exposed on the surface. But, when considering this, it is necessary to remember that the cells do not have homogenous surfaces i.e. there are many different groups which contribute to the surface charge. The net charge at any pH will depend on the pH at which these different chemicals dissociate. Therefore, from what is known of the dissociation constants for various chemical moieties, it is possible to determine what groups are on the surface. As can be seen on the graphs (Figure 2) MP has at least two regions of dissociation, one at approximately pH 4.0 and the other near pH 7.0. On the other hand, HR exhibits only one, at pH 4.5. This probably means that HR and MP share many of the same chemical groups on their surfaces. The data from the pI's indicated that carboxyl groups (COO^-) are probably the major contributor to the charge on both cells; however, it

must be considered that there are several different molecules that have this group attached e.g. sialic acid, proteins and lipids. The curves for pH vs. mobility of the cells will be influenced by which of these is present. It also must not be overlooked that the dissociation of sialic acid could be influenced by proteins and lipids because it has been found that sialic acid is the terminal group of many glycoproteins and glycolipids. In this case the lipid and the protein parts of the molecule would have a definite influence on dissociation properties. Since the carboxyl of sialic acid has the lower pKa, it is probably the primary contributor to the negativity of the surface. Carboxyl groups of proteins however cannot be ignored completely because they have a pKa ranging from pH 3.0-4.5. One must be careful in just looking at the pI's of the cell for even though HR has the lower pI, MP has more sialic acid (Table 6). The apparent contradiction between the two pieces of data can be explained by considering to what the sialic acid is bound. Also, it appears that MP has at least one other large group of molecules on its surface which influence more directly the net surface charge. Most likely, this is the NH_2 groups either contributed by proteins or amino groups of carbohydrates such as glucosamine, galactosamine or their derivatives. This is not to say that HR does not have any of these groups but rather that the concentrations on the MP surface are higher, possibly due to the presence of more complex carbohydrates.

Alteration of the Surfaces and the Effect on Adhesion

According to Band and Mohrlok (1969), glutaraldehyde fixed cells maintain adhesive specificity so it was of interest to see how fixation effected the surface properties. Since only the adhesive properties of HR were altered by the various chemicals, it was the choice to be tested by microelectrophoresis. As was pointed out, the pI of the fixed cells was the same as that of the live cells; therefore, one would expect no difference in adhesive properties if ionic interactions are involved in adhesion and indeed there were none. This data also justified the use of the fixed cells as models for adhesion studies. This specificity remained even if the cells were fixed in glutaraldehyde and osmium tetroxide and lipid extracted with a series of alcohols up through propylene oxide. The chemical moieties that are uneffected, then, may be those that contribute to the adhesive properties. Glutaraldehyde is thought to cross-link the proteins and osmium tetroxide the lipids. Moreover, any of the lipids not bound up would be extracted with the alcohols and propylene oxide. This leaves only carbohydrate moieties such as sialic acid, glucosamine, etc. free on the surface to contribute to adhesion. One must note, though, that the carbohydrates are most likely a part of a larger lipid or protein molecule and not a separate entity.

It was also found that neither DNAase, RNAase nor both combined had any effect on the adhesive properties of the two cells. This indicates that nucleic acids do not have a role in the physical clumping of the cells and that they were not leeched out onto the surface during fixation. As with most other cells, nucleic acids were not detected in the membrane preparations so it was not too surprising to find no effect. However, they could be present in such low quantities that they were not detectable by my methods; but if this were so, the concentrations probably would not be sufficient to influence adhesion.

After treatment of the cells with a variety of chemicals such as dimethylformamide (organic solvent) and a mixture of 8M urea, SDS and mercaptoethanol, there was a noted decrease in the electrophoretic mobility concomitant with a lose of adhesive properties. This was not too surprising since these solvents were also used to solubilize the isolated membranes. Therefore, any groups connected to the lipids or the proteins will be dissociated or destroyed. Also, the snake venoms which contain two or more different enzymes gave similar results indicating this same type reaction. The data thus far indicates that there are certain substances on MP which somehow make the surface more conducive to adhesion, while HR either does not have these substances or does not have them exposed, naturally. This hypothesis is further substantiated by the data from the

enzyme analysis done on the fixed models. Essentially there was only one group of enzymes that effect adhesion. These were the ones that destroy or remove carbohydrate moieties. One of the proteases, subtilisin, also effected the specificity but this could be explained by the fact that it was not a pure fraction of the enzyme. This also may account for the effects from the many different carbohydrate enzymes. On the one hand, it would be expected that the enzymes would be quite specific, yet on the other hand, it is hard to believe that all the substances attacked by these enzymes are on the surface. It should also be particularly noted that neuraminidase which removes sialic acid effected the adhesion. This correlated well with the electrophoretic data. Two other solutions, 2M NaCl and 1M NaCl, caused HR to adhere non-specifically. This is due to a reduction in the charge on the surface. As Na^+ ions are added, there would be a binding or a neutralizing of negative sites and subsequently a reduction in electrostatic repulsion, since it must be assumed nothing is being removed from the surface. The possibility that nucleic acids are being extracted with the high salt solutions cannot be overlooked. However, this should present no problem since it was shown that neither DNA or RNA have an effect on adhesion. All of this fits quite well into Curtis' lyophobic colloid model (Curtis, 1967) that any material added that can reduce the charge on the surface (e.g. Ca^{++} and Na^+) would also allow the cells to come in closer contact since the electrostatic

forces of repulsion were reduced. By being able to get closer, the cells may generate other forces which will induce clumping.

Effect of Phytohemagglutinins on the Cells

One way to get at the question of carbohydrates and their role in adhesion was through the use of three phytohemagglutinins. They were: Concanavalin A (Con A), an extract of Jack bean that binds to α-D-glucopyranoside-like sites (Inbar and Sachs, 1969); wheat germ agglutinin (WGA), an extract of wheat lipase that binds specifically to N-acetyl-D-glucosamine (Burger and Goldberg, 1967); and soybean agglutinin which binds to N-acetyl-galactosamine or to dissacharides to which this sugar occupies a terminal position (Lis et al., 1970). As shown in the results, MP was the least effected, only SBA caused a slight agglutination while HR was agglutinated by all three. This means that the surface of HR is covered by carbohydrates composed of α-D-glucopyranosides, N-acetyl-glucosamine and N-acetyl-galactosamine. The MP surface apparently has only the galactosamine-like structure exposed. It has been considered that if glucose is present in glycolipids or glycoproteins, it will occupy the first position after the lipid or protein and almost never found as the terminal group of these type of macromolecules (Ginsburg and Kobata, 1971). With respect to the HR surface, there is the possibility that glucose is the terminal group since the cells agglutinated

very well in the presence of Con A which binds to derivatives of glucose. In conjunction with this are the observations that HR also clumped relatively well in WGA which binds to another glucose derived molecule and very poorly in SDA which binds to galactose derivatives. This could mean that glucose is the last sugar for many of the macromolecules which in turn would imply that the glycoproteins and glycolipids on HR are smaller as seen in the gel electrophoresis patterns (Figures 4, 5 and 7). The clumper, MP, on the other hand, clumps only slightly in the presence of SBA indicating that there are many other types of terminal groups and therefore more complex and larger macromolecules.

Isolation and Composition of Surface Membranes

The isolation of these membranes was carried out by the standard methods of differential and sucrose gradient centrifugation. Gradients were used to separate the nuclei from the surface membranes if need be; however, the nuclei could be eliminated early in the isolation procedure, thus doing away with the need for gradients. The exclusion of Mg^{++} from the isolation solution caused the nuclear membrane to disrupt after which the contents of the nuclei could be washed away. This was evidenced by the fact that no detectable nucleic acids were found in the isolated membranes. The Mg^{++} free solutions also made it easier to solubilize the membranes.

As mentioned earlier, there were predominantly negative charges on the surface of the cell and in the presence of Mg^{++} the different molecules were cross-linked, in this way stabilizing the membrane. Its importance in this role can be realized by considering what happened to the nuclear membrane in the absence of Mg^{++}. This has also been proposed by Curtis (1967) and Weiss (1965) for divalent molecules and their role in cell adhesion. It is not that they bind one surface to another but rather stabilize the membranes in such a way that makes them more suitable for adhesion.

One interesting point concerning the composition of the membranes is the amount of sialic acid present. The membranes of MP according to two different analyses, have approximately twice the amount as HR. Relative to what has been mentioned before about surface charge and complexity of carbohydrate chains, this seems quite probable. HR may have many carboxyl groups on its surface, but they do not belong to the sialic acids. It also should be pointed out that many of the glycolipids and glycoproteins have sialic acid as terminal groups; thus, HR may have incomplete carbohydrate chains on the surface.

Gel Electrophoresis of Membrane Proteins and the Implications on Adhesion

The theory that there may be differences in the amount of carbohydrate bound to the proteins in the membranes is supported by the data from the electrophoretic patterns of

PAU solubilized surface membranes (Figure 7). Although there were many similar bands, the differences cannot be overlooked. Most striking was the large band in HR near the middle of the gel. There was a similar band in MP however, its quantity was obviously much less. This area of the gel corresponds to smaller molecular weight proteins. Near the top, in contrast, the gels of MP stained darker than those of HR and there was an increase in the number of bands. Therefore, it seems that MP has a greater amount of larger molecular weight proteins than does HR and conversely, HR has smaller molecular weight molecules than MP. Since on the SDS gels the protein bands stained with PAS, it was assumed most of the proteins were actually glycoproteins. This difference in size of molecules could indicate a lack of carbohydrate moieties, smaller peptides or both in the HR plasma membrane. Moreover, electrophoretic molecular weight determinations in SDS indicated the proteins of MP were slightly larger. However, carbohydrates on the protein can alter the migration in the gel; therefore, the molecular weight may not be extremely accurate and the observed differences may even be attributable to the presence of different carbohydrates. Yet, the gels do indicate differences which is the most significant point.

The discrepancy in the number of bands between the two methods of electrophoresis can be accounted for either by the similarity in molecular weights of the different proteins

or by the presence of carbohydrates. In the SDS system, separation is determined by molecular weight solely, while in the PAU methods, separation depends on the charge, size and shape of the molecule. Yet, one reason to suppose that the two or three bands in the SDS were the same as the 15-20 bands in the PAU was the observation that when PAU solubilized membranes were dialyzed into a SDS-urea system and electrophoresis was carried out, 2-3 bands resulted. The reverse of this was also true i.e. when the SDS-urea membranes were dialyzed into a PAU system, there were numerous bands after electrophoresis. It can be concluded that there are many different proteins in the surface as might be expected but that they based on molecular weight are probably grouped into 2-3 general classes.

The membranes of these amoebae differ from other eucaryotic cells in that they do not exhibit a large variety of enzymatic activities. Prior to the experiments for various glycosyltransferases, I had been able to find only two enzymes--5'-nucleotidase, a marker for surface membranes of Acanthamoeba (Schultz and Thompson, 1969) and other cells and Ca^{++}-Mg^{++} dependent ATPase. There did not appear to be any Na^{+}-K^{+} dependent ATPase as found in many other cells, even though two different analyses were used. Possibly it was present in such low concentrations that it was not detectable by these assay methods.

Membrane Glycosyltransferase Activity and Its Role in Adhesion

Because of the universal interest in glycolipids and glycoproteins and the role of carbohydrates in adhesion, it was of interest to look at the presence of the glycosyltransferase activity in these membranes. It is thought that most of the carbohydrates are added to the surface membrane at the level of the Golgi before insertion into the plasma membrane (Whaley et al., 1972; Wise and Flickinger, 1970). Recent work (Roth et al., 1971) has shown that there are glycosyltransferases on the surface of chick embryo neural retina cells. As seen from my results, this was also true for the amoebae, although the activity here was not as high as in the embryonic cells. It was also interesting to note the differential disposition of the enzymes. The clumper, MP, had no glucosyltransferase activity on the surface but did in the Golgi, while the non-clumper, HR, had it on the surface and not in the Golgi. Both membranes contain galactosyltransferase activity but that of MP was 2X that of HR. This fits in quite well with the results from the agglutinins and supports the hypothesis of incomplete synthesis of glycoproteins and glycolipids in nonadhesive cells. The fact that Con A bound quite readily to HR implies that a glucose residue is the terminal sugar; therefore, an enzyme would have to be present which attaches the glucose to the existing molecule--glucosyltransferase. As for MP either of two

hypotheses could explain its absence. (1) As in embryonic chick brain, the glucosyltransferase could be degraded upon the addition of glucose and the next carbohydrate to the macromolecule (Basu et al., 1968). Or (2) the glucose could be added elsewhere in the cell. Since glucosyltransferase activity was found in the Golgi of MP and not HR, the data supports the latter hypothesis but does not necessarily rule out the other. The presence of galactosyltransferase activity in both membranes indicates longer chain carbohydrates attached to the protein and lipid bases. Since there is more (2X) activity in MP than HR, it can therefore be assumed that there are more large macromolecules in MP than HR which is in complete agreement with the gel electrophoresis data, indicating larger proteins in MP membranes. Prior evidence has shown that transformed cells which are less adhesive than normal cells (Edwards et al., 1971) contain smaller glycolipids and glycoproteins than normal cells (Hakomori et al., 1968 and Chiarugi and Urbano, 1972). Also, as mentioned before, there is very little glucose present in macromolecules and its position is usually next to the lipid or the protein. This then would imply that most of the HR glycomolecules consist of one or at least very few different sugars. Yet, there is also the possibility that there are other transferases present such as sialyltransferase but these have not been assayed here.

CONCLUSIONS

From all this, what can be said about adhesion and the role of surface membranes? Which hypothesis is correct? Adhesion probably occurs as a product of all the above mentioned theories but one factor must be kept in mind. There is the universal requirement for a living component to be present in order for adhesion to occur, e.g. the fixed cells (HR and MP) need a living cell in order to clump. Even the macromolecular or transferase theories are applicable only to living cells and when considering the rest of the discussion, this should be kept in mind.

Roseman (1970) postulated that transferases produce adhesion by interlocking the substrate and enzyme, but as seen here, both cells have transferases but both do not clump. What is probably more relevant in this respect is what transferases are present. It could be that the glucose which apparently is prevalent in HR may not have the ability to adhere as does other carbohydrate moieties. This also related to the size differences in the proteins found in the two surfaces. Proposals have also been made which have sialic acid acting as a mediator between Ca^{++} and the cell membranes; however, this is nullified here because the

addition of Ca^{++} had no effect on clumping of HR or the removal had no effect on breaking up clumps of MP. The observation that the cells do not come close enough together for Ca^{++} bridging (Curtis, 1967) also rules out adhesive role for sialic acid and Ca^{++}. As mentioned earlier, though, Ca^{++} may act to stabilize the membranes or it may depress electrostatic charges between cells, allowing them to get closer and adhere. It has been suggested by Weiss (1965) that Ca^{++} functions in maintaining clumps once they have been formed, i.e. Ca^{++} prevents separation but does not influence adhesion. I did not study this aspect but Band and Mohrlok (1969) did using this same system and their data would support Weiss' hypothesis.

The hypothesis that macromolecules enhance or initiate adhesion is a very popular one (Moscona, 1965; Margoliash et al., 1965; Humphreys, 1963; and Oppenheimer and Humphreys, 1971). Most of these substances are thought to be glycoproteins. When cells are dissociated by a variety of means, certain chemicals are released which ultimately influence adhesion. For instance, if the cells after dissociation are put into the cold, inhibiting protein synthesis and movement, nothing occurs, but with the addition of the extracted substance aggregates will form (Humphreys, 1963). It was assumed this was a surface phenomenon because nothing else occurred. It is interesting to note that besides adding the macromolecules, it was also necessary to add calcium before the reaction

would be completed. In my own observations, I found that cold cells and fixed cells did not clump and that there had to be a living component present to initiate adhesion. Under the above conditions the cells are spherical and rigid, not able to put out pseudopodia or move over other cells. Most likely there are molecules or groups of molecules on the surface which somehow effect the adhesion, whether it be by electrostatic charge reduction, by macromolecular contacts or by Ca^{++} bridging. But because of the rigidness of the cell, only small areas of contact can occur between the cells--not enough for a stable adhesion. In the case of the fixed cells, the addition of the live clumper initiated aggregate formation because the areas of contact were increased and because the clumper has something different on its surface that makes it more conducive to adhesion. Yet, two other factors are very important--the ability to form pseudopodia and the ability to move. Jones (1965) has also shown that hardening the cell membrane with benzoquinone decreased adhesion as a result of inhibiting ATPase activity and contractility. Likewise, it seems that a living, viable factor must be necessary here in order for adhesion to occur.

As mentioned in the introduction the implications of this research with regard to medical research are great. Cancer cells are known to be less adhesive (Edwards et al., 1971) and no longer contact inhibited, thereby they are able to spread easily throughout their host. Some workers have

found that transformed cells have smaller glycoproteins and glycolipids (Hakomori et al., 1968; Meezan et al., 1969; and Chiarugi and Urbano, 1972) as I have found here with the non-clumper. A differential activity of transferases has also been found in the cell of normal and malignant cells (Kijimoto and Hakomori, 1971). Others have found that transformed cells in the presence of Con A, SBA and WGA agglutinate but normal cells do not (Sheppard et al., 1971; Kapeller and Doljanski, 1972; and Sela et al., 1970). This is quite similar to what I found with HR and MP. The electrophoretic mobilities (i.e. pI's) of cancerous cells have also been reported different from normal cells (Ambrose et al., 1956), again as I found with HR and MP. The results found here, then, could be directly applicable to cancer research and the problems of control and prevention.

Also as mentioned in the introduction, the penetration of parasitic organisms not only depends on the nature of the host as a substrate but also the adhesiveness of the cell. Therefore, any conclusions that can be drawn about the causes of cell adhesion can also be used in the study of this host-parasite relationship.

SUMMARY

The major results of these experiments are seen in Table 9 where a comparison between the HR and MP cells is made.

In conclusion, I would agree with Curtis' hypothesis of adhesion and the lyophobic colloid model. All cells have a net negative charge on them at the physiological pH's but because of the physical or biochemical factors, the charges can be altered. The addition of ions, the complete synthesis of a molecule or even the ability of the cells to form pseudopodia decreases the negativity and likewise the electrostatic charges. This allows the cells to come closer and depending on the degree of charge reduction and the closeness of the surfaces, other factors can become involved such as Ca^{++} bridging or even covalent binding of macromolecules. Of course the nature of the groups on the surface will influence the charge that is being produced and determine how easily the surface can be altered. In this respect, it is possible to combine the macromolecular and lyophobic colloid theories since the effect of the macromolecules may be outlined by the lyophobic colloid model.

Table 9. A comparison of HR and MP surfaces and cells.

Experiments	HR	MP
Electrophoretic mobility at physiological pH, 7.2	-.8059	-.8086
pI	2.9	3.4
Size of proteins	50-80,000 Those of HR smaller than those of MP	50-80,000
Carbohydrate composition	10%	10%
Sialic acid composition	3.5%	6.8%
Binding of phytohemagglutinin		
Con A	+	-
WGA	+	-
SBA	+	+
Glucosyltransferase activity		
Surface membrane	42dpm/μg	-
Golgi	-	60dpm/μg
Microsomes	36dpm/μg	-
Galactosyltransferase activity	37dpm/μg	77dpm/μg

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