A Biochemical Study of the Surface of an Adhesive Variant of BHK 21 Cells

By Jean McK. Dysart B.Sc.

A Thesis Submitted to the University of Glasgow for the Degree of Doctor of Philosophy

December 1975

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789 East Eisenhower Parkway
P.O. Box 1346
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SUMMARY

A variant of BHK 21/C13 cells, which arose spontaneously in culture, was isolated and cloned. The aggregative behaviour of the variant was studied by means of a reciprocating shaker/electronic particle counting technique and found to differ from that of the parent line. Freshly trypsinized C13 cells aggregate rapidly at 37°C while the variant and a polyoma virus transformed derivative show little aggregation after forty minutes. Neither neuraminidase nor dibutyryl cyclic AMP increased the aggregation of the variant to the level attained by C13 cells.

When the variant cells were removed from the culture flasks by ethylene-diamine-tetra-acetic acid (EDTA), rather than trypsin, they aggregated in a manner similar to C13 cells while the transformed derivative still showed little aggregation.

The variant had a lower sialyl transferase activity towards endogenous acceptors but the same activity towards exogenous acceptors as C13 cells. The cells were also assayed for galactosyl transferase activity and no differences were found between the variant and the parent line. Isolated plasma membranes were also assayed and again no differences were found.

The proteins of the isolated plasma membranes were analysed by polyacrylamide gel electrophoresis. Two proteins of estimated molecular weights 182,000 and 47,500 were absent from the membranes of polyoma virus transformed cells. These same two proteins were released from C13 and the variant cell membranes by mild trypsinization. The possible role of these proteins in cell adhesion is discussed.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Professor A.S.G.Curtis and Dr.G.R.Moores for all their help and advice. I am also indebted to Drs. A.J.Lawrence and J.G.Edwards for many stimulating discussions and to the Faculty of Science of the University of Glasgow for financial support during the course of this work.

INTRODUCTION

The surfaces of eucaryotic cells are thought to play a dominant role in various complex physiological phenomena including cell recognition (Roth et al, 1971), contact inhibition of motility (Abercrombie and Heaysman, 1954) and growth (Dulbecco, 1970), morphogenesis (Curtis, 1967) and metastasis (Abercombie and Ambrose, 1962). Intercellular adhesion, or lack of adhesion, is probably a component in many of these phenomena.

The early embryogenesis of vertebrates proceeds through a series of complex morphogenetic movements which eventually lead to the formation of the three primary germ layers. A variety of embryonic tissues can be dissociated to give suspensions of single cells. If these cells are allowed to reaggregate they will often organise themselves so that the cells from different organs occupy different positions within the aggregate (Townes and Holtfreter, 1955; Moscona and Moscona, 1952). This sorting out of cells in mixed aggregates has been used as a model of morphogenetic movement and it has been proposed that differential cell adhesion could govern the final positions adopted by the various cell types (Steinberg, 1958, 1964, 1970).

The motility of cells must also be a factor in deciding their final positions in both embryos and in aggregates. Differences in motility may be a reflection of differences in adhesiveness. Increased adhesion of cells to a substrate has been shown to be associated with decreased motility (Carter, 1965; Gail and Boone, 1972).

The passive dissemination of cancer cells and the subsequent establishment of secondary tumours probably involves changes in both

the adhesiveness and the motility of the cancer cells. In order to form successful secondary tumours cells must first detach from the primary tumour. A decreased mutual adhesiveness would obviously facilitate this process and Coman (1961) has shown that mutual adhesion is much lower between carcinoma cells than between normal epithelial cells. A detached cell must then settle down in a place favourable for growth. Where it settles may depend upon a preferential adhesion for that site. Nicolson and Winkelhake (1975) isolated variants of a melanoma cell line with increasing ability to form lung metastases and found a preferential adhesion to lung cells by those variants which showed high lung metastases. These results imply that cell recognition and adhesion are important in determining the organ specificity of metastatic tumour spread. Malignant cells have the ability to infiltrate normal tissues which indicates that they are less contact inhibited than normal cells. This has been demonstrated in the case of sarcoma cells by Abercrombie and Heaysman (1954). Contact inhibition of movement appears to be an adhesion mediated phenomenon. Trinkaus et al (1971) using time lapse cinematography observed that inhibition of ruffling on cell contact was completely local and proposed that the inhibition was due to the adhesion of a lamellipodium to the surface of the contacted cell.

Thus an understanding of intercellular adhesion in molecular terms could shed light on a number of interesting biological problems. Since adhesion is primarily a surface phenomenon, a knowledge of the precise chemical composition and the structural nature of the cell surface should enable us to determine the types of adhesions that could form.

(1) Membrane Composition and Structure

Early models of the plasma membrane were based on the theoretical requirements of a permeability barrier controlling the exchange of water and solutes between the cell and its environment. The permeation of many solutes was shown to be related to their lipid solubility and so the idea of the lipid plasma membrane was established. An early chemical fractionation of the membrane from red blood cells was carried out by Gorter and Grendel (1925). They found that the area occupied by the extracted lipids when spread as a monomolecular film on water was twice the surface area of the total number of red cells used for the extraction. They concluded that the lipids were arranged as a bilayer with the polar ends of the molecules oriented towards the aqueous environment and the apolar ends towards each other.

Surface tension studies showed that the values obtained at cell surfaces were considerably lower than was thought possible for a lipid surface and Danielli and Davson (1935) proposed a model in which the lipid core was sandwiched between layers of protein.

It has since been shown that the results of Gorter and Grendel were purely fortuitous (Bar et al, 1965; Westerman et al, 1962) and that phospholipids alone can produce low surface tension values (Haydon and Taylor, 1963) but the concept of the bimolecular lipid leaflet has received support from other sources. Electron micrographs of the myelin sheath of Schwann cells (Robertson, 1957) showed a trilaminar structure which was interpreted as a bimolecular lipid leaflet with protein in the β-pleated sheet form on either side and some x-ray diffraction data from dispersions of isolated membranes (Engelman, 1970) and calculations of the molecular dimensions of

phospholipids, cholesterol and extended protein chains (Finean, 1962) were compatible with a bimolecular lipid leaflet coated with protein.

Most of the early work was done with membranes which are probably atypical and it is only recently that membranes from cells other than red blood cells or Schwann cells have been isolated and chemically analysed. Recent reviews on the techniques for isolating plasma membranes include those by Warren and Glick (1971), Neville and Kahn (1974) and Wallach and Lin (1973).

Analyses of membranes from many sources have confirmed that the major components are lipid and protein but the lipid protein ratio varies from cell type to cell type. Three classes of lipid are almost always present: neutral lipids, phospholipids and glycolipids. Cholesterol and triglycerides are the main neutral lipids (Fleischer and Rouser, 1965; Ashworth and Green, 1966; Weinstein et al, 1969) while of the phospholipids phosphatidylcholine is predominant (Klenk and Choppin, 1969; Weinstein et al, 1969) with sphingomyelin, phosphatidyl-ethanolamine, -inositol and -serine making up the bulk of the remainder. Glycolipids mainly occur as glycosylceramides and gangliosides. Interest in glycolipids has intensified in recent years since Hakamori and Murakami (1968) showed differences in the glycolipids of normal and transformed cells. They found that the level of haematoside was decreased in a line of BHK cells transformed with polyoma virus and this was accompanied by an increase in lactosylceramide. This has since been confirmed for a number of cell types transformed with different viruses (Cumar et al, 1970; Hakamori et al, 1971; Robbins and MacPherson, 1971; Steiner and Melnick, 1974) and Robbins and MacPherson also found that the glycolipid pattern of growing normal cells was similar to that of transformed cells. This has led to

speculation on the role of glycolipids in contact inhibition of growth.

The role of proteins in membranes has recently been the topic of considerable discussion and the subject has been reviewed by Guidotti (1972). Controversy has centred round the possible presence of structural proteins in plasma and other cell membranes. The concept of structural protein was first suggested by Green et al (1961) who isolated a seemingly enzymatically inactive homogeneous protein of 20,000-30,000 molecular weight from beef heart mitochondria. However, as Winzler (1970) points out, whenever careful studies of solubilized membrane proteins have been made an exceedingly heterogeneous group of some twenty different proteins is revealed. This example illustrates the difficulties inherent in the elucidation of the nature of membrane proteins. A major difficulty has been the insolubility of most membrane proteins in aqueous solutions. Current techniques for solubilization include the use of detergents such as sodium dodecyl sulphate (SDS), organic solvents such as n-butanol and organic acids (Kaplan and Criddle, 1971). Using these techniques in conjunction with polyacrylamide disc gel electrophoresis various studies on the characterisation of membrane proteins have been reported (Rosenberg and Guidotti, 1969; Gahmberg, 1971; Hogg, 1974; Bussell and Robinson, 1973; Greenberg and Glick, 1972; Stone et al, 1974). Although each cell type appears to have a unique electrophoretic pattern the range of molecular weights (10,000-250,000) is similar.

Several groups have used membrane impermeable agents to exclusively label proteins on the outer surface of the membrane (Critchley, 1974; Gahmberg and Hakamori, 1973; Hogg, 1974; Hynes, 1973). These studies have revealed a high molecular weight protein

which is sensitive to trypsin and disappears on transformation by oncogenic viruses. This protein is possibly a specific fibroblast antigen (Ruoslahti and Vaheri, 1974).

The enzymic nature of the plasma membrane is well established. Numerous enzymic activities have been found associated with isolated membranes including an ATPase (Kamat and Wallach, 1965; Emmelot and Bos, 1962), 5' nucleotidase (Bingham and Burke, 1972; Bosmann et al, 1968; Gahmberg and Simons, 1970), glycosyl transferases (McLean and Bosmann, 1975; Lloyd and Cook, 1974), nucleotide pyrophosphatase (Touster et al, 1970) and cyclic AMP phosphodiesterase (Russell and Pastan, 1973) but information on the arrangement of these enzymes in the membrane and their possible requirement for specific lipids is sketchy. Further work in this field could yield valuable clues as to the structure of the plasma membrane.

The classical model of the membrane took little account of the carbohydrate which is apparently present on the outer surface of the membrane. The presence of this carbohydrate has been demonstrated by various techniques. Several histological stains have been employed including the periodic acid-Schiff (PAS) reaction (Rambourg et al, 1966; Gasic and Gasic, 1963) or periodic acid-silver methenamine (Rambourg and Leblond, 1967). Using the latter stain with electron microscopy Rambourg and Leblond detected carbohydrate at the surface of 50 different types of rat cells implying that the presence of carbohydrate is the rule rather than the exception. Colloidal iron, colloidal thorium, colloidal ferric hydroxide and ruthenium red have been used to demonstrate the presence of acidic groups such as sialic acid in membranes.

The presence of carbohydrate at the cell surface has also been demonstrated by treatment of intact cells with various enzymes.

Trypsin treatment releases sialic acid containing (Cook et al, 1960) and fucose containing (Warren et al, 1972) glycopeptides from the surface of cells and treatment of intact cells with neuraminidase reduces their electrophoretic mobility (Forester et al, 1962; Kraemer, 1966; Langley and Ambrose, 1967; Ward and Ambrose, 1969) presumably due to the removal of sialic acid.

Plant lectins, specific for certain sugar residues, have been used to show differences in the surface components of transformed cells. The best characterised of these is the protein isolated from the Jack bean, concanavalin A. This protein has a high affinity for carbohydrates containing α-D-glucopyranosyl or α-D-mannopyranosyl residues (Inbar and Sachs, 1969). Other lectins which have been used include wheat germ agglutinin which binds to N-acetyl-glucosamine (Burger and Goldberg, 1967) and ricin which binds to β-galactose residues (Nicolson and Lacorbiere, 1973).

Lectin binding sites have been visualised by light microscopy by conjugating the lectin with fluorescein isothiocyanate (Shohan and Sachs, 1972) and by electron microscopy by complexing either horse radish peroxidase (Bernhard and Avrameas, 1971) or haemocyanin (Smith and Revel, 1972) to lectins previously bound to cell surfaces. These techniques have been used to demonstrate the inhomogeneous distribution of lectin binding sites on the cell surface and also their asymmetrical distribution between the inner and outer faces of the membrane (Nicolson and Singer, 1971).

Experiments by Steck (1972) further emphasized the asymmetry of plasma membranes. He isolated human red cell membranes in the form of right side out ghosts and inside out vesicles. Each membrane face

could be selectively reacted with probes which do not cross the permeability barrier. Sialic acid and acetylcholinesterase were found only on the external face while a diaphorase and glyceraldehyde-3-phosphate dehydrogenase were confined to the internal face. The membrane phospholipids may also be asymmetrical. Bretscher (1972) showed that erythrocytes have few lipid amino groups available on the outer surface and postulated that phosphatidylcholine and sphingomyelin are chiefly located in the outer half of the bilayer while phosphatidyl-serine and phosphatidylethanolamine are located in the inner half.

This evidence of asymmetry is at variance with the classical model of membrane structure proposed by Danielli and Davson and other evidence which is inconsistent with a simple lipid bilayer coated with protein has been accumulated. Using the techniques of optical rotary dispersion and circular dichroism Wallach and Zahler (1966) and Lenard and Singer (1966) found evidence that a high percentage of membrane protein is present in helical formation as opposed to random coil or pleated sheet. Wallach and Zahler proposed that the protein in random coil was present on the surface while that in helical form was predominantly within the membrane. This was in agreement with the finding that interactions between membrane lipids and protein could be of a hydrophobic nature (Lenaz, 1969) and that proteins can span the entire thickness of the membrane (Bretscher, 1971). Furthermore Branton (1969) demonstrated by a freeze cleave technique the presence of globular structures in the interior of the membrane. The results from a number of experiments have indicated that these globular structures are associated with protein. When isolated erythrocyte membranes are treated with trypsin these globular particles clump into irregular patterns. Marchesi et al (1971) have suggested that the

particles are normally anchored in the membrane possibly by other proteins that are hydrolysed by trypsin. Further evidence that at least some protein is embedded in the membrane is suggested by the work of Phillips (1972) who showed that three major glycoproteins of the erythrocyte membrane could not be iodinated by the lacto-peroxidase technique after trypsinisation but if they were labelled and then trypsinised the iodine label was found associated with the membrane. This implies that a part of the protein is buried in the membrane and so protected from enzymatic hydrolysis. Thus the general consensus emerges that, at least in the erythrocyte, some parts of the membrane proteins are exposed at the surface while others are buried within the membrane.

The experiments of Frye and Edidin (1970) drew attention to the possibility that the plasma membrane is in a fluid state. Using the indirect fluorescent antibody technique they followed the distribution of surface antigens in heterokaryons formed between mouse and human cells. The mouse and human antigens were detected by rhodamine and fluorescein labelled antibody respectively. After 40 minutes at 37°C the mouse and human antigens were completely intermixed. Frye and Edidin suggested four possible mechanisms to account for this intermixing: a rapid metabolic turnover of antigens, integration into the membrane of material from a cytoplasmic pool, movement of antigen into the cytoplasm and then into a new membrane site or diffusion of antibody in the plane of the membrane. Based on the results of experiments done at low temperatures or in the presence of metabolic inhibitors they favour the explanation that at 37°C the membrane is fluid and antigens are free to diffuse but at temperatures below 15°C the membrane is frozen and diffusion is inhibited.

The addition of antibody directed against surface antigens can induce a redistribution or "capping" of these antigens (Taylor et al, 1971; Edidin and Weiss, 1972). This phenomenon is not observed if the experiments are performed at $0^{\circ}C$ or if univalent antibody is used. These results can be explained by the antigens being free to diffuse at $37^{\circ}C$ and when bivalent antibody is used cross-linking can occur resulting in the formation of a cap.

Based on this recent experimental evidence Singer and Nicolson (1972) suggested a model of membrane structure which they called the "fluid mosaic model". In this scheme of membrane structure the membrane matrix is formed by a discontinuous lipid bilayer interrupted by intercalated amphipathic globular protein and glycoprotein components. This model can easily accomodate the physical asymmetry required by a membrane which is functionally asymmetric.

(ii) Morphology of the Cell Surface

The morphology of the cell surface may be important in adhesive interactions. Since cells are negatively charged under physiological conditions a repulsive force will exist between them making close approach difficult. Bangham and Pethica (1960) have suggested that cells could overcome this electrostatic resistance by putting out narrow projections. These probes of low radius curvature might receive sufficient Brownian energy to overcome the repulsion forces whereas a sphere of large radius would not. Curtis (1967) however has argued that a microvillus connected to a cell would have to experience higher thermal energy than that calculated by Bangham and Pethica for a freely mobile particle and also that it is unlikely that two microvilli will frequently be projected towards one another. They may however lessen the mechanical work involved in draining the gap between the two

approaching surfaces. Furthermore micrographs have been published which appear to show intercellular microvillus contacts (Lesseps, 1963; Spring-Mills and Elias, 1975) although these may be artifacts of the specimen preparation.

Rajaraman et al (1974), looking at cell to substrate adhesion, suggested that adhesion was effected by microvilli which only appeared at points of contact. Cells which had been left in a pellet had microvilli over the whole surface. This evidence is more suggestive of contact inducing the formation of microvilli rather than contact being made through microvilli although O'Neill and Follett (1970) have produced evidence to the contrary. They found that the number of microvilli on the surface of BHK cells was characteristic of the cell density, cells from sparse cultures having a high number of microvilli and those from confluent cultures very few, and they proposed that microvillus formation may be inhibited by cell contact. These apparently conflicting results could be accomodated by postulating that microvilli are formed all over the cell surface but are withdrawn except at points of contact with either other cells or the substrate. As extensive contacts are formed a "zipping up" process takes place so that the microvilli are replaced by larger areas of contact. This would be in agreement with the observations of Curtis (1964). Using interference microscopy he found that the cell-substrate separation remained fairly constant over large areas suggesting that the whole underside of the cell formed a single large attachment site. The separation distance however was 10-20 nm and not the close molecular contact that microvilli are proposed to make.

(iii) Cell Junctions

Morphological studies of cell junctions are of importance when considering the mechanisms of cell adhesion. At least four

morphologically different types of junction have been observed in electron micrographs: the zonula adherens, the zonula occludens, the macula adherens (or desmosome) and the gap junction.

In the zonula adherens there appears to be a gap of at least 10 nm. Farquar and Palade (1963) showed that haemoglobin could penetrate into the space between opposed plasmalemmae while Brightman (1965) showed that ferritin could penetrate into the intercellular gap. Permeation halted when the tracers reached the zonula occludens.

In the zonula occludens the opposing plasmalemmae appear to be in direct contact. From the work of Farquar and Palade (1963) junctions of this type would appear to completely encircle cells but Brightman (1965) found that ferritin could bypass them.

In the macula adherens or desmosome plasmalemmae are 20 nm apart. The gap between is filled with dense staining material. Bundles of microfilaments within the cytoplasm are associated with this gap.

The gap junction appears to have an intercellular separation of 2 nm. Freeze cleave preparations of cells reveal a hexagonal array of subunits (Goodenough and Revel, 1970). Lowenstein (1968) considers this to be the site of electrical coupling between cells.

The diversity of morphological contacts observed by electron microscopy suggests that there may be more than one adhesive mechanism. The different methods used to elucidate the mechanism of adhesion are probably not all looking at the same surface property. This has been demonstrated by Gershman (1970) who looked at four different techniques: sorting out, electrophoretic mobility, aggregation rate and aggregate size as a function of embryo age and found different patterns with age for all four.

(iv) Models of Adhesion

Most models of adhesion fall into one of three general categories:-

1. The bridging system. There are two main classes of bridging system. In the first divalent cations, generally calcium, bind to anionic groups on the opposing cell surface. This method of adhesion has been favoured by Pethica (1961) and Steinberg (1958). Support for this theory comes from the observation that calcium chelating agents can cause tissues to dissociate to single cells. In the second class macromolecules in the medium adsorb to the cell surface and effect adhesion. Moscona has argued strongly in defence of this theory. He has further proposed that the bridging agents (or "cement") may be specific for each cell type (Moscona, 1961). The action of proteases in separating tissues has been cited as evidence for this cell cement theory.

2. Direct molecular contact. Tyler (1946) and Weiss (1947) proposed that cells could adhere by an antigen-antibody like complex. Roseman (1970) has suggested that rather than antigen-antibody interactions an enzyme-substrate complex could bind cells together. This theory was based on observations by Oppenheimer et al (1969) that the aggregation of trypsinised cells was dependent on the presence of L-glutamine indicating that the synthesis of glycosidic compounds was required for adhesion. Several other workers had observed that high levels of glucosamine hydrochloride inhibited the formation of large aggregates of embryonic cells (Garber, 1963; Glaesser et al, 1968). Subsequent work by Jamieson et al (1971) and Roth et al (1971) demonstrated the presence of glycosyl transferases at the cell surface of platelets and neural retina cells respectively.

3. Long range forces. Curtis (1960) has proposed that adhesion is a consequence of the bulk properties of the membrane. He likens cell adhesion to the flocculation of lyophobic colloids. Deryaguin and Landau (1941) and Verwey and Overbeek (1948) formulated a theory of the stability of lyophobic colloids in which electrostatic forces of repulsion act together with the London force of attraction. This theory is commonly called the DLVO theory. This theory allows for non-adhesion or adhesion at two major separation distances due to the fact that electrostatic and London forces decline at different rates. At very small values of separation (less than 1 nm) the London force will always exceed the electrostatic force of repulsion and particles will be strongly attracted to each other at this distance of separation in the primary minimum. However particles are normally prevented from entering into such close contact due to the electrostatic barrier. At large separations (10-20 nm) the London force is again greater than the repulsive force and particles may adhere at this distance in the secondary minimum. If sufficient energy can be supplied by, for example, Brownian motion particles may overcome the electrostatic barrier and enter the primary minimum. Curtis (1967) has advanced the idea that the zonula adherens and the zonula occludens represent secondary and primary minimum situations respectively.

Tests of these theories require measurements of cell adhesion and many experimental approaches have been evolved.

(v) Measurement of Adhesion

Adhesion has generally been measured by either the force required to break an adhesion or by the ability of single cells to form adhesions. There are difficulties inherent in both methods.

When measuring de-adhesion it is difficult to separate the force required to break the adhesion from other forces involved such as that required for cell deformation (Weiss, 1961) and drainage (Curtis, 1962). When looking at the formation of adhesions the cells have often been recently dissociated from complete tissues. Different disaggregation techniques will result in an unknown amount of surface damage.

1. Dispersion techniques. One of the earliest methods for obtaining a semi-quantitative value for the adhesion of cells was that used by Dan (1936) who counted the number of echinoderm cells remaining on a glass plate after it had been subjected to gravity by inverting it. Coman (1944) measured the force required to separate two adhering cells using microneedles. The microneedles were calibrated so that the deformation of the needles gave a measure of the force required to separate the cells. This technique suffers from the defect that there is no way of knowing how much energy is required to separate the cells and how much is involved in deforming the cells. Weiss (1961) attempted to make measurements more quantitative by using a known shear rate to dislodge cells from a flat plate. However he used a parallel plate system to apply the shear which results in a variation in shear from place to place over the sheet of cells. Gail and Boone (1972) measured the percentage of cells removed from a flat surface by a blast of compressed air. All of these methods involving removal of cells from a substrate are complicated by the fact that the degree of spreading of a cell probably influences its strength of attachment.

2. Aggregation techniques. The techniques used to study aggregation are based on the necessity to bring about cell encounters. These fall into two main categories:

a) Single cell suspensions are agitated in a manner likely

to bring about cell collisions.

b) Single cell suspensions are seeded on to prepared monolayers of cells.

Moscona (1961), Garber (1963) and Humphreys (1963) aggregated cells in a gyratory shaker and measured the diameter of the aggregates that formed. The size of an aggregate should in theory be related to the adhesiveness of the cells since as aggregates increase in size shear will tend to pull cells off the aggregates. The greater the adhesiveness of a cell the less likely is the chance that it will be pulled off and so the greater will be the diameter of the final aggregate. This approach implies that when the final aggregate size has been reached an equilibrium situation will exist in which cells will be added and removed from the aggregate at the same rate. Curtis (1973) however has found that aggregates do not appear to be in equilibrium, very high shear rates being required to remove cells from preformed aggregates. This has been confirmed by Edwards (1973) who mixed labelled and unlabelled aggregates and found that no exchange occurred between the two. These findings mean that the relationship between aggregate size and adhesiveness is in doubt.

Curtis and Greaves (1965) introduced the measurement of the rate of aggregation as a means of obtaining a quantitative measurement of adhesiveness. The reasoning behind the technique is that the more adhesive a cell is then the greater is the chance that a collision will result in the formation of an adhesion and so the initial rate of aggregation will be faster.

Two different methods of shaking have been used to generate collisions: reciprocating motion and gyratory rotation. Roth and Weston (1967) used gyratory rotation to collect labelled single cells by preformed aggregates. Rather surprisingly they found that small

aggregates collected more cells than large aggregates and they attributed this to zoning of cells and clusters by size due to the gyratory action. Single cells and small clusters concentrated at the centre while large aggregates were found further out. This problem can be avoided by using a reciprocating shaker although Curtis (1969) has pointed out that in this system, as well as the gyratory shaker, shear rates will vary in different parts of the flask and that changes in viscosity of the medium will lead to changes in shear rate. Curtis (1969) introduced the use of the Couette viscometer. In this machine a laminar shear flow of known value can be set up. An increase in the viscosity of the medium, which would reduce the shear rate, can be compensated for by increasing the power to the viscometer so that the shear rate remains constant. Using this instrument the aggregation kinetics of cell suspensions can be measured under closely controlled conditions. With some cell types however problems of settling occur.

In all of these methods aggregation is assessed by periodic sampling and counting using either haemocytometry or electronic particle counting (see Edwards, 1973, for a review) or continuously using changes in the transmission of light by the cell suspension as aggregation proceeds (Born, 1962; Jones and Morrison, 1969).

The technique of monolayer collection was introduced by Walther et al (1973). In this technique radioactively labelled cells are seeded on to a confluent monolayer. At various time intervals non-adhering cells are washed off and the percentage adhering is calculated by counting in a scintillation counter. This technique has the advantage that the monolayers and the suspensions can be of different cell types so that specific adhesion can be investigated

or the monolayers and suspensions can be treated in different ways.

(vi) Biochemical Studies

Using the above techniques attempts have been made to distinguish between the various models of adhesion that have been postulated either by enzymic modification of the cell surface or by changes in medium composition.

1. Surface charge and adhesion. If electrostatic forces of repulsion are involved in adhesion it would be expected that a reduction in the surface potential of the cell would be accompanied by an increase in cell adhesion. Garrod and Gingell (1970) found a progressive decrease in electrophoretic mobility of cells of Dictyostelium discoideum as they approached the aggregation stage. However they point out that although their results were always qualitatively the same they were quantitatively dissimilar, for example some cells had a higher surface charge density at six hours than others at twelve hours. Lee (1972) obtained similar results and also found that the reduction in charge was abolished by reagents which abolished cell aggregation. However under starvation conditions aggregation proceeded with no change in surface charge density and treatment of aggregated cells with disaggregating agents failed to alter their electrophoretic mobility.

Neuraminidase treatment removes sialic acid from the cell surface and so should result in a lowering of the surface charge density. Berwick and Coman (1962) found that neuraminidase decreased the adhesion of buccal epithelial cells to glass but had no effect on cell to cell adhesion. McQuiddy and Lilien (1971) also found that neuraminidase had no effect on the aggregation of chick embryo neural retina cells. Kemp (1968) found that neuraminidase inhibited the aggregation of chick muscle cells. Most other workers have found a stimulating effect. Lloyd and Cook (1974) showed that neuraminidase

increased the aggregation of malignant rat cells. The stimulation was reversed by desialysed-agalacto fetuin and by desialysed bovine submaxillary mucin. These glycoproteins also acted as acceptors for sialyl- and galactosyl-transferases. Based on these results they proposed that neuraminidase increases aggregation by generating acceptor sites for cell surface transferases. Vicker and Edwards (1972) reported that neuraminidase increased the aggregation of BHK cells but had little effect on a polyoma transformed derivative. These rather contradictory results argue against a purely electrostatic explanation although several workers have pointed out that removal of equal amounts of sialic acid from different cell types does not necessarily cause an equal reduction in their surface charge. Wallach and Esandi (1964) discovered that treatment of three tumour cell types with neuraminidase led to the removal of similar amounts of sialic acid but reduced the electrophoretic mobility of one type linearly with linear release of sialic acid, reduced that of another sharply until a certain amount of sialic acid was removed when there was no further change and had no effect on that of the third. Similar observations were made with the solid and ascites forms of some tumours. Neuraminidase removed similar amounts of sialic acid from both but only reduced the surface charge of the ascites cells (Kojima and Maekawa, 1972; Cook et al, 1963). These results imply that some molecules of sialic acid contribute only partially, or not at all, to the electrokinetic properties of the cells or removal of sialic acid may alter the conformation of the plasma membrane and change the contribution of other charged species to the electrokinetic potential of the cells. Thus changes in adhesion following neuraminidase treatment cannot be assumed to be due to a reduction in the surface charge of the cell unless the electrophoretic mobility of the cell has also been measured.

Divalent cations are believed to exert their effect on adhesion by virtue of their ability to reduce the magnitude of the net negative charge at the cell surface (see Curtis, 1962, 1966). Dan (1947) measured the zeta potential of echinoderm eggs and found that increasing the concentration of cations lowered the zeta potential and increased their adhesion to a flat plate. Increasing the divalent cation concentration has also been shown to decrease the separation distance between cells and substrate (Curtis, 1964). Lowering the cation concentration increased the separation distance and Curtis has proposed that these results can only be explained by the hypothesis that electrostatic forces of repulsion act in controlling cell adhesion. Other workers have proposed that cations may act as bridges between cells (Pethica, 1961; Armstrong and Jones, 1968). Armstrong (1966) examined the effect of calcium, magnesium, strontium and barium on cell adhesion. He suspended embryonic chick limb bud cells in concentrations of the ions that would bring the cell surface to a chosen charge density. When the cells were aggregated at these concentrations they did not show equal adhesiveness, magnesium being more effective than calcium which was more effective than either strontium or barium. He concluded that an electrostatic explanation could not account for his observations. Curtis (1973) has pointed out however that the higher concentrations of magnesium and calcium used would result in the extent of double-layer repulsion being reduced in these media allowing close approach of the cells. In another paper Armstrong and Jones (1968) reported that calcium specifically protected the tissues of Rana pipiens embryos from dispersal by EDTA. Magnesium, barium and strontium ions at the same concentration were less effective. However if Stern-layer adsorption of calcium is stronger than that of the other ions an electrostatic interpretation could be applied. Collins (1966) has

shown that Stern-layer binding of calcium does take place in chick neural retina cells.

Cations may of course be required for intracellular reactions which have a secondary effect on adhesion such as activation of an actomyosin system beneath the membrane, or of some enzyme system or they may be involved in the biosynthesis of cell surface components. Rabinovitch and De Stefano (1973) have shown that manganese stimulates the adhesion and spreading of sarcoma cells on serum coated glass and have proposed that this may be due to manganese activation of a nucleotide cyclase or to an inhibitory action on calcium fluxes. It may be of relevance that some glycosyl transferases are activated by manganese.

2. Metabolic inhibitors. Many papers have been produced which show that cell adhesion is apparently dependent upon a metabolic process. Moscona (1961) found that trypsin dissociated chick embryo cells would not aggregate in the cold or in the presence of puromycin (Moscona and Moscona, 1963) and proposed that metabolism was required for the synthesis of a bridging molecule. Curtis (1963) however showed that cells dissociated with EDTA would aggregate in the cold in serum free medium. In the presence of horse serum the aggregation of the same cells was inhibited. A factor was purified from horse serum (Curtis and Greaves, 1965) which inhibited aggregation at low temperatures but not at 37°C and Curtis and Greaves concluded that this factor was responsible for the lack of adhesion observed by Moscona. They further postulated that the factor was destroyed by cell metabolism at 37°C. Moscona and Moscona (1966) found however that in their system serum had no effect. Therefore trypsin treatment seems to be the determining factor in whether or not cells will aggregate in the presence of

metabolic inhibitors. This theory is supported by the findings of many other workers. Edwards and Campbell (1971) showed that trypsinized BHK cells would not aggregate in the cold and Weiss and Maslow (1972) found that the aggregation of freshly trypsinized cells was more sensitive to cycloheximide than that of cells which had been given time to recover. Furthermore Glaeser et al (1968) reported that the aggregation of trypsin dissociated cells was inhibited by puromycin whereas that of cells dissociated with EDTA was not. These results suggest that trypsin damages the cell surface. Trypsin has been shown to release macromolecules containing amino sugars and sialic acid presumably from the cell surface (Snow and Allen, 1970; Winzler, 1967; Cook et al, 1960) and there is also some evidence that it can cause a redistribution of proteins in the membrane (Nicolson, 1972). The question remains whether trypsin is causing non-specific damage to the cell surface which must be repaired before adhesion can ensue or whether it is releasing molecules specifically required for adhesion from the surface.

3. Aggregation factors. Aggregation promoting factors have been isolated from the disaggregation medium after the dispersal of cells, from the culture medium in which cells have been grown, from isolated plasma membranes and from serum. It is often assumed that all of these factors originated in the cell surface and a certain amount of evidence has accumulated which supports these assumptions. It has been reported that growing and non-growing cells synthesise similar amounts of membrane material (Warren and Glick, 1968). In growing cells the new material is incorporated with a net gain in membrane whereas in non-growing cells incorporation is accompanied by a corresponding amount of membrane material appearing in the medium. A similar finding was reported by Hubbard and Cohn (1975). They labelled proteins on the

external surface of the membrane of L cells with 125-iodine and found that 80-90% of the label was released into the medium within 24 hours. Most of the labelled material was degraded to amino acids but a small amount remained as acid insoluble material. Kapeller et al (1973) have suggested that trypsin releases material from the surface which is similar to the material released during normal turnover. This suggestion was based on their observations that glucosamine labelled macromolecules released by trypsinization had similar profiles on DEAE cellulose as material released into the medium during normal cell culture. Results from experiments such as Kapeller's may have to be reassessed however since the discovery that commercial preparations of 14C-glucosamine bind non-enzymically to macromolecular components in serum. Angello and Hauschka (1974) found that the chromatographic profile of culture medium which had been exposed to 14C-glucosamine in the absence of cells was very similar to the profile of trypsin released cell surface material and culture medium in which cells had been grown for 24 hours.

Further evidence that material is released from the cell surface into the medium can be found in the studies of Ruoslahti et al (1973) and Ruoslahti and Vaheri (1974) which describe an antigen that is exposed at the surface of fibroblasts and is also present in homologous serum. This antigen can be released from the surface by mild trypsinization.

Several workers have described cellular exudates which may be involved in cell adhesion. Cells will attach to clean glass very rapidly and are resistant to removal by trypsin or EDTA (Takeichi, 1971). After incubation at 37°C for 24 hours they are easily detached

by these agents. They are also easily detached when the substrate has been pretreated with conditioned medium. These results suggest that it is an extracellular material produced by the cell that is sensitive to trypsin. Taylor (1961) observed a similar phenomenon and proposed that attachment to clean glass, which is independent of cations, is due to strong Van der Waal's attraction but that attachment to serum coated substrates is by a bridging mechanism involving protein molecules and cations. The production of this exudate may be dependent on cell contact as Maslow and Weiss (1972) have reported that Ehrlich ascites cells will release 51-chromium when they are adherent to a fibroblast monolayer. If adhesion is prevented by gentle agitation then the release of 51-chromium is considerably diminished.

Lilien (1968) found aggregation promoting material (APM) in the growth medium of several cell types. Antisera against this material agglutinated cells that had been treated with APM which implies that APM is bound to the cell surface. A third component also seems to be necessary before stimulation of aggregation occurs (Balsamo and Lilien, 1974a). This is demonstrated by the use of mixtures of live and dead cells. Glutaraldehyde fixed cells do not aggregate even in the presence of APM. If however cells are treated with APM then fixed with glutaraldehyde they will enhance the aggregation of unfixed cells. Balsamo and Lilien have suggested that this third component is labile and must be synthesised continuously.

Pessac and Defendi (1972a) have suggested that these factors may be hyaluronic acid. They have isolated factors, from conditioned medium and from serum, that induce the aggregation of mouse lymphoblasts. These factors are inactivated by hyaluronidase and hyaluronic acid

itself will promote the aggregation of these cells.

The factors appear to be mucopolysaccharides with a molecular weight of about 10^6 daltons with hyaluronic acid as a major component. The regulated release of hyaluronate has also been implicated in the control of morphogenetic events (Toole et al, 1972) which may be related to changes in cell adhesion.

Many of the factors cited in the literature have been shown to specifically enhance the aggregation of one cell type. Attempts have been made to elucidate the chemical basis of this apparent specificity. Moscona (1963) and Humphreys (1963) reported that cells of the sponges Microciona prolifera and Haliclona occulata would aggregate in the cold if they were mechanically dispersed but not if they were chemically dispersed in medium lacking calcium and magnesium. The addition of supernatent from the chemical dissociation procedure to chemically dissociated cells promoted their aggregation in the cold. They proposed that some factor, normally involved in intercellular adhesion, was released during the chemical dissociation procedure and could be re-adsorbed by dissociated cells permitting them to reaggregate. These factors exhibited species specificity in as much as they supported the aggregation of homologous cells but not heterologous cells. Humphreys (1965) has described the isolation of a factor from Microciona prolifera which contained carbohydrate and protein in roughly equal amounts with a particle diameter of 100-300 Å and a molecular weight of 10^7. Margoliash et al (1965) isolated factors from both Microciona prolifera and Haliclona occulata and found, in agreement with Humphreys, that both preparations contained carbohydrate and protein. These factors however had a particle diameter of only 20-25 Å and a minimum molecular weight of 13,000. Moscona (1963) has argued that it is the carbohydrate moiety

of these factors that is responsible for their specificity on the grounds that their aggregation promoting activity was destroyed by periodate. Unfortunately periodate can also affect protein structure (Gasic and Galanti, 1966).

Two different strains of the fresh water sponge Ephydatia fluviatilis produce factors that inhibit the aggregation of heterologous cells and stimulate the aggregation of homologous cells (Curtis and Van de Vyver, 1971). These cells show no specificity of adhesion, the level of adhesiveness of each cell type being controlled by the factors. Curtis and De Sousa (1973) have isolated factors from B and T lymphocytes which have no effect on homologous cells but greatly reduce the adhesiveness of the heterologous cells. From the dose response curves for these factors Curtis and De Sousa have proposed that the factors are altering surface components rather than binding to the cell surface.

Factors showing age specificity rather than species specificity have also been reported. Kuroda (1968) showed that liver cells from seven day embryos produced larger aggregates than cells from eighteen day embryos. A supernatent fraction from seven day liver cells enhanced the aggregation of cells at the same stage but had no effect on eighteen day cells. Supernatents from eighteen day cells had no effect on the aggregation of either seven day or eighteen day cells.

Several theories have been proposed for the mode of action of aggregation promoting factors. They may promote aggregation by inducing the synthesis of intercellular material or they may be substances released from the surface of the cell and their aggregation promoting action is caused by a net increase in their number at the cell surface or they may cause conformational changes at the cell surface leading to the exposure of adhesive sites. Balsamo and Lilien (1974b) have found

that the binding of their AFM is tissue type specific and the binding curves reveal a cooperative effect. They suggest that the cooperative effect is indicative of a configurational change occuring at the cell surface. This is supported by the observation that AFM inhibits the capping of FITC-con A receptors(McDonough and Lilien,1975). If these factors originated in the cell surface their purpose may be to maintain the surface in a configuration such that stable contacts can be made.

Pessac and Defendi(1972b) have produced evidence that factors bind to specific receptors on the cell surface. They selected murine cell lines which did not aggregate. One of these lines(P388) could aggregate in the presence of a factor from an aggregating cell line. They proposed that P388 cells did not produce factors but did possess receptors. Another line(N2) produced factors but did not aggregate and they suggested that N2 had very few receptors. Further evidence that factors and receptors are distinct entities is that factors are resistant to trypsin whereas receptors are sensitive, factors are more sensitive to treatment with cycloheximide than receptors and finally factors are inactivated by hyaluronidase whereas incubating P388 cells with this enzyme does not decrease their ability to aggregate in active conditioned media.

Several workers have looked at the effect of isolated plasma membrane fractions on cell aggregation. The results, however, are contradictory and difficult to interpret. Rosenberg et al(1969) reported that membranes isolated from chick liver cells enhanced the clumping of other chick liver cells. The opposite result was obtained by Merrell and Glaser(1973) who isolated membranes from chick neural retina and cerebellum cells. Membranes bound specifically to their own

cell types and inhibited their aggregation. The initial rate of aggregation of control cells reported in this paper is much higher than that observed by other workers for the same system however so other factors may be involved. Neuraminidase treated plasma membranes from 16C cells enhanced the aggregation of 16C cells particularly in the presence of neuraminidase(Lloyd and Cook,1974). Many interpretations could be placed on these results. Membranes may enhance aggregation by acting as multivalent ligands or membrane vesicles may be incorporated into the cell surface by fusion thereby increasing the number of adhesion sites. Inhibition of aggregation could occur if membrane fragments bound to adhesive sites on the cells and competitively inhibited the attachment of other cells. Since the mechanism of adhesion is unknown it is difficult to predict which of these possibilities is the more likely and so few real conclusions can be drawn from the above work.

4. <u>The role of surface carbohydrate</u>. Evidence that surface heterosaccharides may be involved in adhesion has come from the work of Oppenheimer et al(1969) who found that mouse teratoma cells would aggregate in complete culture medium but not in Hanks' balanced salts solution. If L-glutamine was added to the Hanks' solution aggregation proceeded. Furthermore the cells would not aggregate in complete medium from which L-glutamine had been omitted. These workers suggested that the amino acid was required for the synthesis of cell surface amino sugars involved in intercellular adhesion. This theory was supported by the observation that D-glucosamine and D-mannosine could replace L-glutamine but other interpretations are also possible. Hexosamines may be required to repair the cell surface and maintain it in a suitably adhesive state without being part of the actual adhesive species. Furthermore this cell type may be an exceptional case in its requirement

for L-glutamine. Other cell types have been shown to aggregate in medium lacking it (Edwards and Campbell, 1971).

The administration of simple sugars to cells in culture may modify the carbohydrate components of the cell surface and alter cellular adhesiveness. Rizki (1961) fed a solution of glucosamine to the larvae of Drosophila melanogaster and found that this decreased the adhesiveness of haemolymph cells and Garber (1963) reported that this sugar also inhibited the aggregation of trypsin dissociated embryonic chick neural retina and liver cells. It was concluded from these experiments that glucosamine inhibits the synthesis of surface materials involved in adhesion. Glucosamine has been shown to have several other effects on cellular metabolism however. Lloyd and Kemp (1971) showed that the reduced aggregation of chick muscle cells was accompanied by increased respiration and changes in the adenine nucleotide ratio. It can not be assumed therefore that changes in adhesion are directly related to a diminished glycoprotein synthesis.

Preparations of mucoproteins have also been found to inhibit adhesion (Allen and Minnikin, 1975). These authors reported that a preparation of mucoprotein from pig gastric mucus would bind equally well to BHK, PyBHK and HeLa cells. The binding was temperature and cation dependent and heavy trypsinization of the cells reduced their binding capacity. This same gastric mucoprotein inhibited the adhesion of single cells to a monolayer. Similarities in the binding of the gastric mucoprotein to cells and the adhesion of cells to monolayers led the authors to propose that the same type of interactions were involved in both processes although the nature of these interactions is unknown.

The cellular slime moulds have been widely used in cell adhesion studies because of the two distinct phases in their life cycle: a non social vegetative phase and a cohesive phase which is initiated under conditions of starvation. Rosen et al (1974) have isolated carbohydrate binding proteins from two of these slime moulds: Dictyostelium discoideum and Polysphondylium pallidum. The proteins are assayed by their ability to agglutinate erythrocytes and have the following properties: they are present on the cell surface; they are present when the cells are differentiated to a cohesive state but absent when the cells are not cohesive; addition of the purified protein promotes cell cohesion; sugars which react with the active site of the molecule block both spontaneous and induced cell cohesion. These findings indicate that the proteins mediate cell adhesion. The authors have also proposed that the proteins are specific for their own cell type as the precise reactivities of the protein from P. pallidum differ from those of the protein from D. discoideum. The acquisition of aggregative competence by D. discoideum is also accompanied by quantitative changes in the iodinability of their surface proteins (Smart and Hynes, 1974) one of which may be the protein described above.

Carbohydrate binding plant lectins have been widely used for studies on the structure of the cell surface and several workers have turned their attention to the effects of these lectins on cellular adhesion. Grinnell (1973) found that concanavalin A increased the strength of attachment of BHK cells to a plastic substrate. The cells became less sensitive to detachment by physical shear, trypsin or EDTA. The effects were reversed by D-glucose or α-methyl-D-glucoside. Pre-treatment of the substrate with conA also increased the strength of

attachment. Grinnell has suggested that this effect may be due to direct cross linking of the cells to the substrate.

The results obtained by workers using "monovalent" conA are somewhat contradictory. Evans and Jones (1974) Found that it inhibited aggregation whereas Steinberg and Gepner (1973) reported that it had no effect on either aggregation or sorting out. These experiments are rather unsatisfactory because the true state of the conA is unknown. Divalent con A is known to induce clustering of the conA receptors probably by cross linking (Edelman et al, 1972). The conA in the above experiments was rendered monovalent by trypsinization which probably results in a mixture of monovalent and divalent molecules. It is more likely that the inhibition observed by Evans and Jones is caused by a conformational change in the membrane rather than conA binding to receptors which are directly involved in adhesion. Similar experiments with succinyl conA, which is believed to be truly monovalent (Gunther et al, 1973), may help to resolve this problem.

Roseman (1970) has outlined a mechanism by which complex carbohydrates at the cell surface could mediate the formation of intercellular adhesions. He has suggested that cells could interlink by the formation of enzyme-substrate complexes, the enzyme on one cell surface reacting with the substrate on a second cell surface. The enzymes and substrates he proposes are glycosyl transferases and complex carbohydrates. In this system adhesions would be temporary, their duration being determined by the supply of sugar nucleotides. Specificity of adhesion could be introduced into the system by cells having different transferases showing a high degree of specificity for particular acceptors.

The investigations of Roth et al (1971) demonstrate that some glycoyl transferases are located in the plasma membrane. Intact chick neural retina cells were able to catalyse the transfer of radioactively labelled galactose from UDP-galactose to high molecular weight endogenous acceptors. Variations in this surface activity following infection with oncogenic viruses have been reported by a number of workers (Bosmann et al, 1974; Grimes, 1970; Warren et al, 1972), and Roth and White (1972) showed that non-adhesive 3T12 cells could glycosylate receptors on the same cell surface whereas the adhesive 3T3 cells could only transfer galactose to receptors on adjacent cell surfaces. McLean and Bosmann (1975) found increased glycosyl transferase activity when (+) and (-) gametes of Chlamydomonas rheinhardii were mixed. Mixtures of vegetative cells did not show any increased activity. Most of the activity appeared to reside in the flagellar membranes. Differences in the activities of a glucosyl transferase and a galactosyl transferase have also been found in clumping and non-clumping strains of Acanthamoeba castellanii (Hoover, 1974).

Transferases need not be involved in adhesion however. They may simply be involved in synthesising complex carbohydrates necessary for adhesion or their activity may be the result of adhesion rather than the cause.

Apart from their postulated role in cell adhesion glycosyl transferases have been implicated in Haemostasis. Jamieson et al (1971) have provided evidence which indicates platelet-collagen adhesion is brought about by glucosyl transferases on the platelet membrane reacting with incomplete carbohydrate chains in collagen and Bosmann (1972) has proposed that platelet aggregation is initiated by

a sialyl transferase on the platelet surface. He also finds neuraminidase activity associated with the platelet which he postulates regenerates acceptor sites for the sialyl transferase. Adhesion is therefore maintained by a cyclic reaction.

Surface heterosaccharides may be involved in cell recognition rather than cell adhesion. Glycoproteins and glycolipids could easily provide the cell with a wide range of different molecular structures ideally suited for recognition processes. The diversity of structure can arise through differences in sequential arrangements and by branching in the oligosaccharide units.

Evidence has accumulated from a number of sources which supports the idea that the integrity of surface oligosaccharides is important in cellular recognition. Gasic and Gasic (1962) injected mice with neuraminidase and then with ascitic cells. They were able to demonstrate that the neuraminidase treatment reduced the number of metastases in these mice compared to the controls. Neuraminidase also alters the homing properties of rat lymphocytes (Woodruff and Gesner, 1968). Treatment with this enzyme causes the cells to accumulate in the liver rather than in the lymph nodes and spleen.

The involvement of glycoproteins in recognition is further reinforced by experiments on the clearance of macromolecules by the liver. Pricer and Ashwell (1971) have shown that rat liver plasma membranes will bind desialysed orosomucoid and Rogers and Kornfield (1971) found that if a desialysed glycopeptide was linked to lysozyme or albumin and injected into rats, it was rapidly removed from the circulation. Removal of galactose from the glycopeptide prevented this clearance.

The response of cells in culture to simple sugars was investigated by Cox and Gesner (1965). They discovered that L-fucose altered the growth and morphological appearance of 3T3 cells and D-mannose had a similar effect with BSC-1 cells. The authors suggest that the sugars

are binding to specific complementary sites on the cell surface and mimicking cell contact.

One system in which interaction by complementary glycosubstances has been clearly demonstrated is the mating of opposite types of the yeast Hansenula wingei (Crandall and Brock,1968). The authors were able to isolate an agglutinating factor from the surface of one strain of yeast which was neutralised by a specific cell surface component from the second strain. Both the agglutinin and the neutraliser were glycoproteins.

5. Fibrillar systems and adhesion. An abundance of cytoplasmic fibres such as microtubules, microfilaments and filaments characterise the ultrastructure of many cultured cells(Goldman and Knipe,1973). These organelles have been implicated in many biological processes including the shaping of cells during spreading(Goldman and Knipe,1973), intracellular transport in fibroblasts(Goldman and Follett,1969), the maintenance of the polarity of fibroblasts(Vasiliev et al,1970) and cell locomotion(Luduena and Wessels,1973). Some recent experiments have indicated that these fibrillar systems may also be involved in cellular adhesion. Cytochalasin B has been shown to inhibit the aggregation of blood platelets(White,1971; Haslam,1972; Kay and Fudenberg,1973), limpet haemocytes(Jones and Partridge,1974) and the attachment of BHK cells to a substrate(Grinnell,1974; Gail and Boone,1972). The effects of cytochalasin B have been attributed to its ability to disrupt microfilaments(Wessels et al,1971). These microfilaments appear to be identical to muscle actin both with respect to the molecular weight of the isolated protein (Spudich, 1974) and their ability to bind heavy meromyosin (McNutt et al, 1973; Perdue, 1973; Goldman and Knipe, 1972). P.C.T.Jones (1966) suggested a theory of cell adhesion involving a

contractile system below the membrane based on his own observations and those of D.M.Jones (1966) that ATP inhibited and ADP stimulated cell aggregation. He prposed that a contractile system could cause folding of the membrane thereby changing the negative charge per unit surface area. These changes in surface charge would lead to changes in the electrostatic forces of repulsion and could in this way change the adhesiveness of the cells.

The presence of myosin at the cell surface has also been reported by a number of workers. Gwynn et al (1974) detected a myosin-like protein at the surface of chick embryonic muscle cells using antibody raised against smooth muscle myosin labelled with peroxidase and Willingham et al (1974) found myosin at the surface of L-cells with fluorescein labelled antisera to L929 cell myosin. This antisera could also agglutinate L-cells. Allison (1973) however using similar techniques could find no evidence of myosin at the cell surface but he found strong fluorescence in the peripheral cytoplasm beneath the membrane.

The evidence for actomyosin-like systems acting in cell adhesion is therfore rather tenuous at the present moment. No doubt further experiments will help to clarify the situation especially with respect to experiments with cytochalasin B. As mentioned earlier this compound is believed to disrupt microfilaments but some cell surface activities also appear to be inhibited for example glucose transport (Zigmond and Hirsch, 1972) and mucopolysaccharide synthesis (Sanger and Holtzer, 1972) either of which could have and effect on adhesion.

If the evidence for the involvement of microfilaments in adhesion is tenuous, that for microtubules is even more so. Cyclic AMP apparently strengthens the attachment of cells to a substrate (Johnson and Pastan, 1972; Grinnell, 1973; Shields and Pollock, 1974). Shields and Pollock found that colcemid reverses this effect and

proposed that cyclic AMP had its effect by stabilising microtubules. Berlin et al (1974) suggested that the mobility of membrane proteins in the plane of the membrane is controlled through their attachment to a cytoplasmic fibrillar network and colchicine disrupts this network thereby releasing these membrane proteins. Part of the function of this network may be to keep the cell surface in a suitable conformation for cellular interactions. Waddell et al (1974) have suggested another role for microtubules in adhesion based on their findings that colchicine and vinblastine inhibit the aggregation of BHK cells. They have proposed that the surface membrane must constantly be replaced for adhesions to be maintained and that this new membrane is transported to the surface by way of microtubules.

6. Miscellaneous observations. Many other agents have been tested for their effects on adhesion. Sulphydryl binding reagents inhibit the attachment of BHK cells to plastic (Grinnell et al, 1973) and abolish the aggregation of chick liver and kidney cells (George and Rao, 1975). These effects can be reversed by the addition of cysteine. Grinnell et al (1973) also found fewer sulphydryl binding groups in attached cells than in suspended cells and proposed that this is due to the sulphydryl groups being actively involved in the adhesive bond.

Cationic anaesthetics will also inhibit the adhesion of cells to a substrate (Rabinovitch and De Stefano, 1974). These same drugs block several processes which appear to be dependent on membrane motility such as the capping of lymphocyte surface macromolecules (Ryan et al, 1974) and Rabinovitch and De Stefano postulate that inhibition of adhesion is a consequence of inhibition of membrane motility by the drugs.

Cell adhesion may be dependent on the state of the plasma

membrane lipids and for this reason agents which change the lipid composition of the membrane ahve been tested for their effect. Hax et al (1974) observed that dense cultures of Acanthamoeba castellanii had an increased phospholipase A activity. Cells from these cultures spontaneously aggregated into multicellular masses. They found that the addition of lysolecithin to sparse cultures would induce the aggregation of these cells. Curtis et al (1975) found the opposite result with chick neural retina cells. In this system phospholipase A diminishes the adhesiveness of the cells and the effect can be mimicked by the addition of lysolipids. In the same paper the authors report that cells will rapidly incorporate exogenous fatty acids into their plasma membranes. Incorporation of long chain fatty acids leads to increased aggregation while short chain acids decrease it. These results can be explained in several ways. Changes in membrane fluidity may affect the aggregation of membrane proteins involved in adhesion or the electrodynamic forces between membranes may be altered leading to alterations in intermembrane forces of attraction and thereby cell adhesion. Alternatively the activity of a membrane bound enzyme may be altered. Several membrane enzymes are known to require particular phospholipids for activity and fatty acid specificity may be as important as the specificity of the head group. The specific activity of a sialyl transferase is increased when HeLa cells are incubated in the presence of short chain fatty acids (Simmons et al, 1975) and the possible involvement of glycosyl transferases in adhesion has previously been discussed.

It is obvious from this review that the mechanism by which cells adhere to each other is still a matter for speculation. Many of the results are contradictory and there is no unequivocal evidence in favour of any one model.

This present investigation has been undertaken in an effort to characterise the molecular species at the cell surface involved in adhesion. Edwards et al (1971) have previously shown that the aggregation of BHK C13 cells is altered when the cells are transformed with polyoma virus. However transformation of mammalian cells by oncogenic viruses is also accompanied by loss of contact inhibition of growth, changes in the composition of the glycoproteins and glycolipids of the cell surface, increased agglutinability with lectins, changes in the intracellular levels of cyclic AMP and changes in the levels of glycosyl transferase activities, but whether a change in any one of these properties inevitably leads to changes in the others is unknown. Therefore a change in any one leading to a change in adhesion does not necessarily mean that that property is involved in the mechanism of cell adhesion. It was considered that a fruitful approach to the resolution of this problem would be the isolation of adhesive variants from cells that had not previously been in contact with oncogenic viruses. Any changes in these cells could be considered to be directly due to, or the cause of, the altered adhesiveness.

MATERIALS AND METHODS

Cells. The cells used were the Syrian hamster fibroblastic line BHK21 clone 13 (Stoker and MacPherson, 1964) and a polyoma virus transformed derivative (Edwards et al, 1971). These cells are referred to throughout as C13 and Py respectively.

Cell Culture. Cells were grown as monolayers in either glass bottles (area 120 cm^2) or in roller culture bottles (area 2,000 cm^2) in modified Eagle's minimal essential medium supplemented with 10% calf serum and 10% tryptose phosphate broth (ECT) at 37°C with 5% CO_2, 95% air as the gas phase.

Cells were subcultured when they reached confluency by pouring off the ECT and washing the monolayer twice with 10 mls of 0.05% Difco 1.250 trypsin (250-300 BAEE units/ml), 0.5 mM EDTA in phosphate buffered saline (pH 7.4) at room temperature. When the cells started to detach from the glass the tryptic activity was stopped by the addition of 10 mls of fresh ECT, and the cells were shaken off into this medium. The cells were counted in a haemocytometer and replated at the required density.

Cells were discarded after one month of serial propagation.

Variant Selection. It had been noticed that if C13 cells were maintained in continuous culture for long periods they often grew to higher cell densities and lost their ability to aggregate. This fact was taken advantage of in the search for adhesive variants.

Cells which had lost their ability to aggregate were plated at very low densities (about 1.5 cells/cm^2) in 90 mm plastic

petri dishes containing sterile 13mm cover slips. The petri dishes were incubated until discrete clones had formed. Cover slips containing only one clone were transfered to 30 mm Falcon plastic petri dishes and allowed to grow to confluency. They were then trypsinized off, transfered to glass culture bottles (60 cm^2) and again grown to confluency. Clones were tested for their ability to aggregate. Those that aggregated normally were discarded. One clone which showed no aggregation was recloned in the same way. All of the subclones derived from this single clone showed the same aggregative behaviour indicating that it had probably arisen from a single cell and not from a small clump of cells. Most of the present work has been done with one of these subclones which has been called NAC8.

Growth of Cells in Soft Agar. An agar underlay was prepared in 50 mm plastic petri dishes with Eagle's medium containing 10% foetal calf serum, 10% tryptose phosphate broth (EFT) and 0.5% Noble agar. This was overlaid with 1.5 mls of EFT containing 0.2% Noble agar and cells in a range of concentrations. The petri dishes were incubated at 37°C until colonies developed.

Preparation of Cells for Aggregation.

1. Trypsin dissociation. Freshly confluent cell monolayers were washed twice with Tris-saline (25 mM Tris-HCl pH 7.4, 0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4) then briefly (one minute) with 10 mls of the trypsin-EDTA solution used for subculturing. The cells were incubated at room temperature for a further four minutes then 10 mls of EGT were added and the cells shaken into suspension. The cells were

washed three times with ice cold Hank's basic salt solution buffered with 10 mM hepes and finally resuspended in Hank's/hepes at a density of 10^6 cells per ml. A single cell suspension was obtained by flushing gently with a Pasteur pipette.

2. EDTA dissociation. Cells were washed with Tris-saline as for the trypsin dissociation then washed briefly with 10 mls of 0.5mM EDTA in phosphate buffered saline (pH 7.4). The cells were then incubated for ten minutes at 37°C then 10 mls of ECT were added and the cells shaken into suspension. Treatment was then as for trypsin dispersion. To obtain a high percentage of single cells the suspension had to be filtered through 15μ Nitex.

Aggregation. 4 mls of cell suspension, in 10 ml siliconized, conical flasks, were shaken in a reciprocating shaker at 92 strokes/minute (Curtis and Greaves, 1965). The progress of the aggregation was followed by taking 100 μl samples at time intervals, diluting them with 20 mls of 0.9% saline and counting them in a Coulter counter using a 200-μm aperture (Edwards and Campbell, 1971). Results were plotted as the ratio of total particles at time t to total particles at time zero, against time. If cells were to be aggregated for long periods of time they were resuspended in ECT rather than Hank's/hepes.

Membrane Isolation. Membranes were isolated by a slight modification of the method of Cahmberg and Simons (1970). Monolayers were washed twice with Tris-saline then the cells were scraped from the glass into 0.25 M sucrose 0.2 mM $MgSO_4$, 5.0 mM Tris (pH 7.4). Cells were collected by centrifugation at 600g for five minutes and washed once with the Tris-sucrose solution. The packed cells were resuspended in five times

their own volume of Tris-sucrose and homogenised in a Potter-Elvejhem teflon grinder at 2,000 rpm by twelve up and down strokes. Homogenisation was monitored by microscopy. The homogenate (H) was made $1x10^{-3}$ M with respect to EDTA and centrifuged at 13,500 g for fifteen minutes in the 10x10 ml titanium rotor in an MSE 65 superspeed. The pellet (N) from this spin contained mainly nuclei, mitochondria and lysosomes. The supernatant was centrifuged in the same rotor at 50,000 g for ninety minutes. The supernatant from this spin was called the soluble fraction (S1). The pellet containing the microsomes was resuspended in 10 mls of 10^{-3} M Tris (pH 8.6) with the aid of a homogeniser and centrifuged at 50,000 g for ninety minutes. The supernatant from this spin was combined with S1 to give a total soluble fraction (S) and the pellet was resuspended in 3 mls of 10^{-3} M $MgSO_4$, 10^{-3} M Tris (pH 8.6) and sonicated briefly then layered over 6 mls of 14.5% Ficoll containing 10^{-3} M $MgSO_4$, 10^{-3} M Tris (pH 8.6). The gradient was centrifuged in the same rotor at 125,000 g for two hours. The white band at the Ficoll interface was the plasma membrane fraction (PM) and the pellet contained the endoplasmic reticulum membranes (ER). The white band was removed with a syringe fitted with a J-shaped needle and diluted with 9 volumes of 10^{-3} M Tris (pH 8.6). The pellet was resuspended in 10 mls of the same buffer and both were centrifuged at 300,000 g for one hour. The pellets were resuspended in 1 ml of 10^{-3} M Tris (pH 8.6).

Chemical Determinations. Protein was measured by the method of Lowry et al (1951) with bovine serum albumin as standard. The reaction mixtures contained 0.1% SDS.

DNA was determined by the diphenylamine reaction of Burton (1956) and RNA by the orcinol reaction of Ashwell (1957).

Carbohydrate was measured by the phenol-sulphuric acid method of Dubois et al (1956) with glucose as standard.

Lipids were isolated by the Folch technique (1957). Two dimensional chromatography of lipids was carried out on silica gel H-HR on 20x20 cm plates. The solvent system in the first direction consisted of chloroform/methanol/ammonia/water in the ratio of 90:54:5.5:5.5 (V/V/V/V) and in the second direction chloroform/methanol/acetic acid/water in the ratio of 90:40:12:2 (V/V/V/V). Plates were air dried between separations. Lipids were visualised by staining with iodine vapour. Phospholipids were quantitated by phosphorus analysis after hydrolysis by a modification of the method of Moores (1970). Dried samples were hydrolysed in 0.25 mls of 10N perchloric acid at 180°C for two hours, then made up to 1 ml with 0.1 M sodium acetate. 2 mls of a solution made up from 95 mls of 0.25% ammonium molybdate, 5 mls of 0.25% ammonium metavanadate in 0.1 M sodium acetate and 1 g of ascorbic acid, were added to each sample and the samples were incubated at 37°C for thirty minutes. The optical density of the resulting blue colour was determined at 660 nm. Air dried potassium dihydrogen orthophosphate was used as standard. The weight of phosphorus was multiplied by twenty-five to obtain the weight of phospholipid.

Enzyme Assays. Na^+-K^+ dependent ATPase was assayed by the method of Franke (1970). The assay medium contained 5 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 20 mM Tris-HCl (pH 7.5) and enzyme. The reaction was started by the addition of ATP to give a final concentration of 5 mM

in 1 ml. The reaction mixture was incubated at 37°C for thirty minutes in the presence and absence 10^{-4} M ouabain and the reaction was stopped by the addition of 100 µl of 50% TCA. Phosphorus was determined as for phospholipids omitting the acid hydrolysis step.

Glucose-6-phosphatase was assayed by the procedure of Franke (1970). The enzyme was assayed in the presence of 20 mM glucose-6-phosphate, 20 mM sodium cacodylate (pH 6) in a total volume of 1 ml at 37°C for thirty minutes and phosphorus was estimated as above.

5' nucleotidase was assayed by the breakdown of AMP. The assay mixture consisted of 5 mM AMP, 10 mM $MgCl_2$, 80 mM Tris-HCl (pH 7.5) and enzyme in a total volume of 1 ml. Incubation was for thirty minutes at 37°C after which free phosphorus was estimated (Bodansky and Schwartz, 1963).

Succinate dehydrogenase was assayed by the method of Greene et al (1955). The reaction mixture contained 10 mg of bovine serum albumin, 1 mM potassium cyanide, 5mM succinate, 10 mM phosphate buffer (pH 7.4) and enzyme in a total volume of 0.9 mls. The reaction was started by the addition of 1 mg of cytochrome C in 0.1 ml phosphate buffer and the change in optical density at 550 nm was observed.

Adenyl cyclase was assayed by the method of Rossomondo and Sussman (1972). The enzyme was incubated in a solution containing 100 mM sodium fluoride, 1 mM dithiothreitol, 0.4% triton X-100, 170 n moles of (^{3}H) ATP at 1.7×10^3 cpm/n mole, 150 n moles cyclic AMP, 20 mM Tris-HCl (pH 7.4) in a total volume of 200 ul for thirty minutes at 37°C. The reaction was stopped by the addition of 50 µl of 5%TCA and the reaction products were separated by chromatography on Eastman

cellulose sheets. The solvent system used was n-butanol/acetone/ acetic acid/ammonium hydroxide/water in the ratio 14:10:6:1:8 (V/V/V/V/V). Spots were located under UV light, cut out and counted in 10 mls of Aquasol (New England Nuclear) in a Beckman model L-200 scintillation counter.

Galactosyl transferase.

1. The assay using whole cells and endogenous acceptor was a modification of the method of Roth et al (1971). The cells were scraped off the culture bottles with a rubber policeman and washed twice in glucose, phosphate, and bicarbonate free Hank's basic salts solution supplemented with 10 mM $MnCl_2$, 10 mM NaN_3 and buffered with 10 mM hepes to pH 7.2 (medium "J"). $5x10^6$ cells were resuspended in 600 µl of medium "J" with 0.1 µCi of ^{14}C-UDP-galactose and incubated at 37°C for two hours during which time the cells settled at the bottom of the tube in a loose pellet. In some experiments the cells were kept in suspension by means of a miniature magnetic stirring bar. The reaction was stopped by addition of 2 mls of cold 5% TCA. The precipitate was washed twice with 5 mls of 5% TCA and once with 5 mls of ethanol and then dissolved in 1 ml of ammonium hydroxide at 37°C overnight and counted in 10 mls of Aquasol.

2. The transferase activity in isolated membranes was determined by the method of Fleischer et al (1969). The assay mixture contained 40 mM N-acetyl-glucosamine, 40 mM mercaptoethanol, 40 mM $MnCl_2$, 80 mM sodium cacodylate (pH 6.75), 50 µg of membrane protein and 0.05 µCi of ^{14}C-UDP-galactose in a total volume of 100 µl. Incubation was for one hour at 37°C and the reaction was stopped by the addition of 20 µl of 0.3 M EDTA neutralised to pH 7.4 with NaOH. The reaction mixture was passed through a column (0.8x2 cm) of

Dowex-2X8, 200-300 mesh, in the Cl^- form. Free galactose was retained in the column while galactose bound to N-acetyl-glucosamine was released by washing the Dowex with two 0.5 ml aliquots of distilled water which were collected directly into scintillation vials. Samples were counted in 10 mls of Aquasol.

Sialyl transferase.

1. Endogenous acceptor:- The reaction mixture used was that of Grimes (1970). $5x10^5$ cells were incubated in 100 µl of potassium phosphate buffer (pH 6.5) containing 10^{-3} M $MgCl_2$, 0.1% Triton X-100 and 100,000 cpm of ^{14}C-CMP-sialic acid for one hour at 37°C. The reaction was stopped with 1 ml of 0.5N HCl containing 1% phosphotungstic acid. The precipitate was washed three times with 5% TCA and once with absolute ethanol. The pellet was dissolved in 1 ml of ammonium hydroxide at 37°C overnight and counted in 10 mls of Aquasol.

2. Exogenous acceptor:- The assay medium was the same as above with the addition of 0.5 mg of desialysed fetuin. At the end of the incubation period the cells were centrifuged at 600 g for ten minutes and the supernatant carefully removed. The protein in the supernatant was precipitated with 1 ml of 0.5N HCl containing 1% phosphotungstic acid and the same procedure as above was followed.

Membrane Solubilisation. Isolated membranes were solubilized by dialysis against 10 mM sodium phosphate buffer (pH 7.2) containing 1% SDS, 1% mercaptoethanol and 6 M urea for twenty-four hours.

Polyacrylamide Gel Electrophoresis. Samples containing 200-300 µg of protein were analysed on 0.6x10 cm gels. The gels were made up from 7½% acrylamide, 0.19% bis acrylamide, 0.1% SDS, 0.05% TEMED, 6 M urea, 0.075% ammonium persulphate and 0.1 M sodium phosphate buffer (pH 7.2).

The electrode buffer was 0.1 M sodium phosphate (pH 7.2) containing 0.1% SDS. Electrophoresis was at 3½ mA/gel for twenty hours.

Gels were fixed overnight in a solution of 50% methanol, 7% acetic acid then stained for one hour with 0.2% Coomassie blue in 50% methanol, 7% acetic acid. Destaining was by diffusion in 7% acetic acid overnight. Gels containing radioactively labelled samples were frozen in dry ice then cut into 1 mm slices with a Mickle gel slicer. The slices were solubilised in 100 µl of 30% hydrogen peroxide at 37°C for twenty-four hours then counted in 10 mls of Aquasol.

Reagents. Neuraminidase was obtained from Behringwerke as a solution containing 500 units/ml. Dibutyryl adenosine cyclic 3':5'-monophosphate, adenosine cyclic 3':5'-monophosphate, adenosine triphosphate, theophylline, diphenylamine, DNA, RNA, orcinol, bovine serum albumin, glucose-6-phosphate, adenosine monophosphate, cytochrome C, triton X-100 and fetuin were from Sigma. Ficoll was from Pharmacia. Lipid standards were purchased from Field Instruments. Acrylamide-bis-acrylamide and cellulose chromatogram sheets were obtained from Eastman and Polygram Sil N-HR sheets for TLC from Macherey-Nagel. All other reagents were from British Drug Houses (AR grade whenever possible).

Radiochemicals. (2-^{3}H) Adenosine 5' triphosphate, Na salt, 500 mCi/mmol; L-leucine-4, 5 H^{3}, 1Ci/mmol; L-(U-^{14}C) leucine, 348 mCi/mmol; Uridin diphospho-D(U-^{14}C) galactose, ammonium salt, 294 mCi/mmol; Cytidine 5'-monophospho (^{14}C) sialic acid, ammonium salt, 250 mCi/mmol; all from the Radiochemical Centre, Amersham.

RESULTS

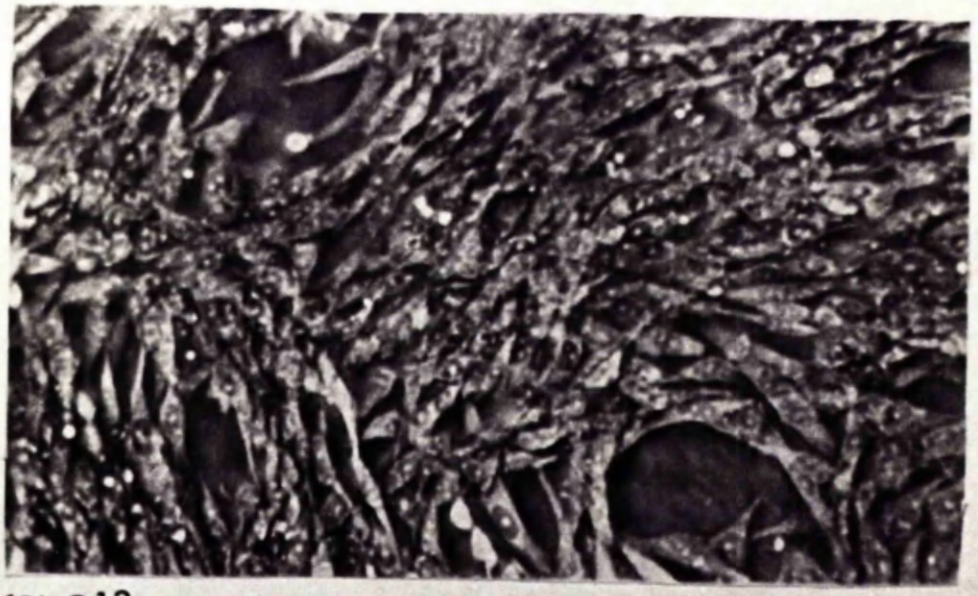
The selected variant NAC8 appears to have some of the properties of normal C13 cells and some of the properties of Py cells. Fig.1 shows that in monolayer culture C13 and NAC8 cells have a similar morphology. Both have long spindle shaped cells aligned to give the characteristic "watered silk" pattern. However unlike C13 cells the variant line forms colonies in soft agar (Table 1). The efficiency of colony formation is an order of magnitude lower than that for Py cells and the uncloned variant has a higher cloning efficiency than the subclone NAC8. The aggregation pattern of trypsinized NAC8 cells over a period of forty-five minutes closely parallels that of trypsinized Py cells (Fig.2).

Effect of Neuraminidase.

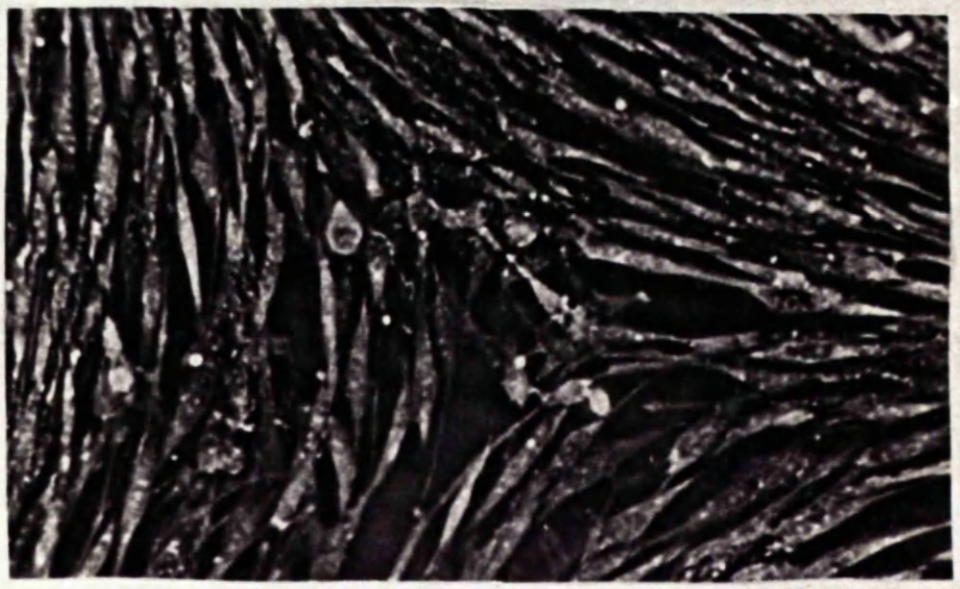
Several workers have reported that neuraminidase increases cell aggregation (Vicker and Edwards, 1972; Lloyd and Cook, 1974; Deman et al, 1974) and so this enzyme was tested to see if it could restore the aggregation of NAC8 cells. Cells were pre-aggregated for forty minutes in Hank's hepes then 1.25 units per ml of neuraminidase were added and the aggregation continued. The results of this experiment are shown in Fig.3. These results show that neuraminidase greatly enhances the aggregation of C13 cells and has little effect on Py cells. This is in complete agreement with the findings of Vicker and Edwards. NAC8 cells were stimulated only slightly.

Lloyd and Cook (1974) found that the neuraminidase induced stimulation could be reversed by certain glycoproteins and these glycoproteins also acted as acceptors for a sialyl transferase. They therefore postulated that neuraminidase increases aggregation by

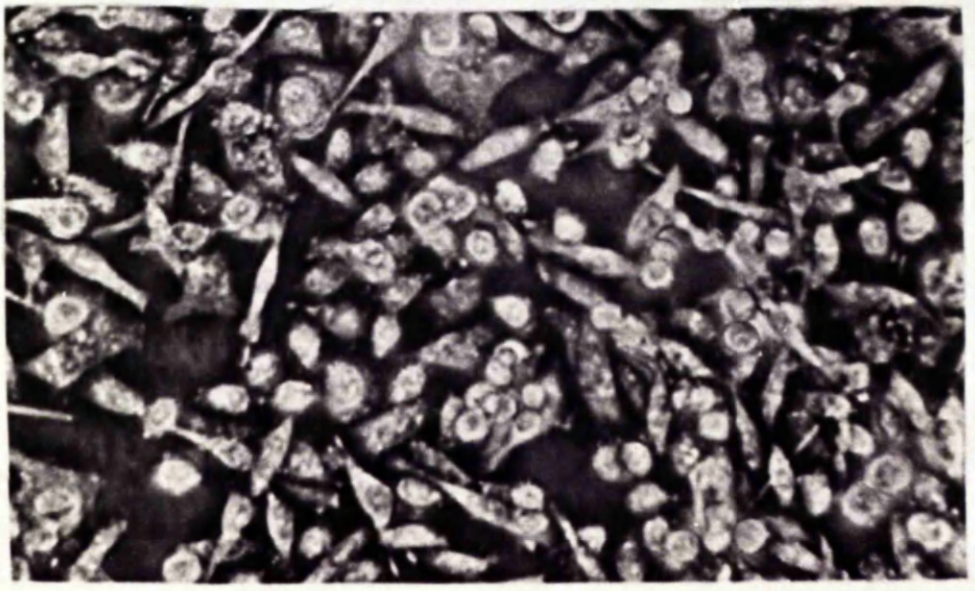
Fig 1 Magnification x 1300



(a) C13



(b) NAC8



(c) Py

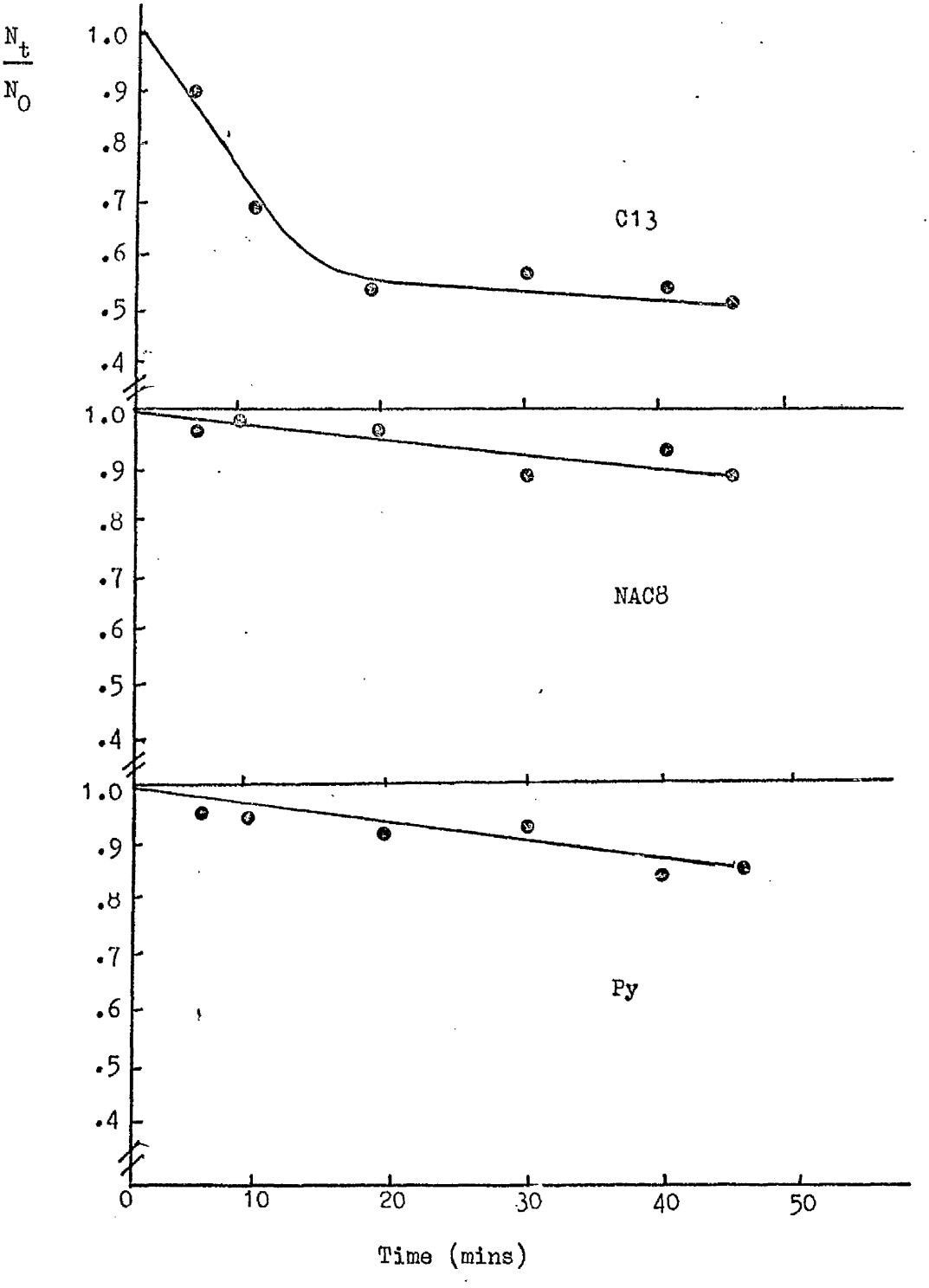
Table 1. Colony formation in soft agar.

	Number of clones		
Cell type	10^2 cells / plate	10^3 cells/plate	10^4 cells/plate
C13	0	0	0
Py	19	124	>1000
NA*	2	38	228
NAC8	0	18	170

* Before cloning

Cells were plated in triplicate at the densities indicated in the table. The values quoted are the means of the three plates.

Fig 2. Aggregation of trypsinized cells

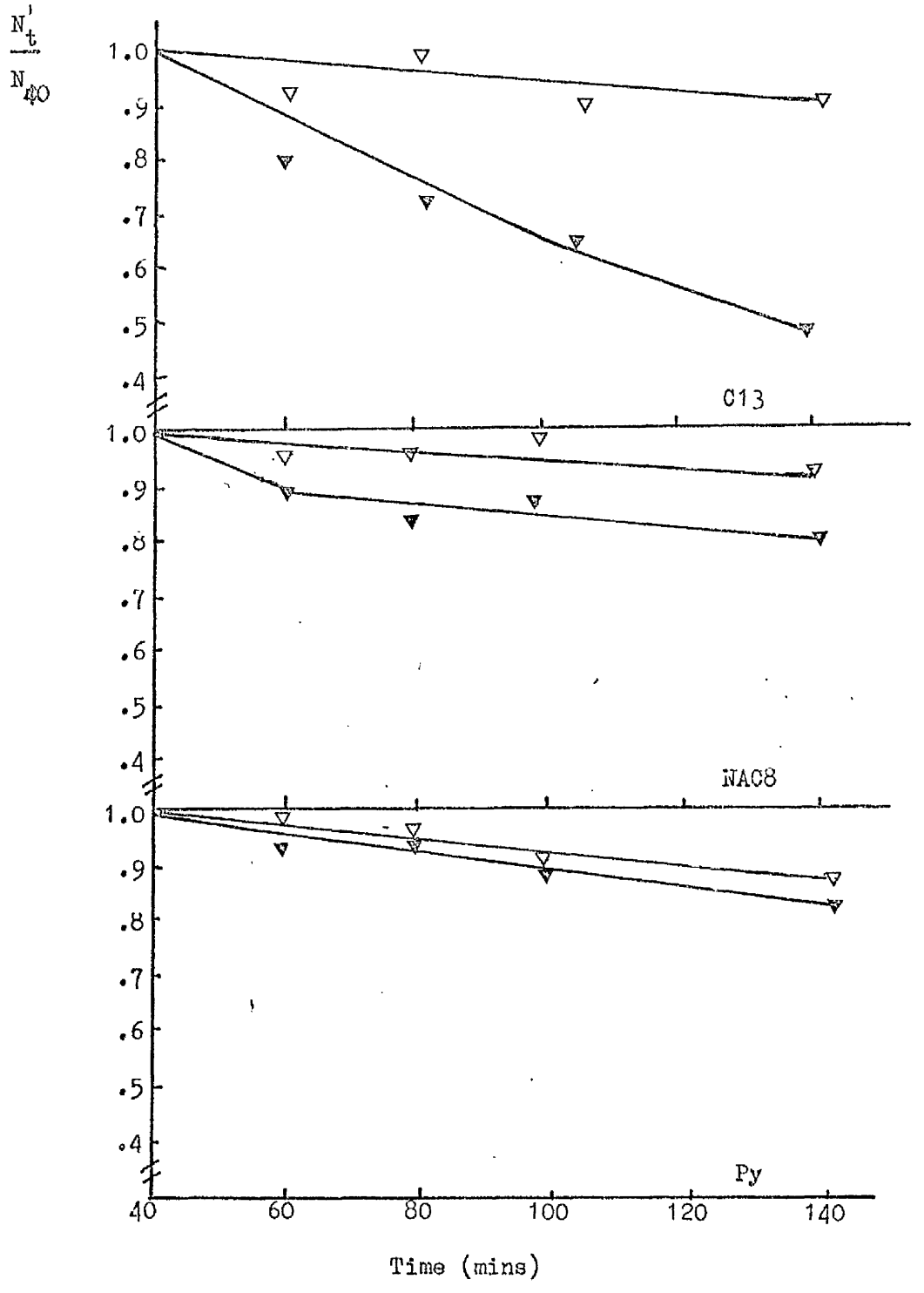


Points plotted are mean values of at least 10 determinations.

Maximal standard deviation $\pm$0.118.

generating acceptor sites for a surface transferase. Based on this theory the reason for the failure of Py and NAC8 cells to aggregate could be the lack of a specific sialyl transferase. Den et al (1971) reported that polyoma transformed BHK cells had a much lower level of a specific sialyl transferase than the normal cells. It would therefore be of interest to know if NAC8 cells had a reduced sialyl transferase activity. The sialyl transferase activity of intact cells towards both endogenous and exogenous acceptors was tested. The results are shown in Table 2. The table shows that the transformed cells have a lower activity in accordance with the results of Den et al. The decrease in activity is less than that observed by Den but this is probably due to the different acceptors used. Den used haematoside as acceptor whereas in the present work desialylized fetuin was used. She also used cell homogenates as the source of enzyme rather than intact cells. NAC8 cells had the same activity as C13 cells towards exogenous acceptors but a decreased activity towards endogenous acceptors. This result suggests that NAC8 cells have a similar amount of enzyme to C13 cells but have fewer sites available for sialylating. Since neuraminidase does not increase the aggregation of NAC8 cells to the same extent as C13 cells this would imply that it does not generate sites that are in a cryptic position on NAC8 cell surfaces.

Fig 3. Effect of neuraminidase



1.25 units/ml of neuraminidase added 40 minutes after the commencement of aggregation. Points plotted are mean values of 4 determinations. Maximal standard deviation $\pm$0.063.

▽ Control

▼ 1.25 units/ml neuraminidase

Table 2. Sialyl transferase activity of intact cells

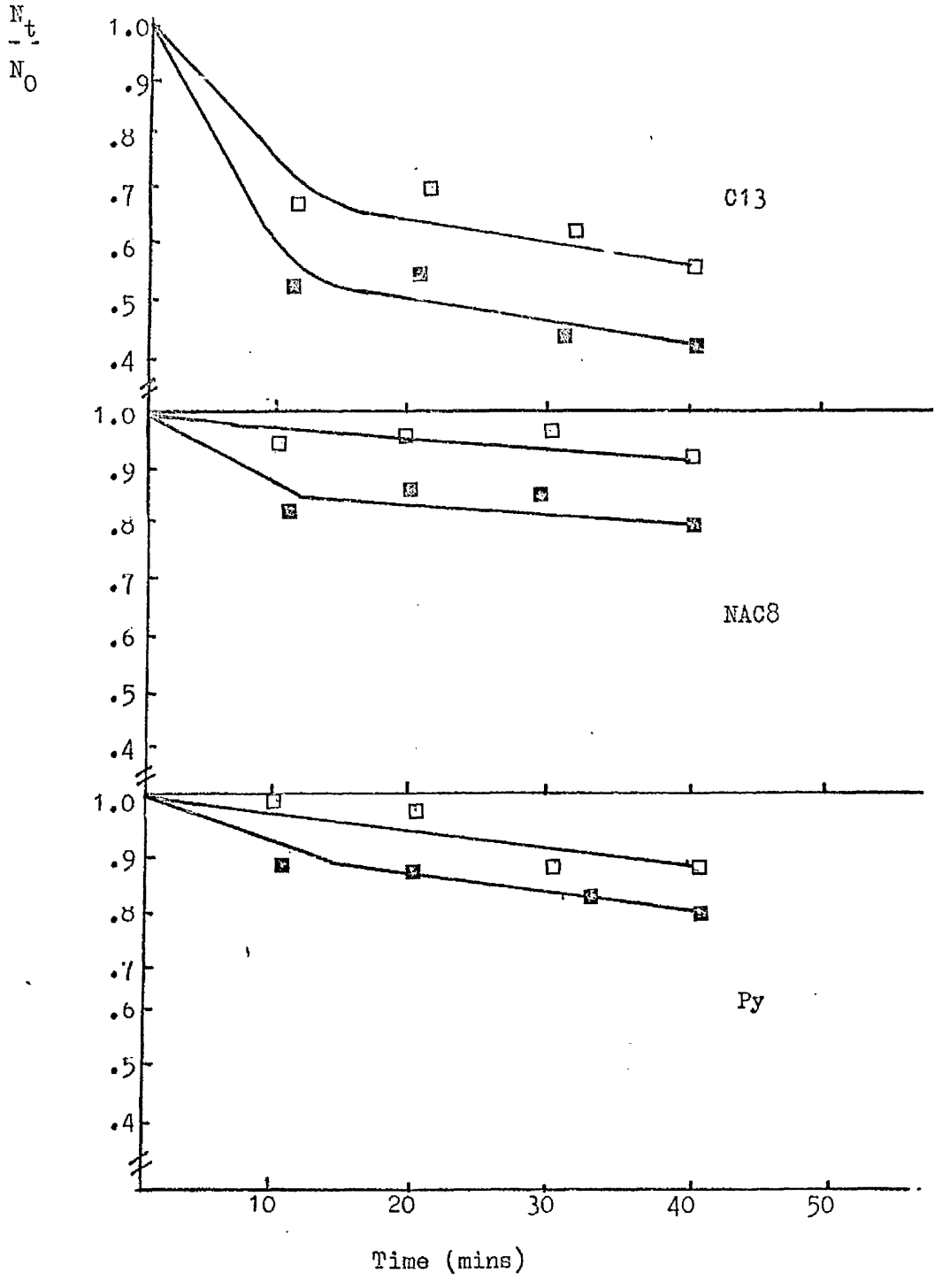
Cell type	endogenous acceptor	exogenous acceptor
C13	5339 ± 404	955 ± 86
NAC8	4055 ± 239	982 ± 71
Py	3721 ± 313	735 ± 24

5×10^5 cells were incubated in 100µl of assay medium containing ^{14}C-CMP-sialic acid for 1 hour at 37°C and the amount of sialic acid incorporated into the cell was measured. Results are means and standard deviations of 3 determinations expressed as cpm/10^6 cells/hour.

Effect of Cyclic AMP.

Low steady state levels of cyclic AMP are characteristic of transformed cells (Sheppard, 1972) and incubating CHO cells in the presence of this nucleotide has been reported to alter their morphology from compact, randomly oriented to fibroblast-like cells (Hsie and Puck, 1971). It has also been suggested that cyclic AMP can restore some of the properties of normal cells to transformed cells. Furthermore it can apparently increase the strength of adhesion of both normal and transformed cells to a substrate (Johnson and Pastan, 1972; Grinnell et al, 1973; Shields and Pollock, 1974). Since NAC8 cells have some of the characteristics of transformed cells the possibility existed that this was due to lowered levels of cyclic AMP. The cells were therefore aggregated in the presence of dibutyryl cyclic AMP and theophylline. The results of this experiment can be seen in Fig.4. The aggregation of all three cell types is slightly increased but Py and NAC8 cells are not stimulated to even control levels of C13 cells. The adenyl cyclase activity of the three cell types was also measured. The results are shown in Table 3. Py cells have a greatly reduced activity and NAC8 cells have a value intermediate to that of Py and C13 cells. These results suggest that some of the properties of NAC8 cells, for example their ability to grow in agar, may be due to a reduced level of cyclic AMP but their reduced adhesion is due to some other factor.

Fig 4. Effect of dibutyryl cyclic AMP and theophylline



Cells were aggregated in the presence of 1mM dibutyryl cyclic AMP and 1mM theophylline. Points plotted are mean values of 4 determinations. Maximal standard deviation $\pm$0.069.

□ Control

■ 1mM cyclic AMP, 1mM theophylline

Table 3. Adenyl cyclase activity of isolated membranes

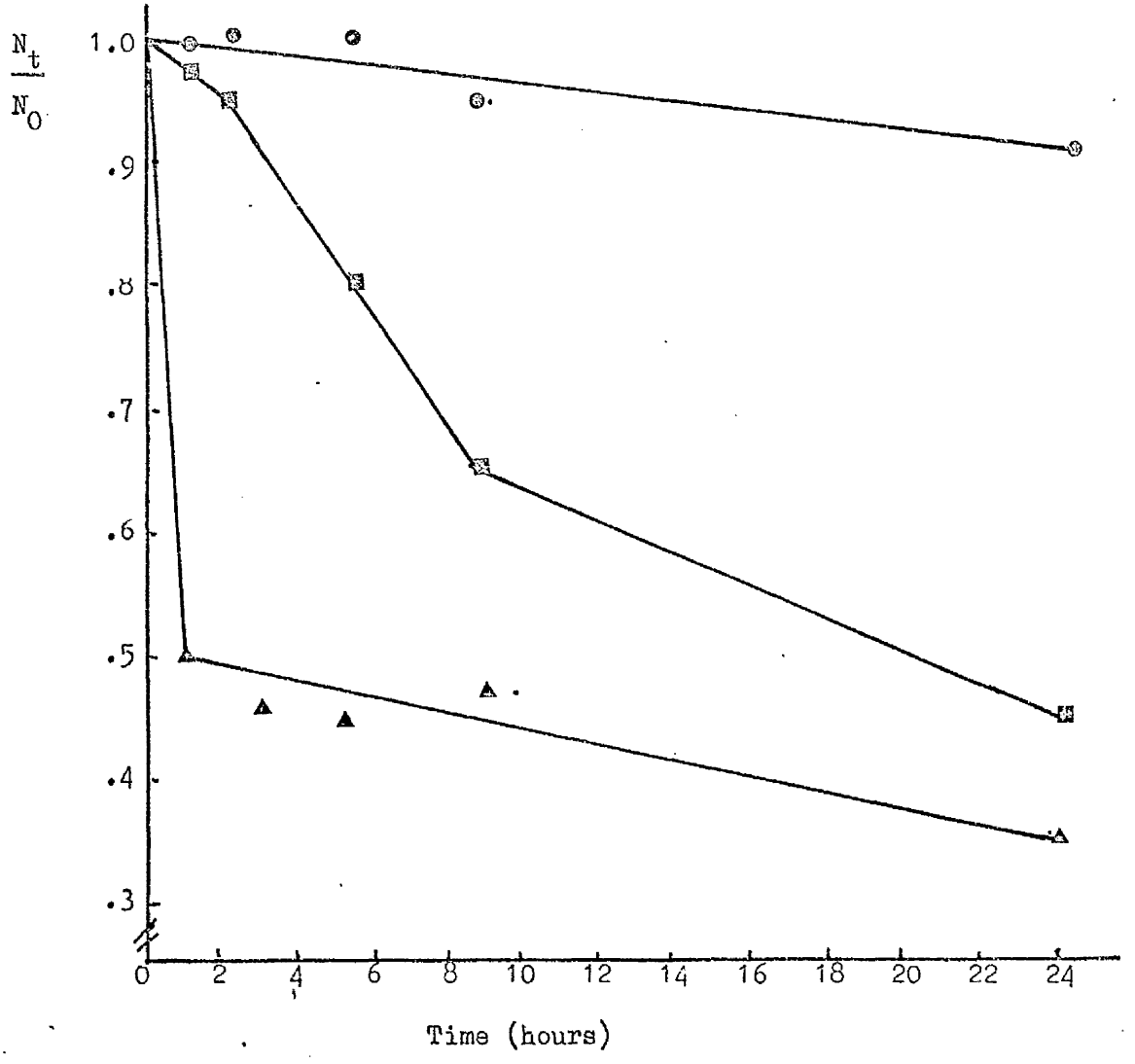
Cell type	Exp 1	Exp 2	Exp 3
C13	2900	1875	4190
NAC8	1958	1609	2980
Py	500	264	490

Membranes were incubated in the presence of ^{3}H-ATP and the reaction products separated by chromatography on cellulose sheets. Results varied quantitatively but not qualitatively from day to day. The results of three separate experiments are shown expressed as cpm/mg protein/30 minutes.

Sensitivity to Trypsin.

The reduction in adhesion of NAC8 cells could be due to an increased sensitivity to trypsin of the molecular species involved in adhesion. If this is the case then giving the cells time to recover from trypsinization or dissociating them without the use of trypsin should result in increased aggregation. Figs.5 and 6 show this to be the case. These figures further illustrate that this is not the case for Py cells. Figs. 7A, 7B and 7C show the results of experiments designed to find what concentration of trypsin completely abolishes aggregation over a forty minute period. Even at the lowest concentration used (0.01%) aggregation is reduced and is virtually abolished by concentrations above 0.025%.

Fig 5. Long term aggregation



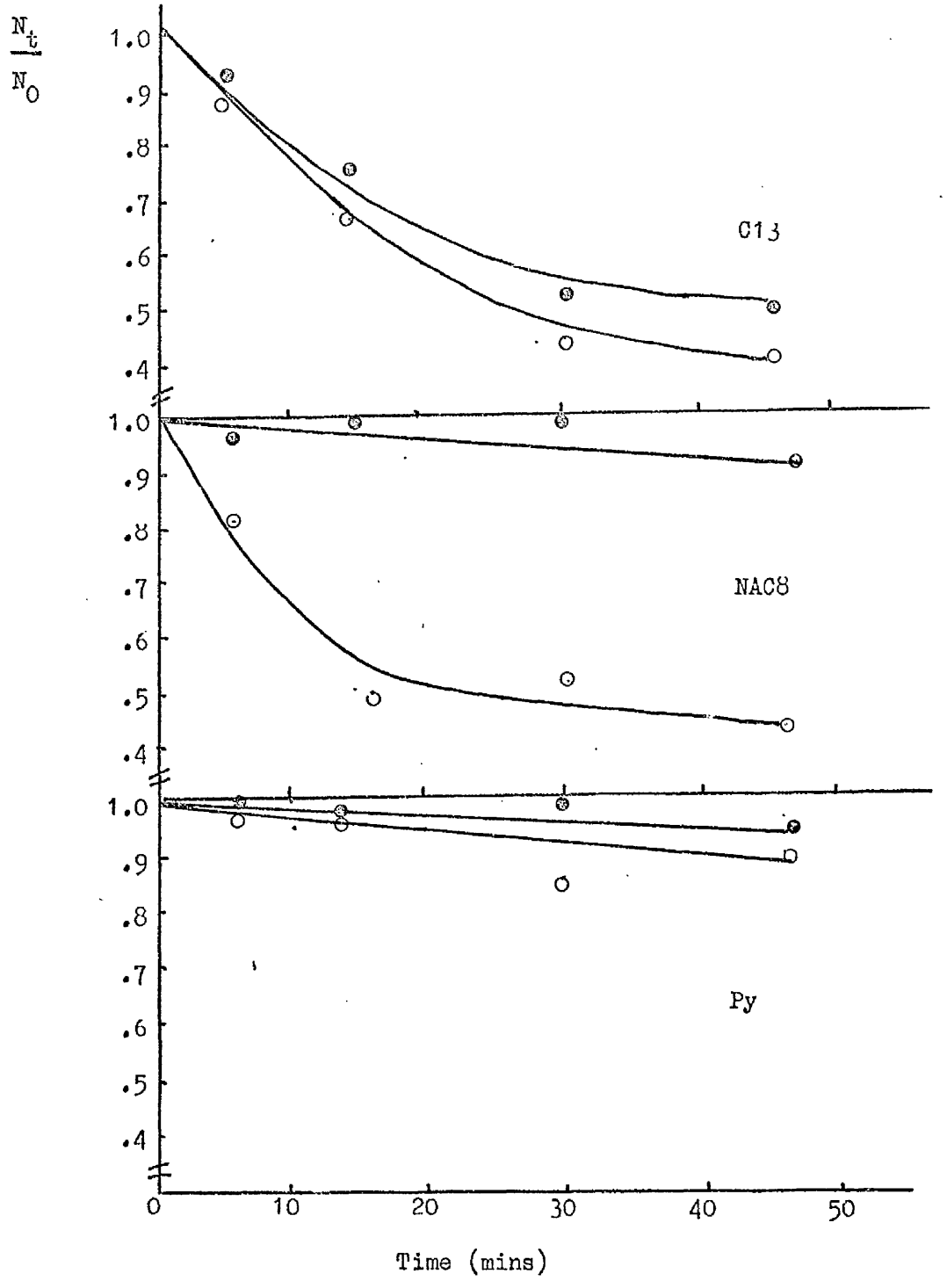
Cells were prepared as for short term aggregations except that they were resuspended to a final concentration of 0.5×10^6 cells/ml in ECT. 4mls of this suspension were aggregated in a gyratory shaker for 24 hours.

▲ C13

▣ NAC8

⊙ Py

Fig 6. Aggregation of cells dispersed with EDTA.

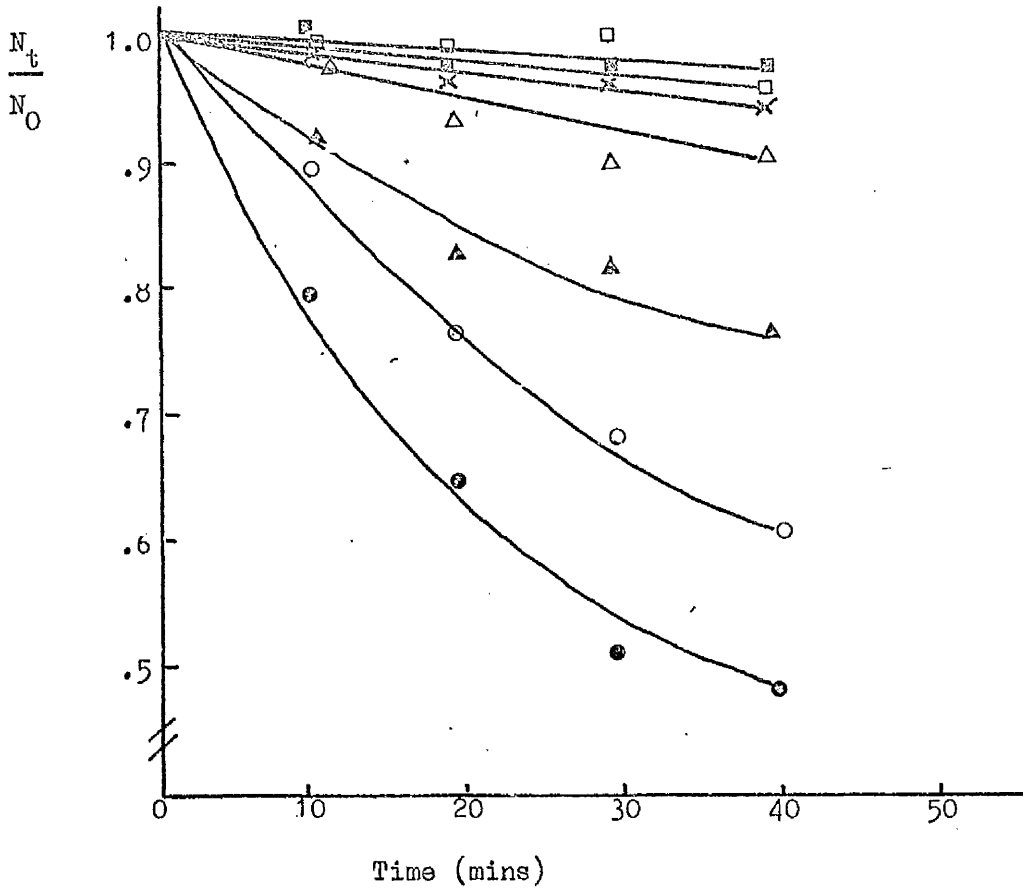


Cells were removed from glass with EDTA as described in the methods then aggregated as for trypsinized cells. Points plotted are mean values of 4 determinations. Maximal standard deviation ±0.065.

● Trypsin dispersion

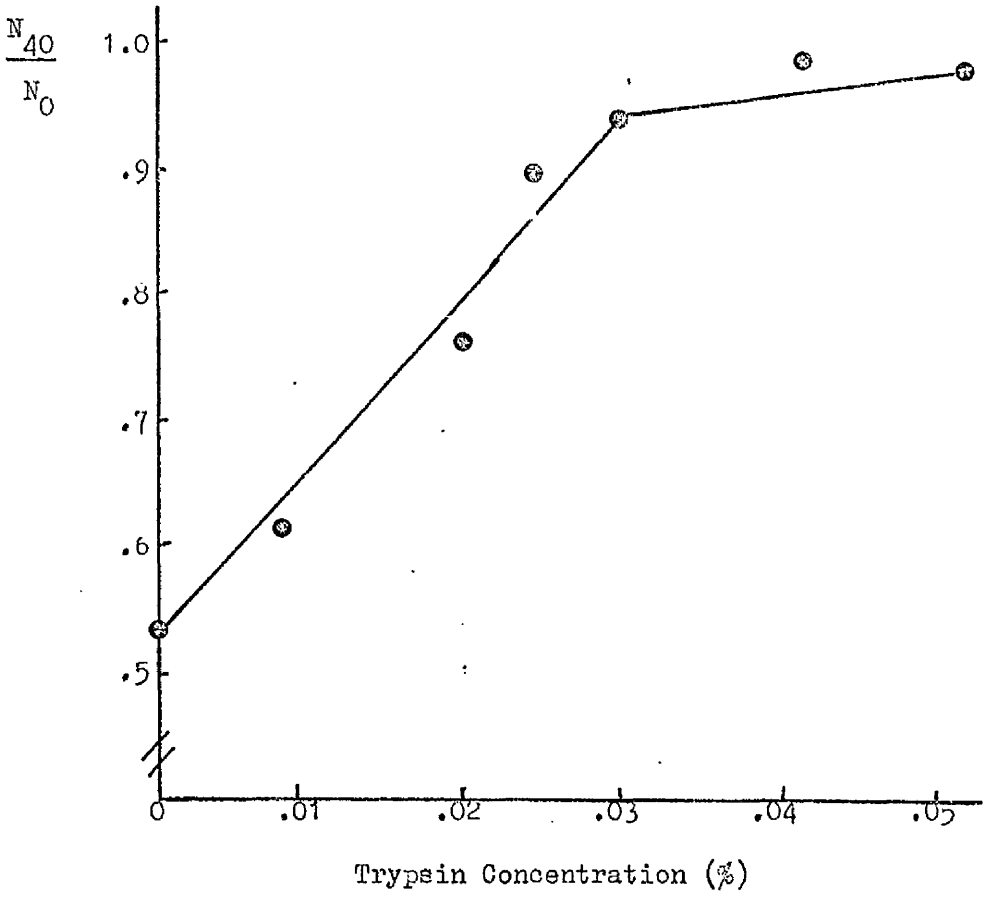
○ EDTA dispersion

Fig 7A. Aggregation of NAC8 after dispersal by increasing concentrations of trypsin.



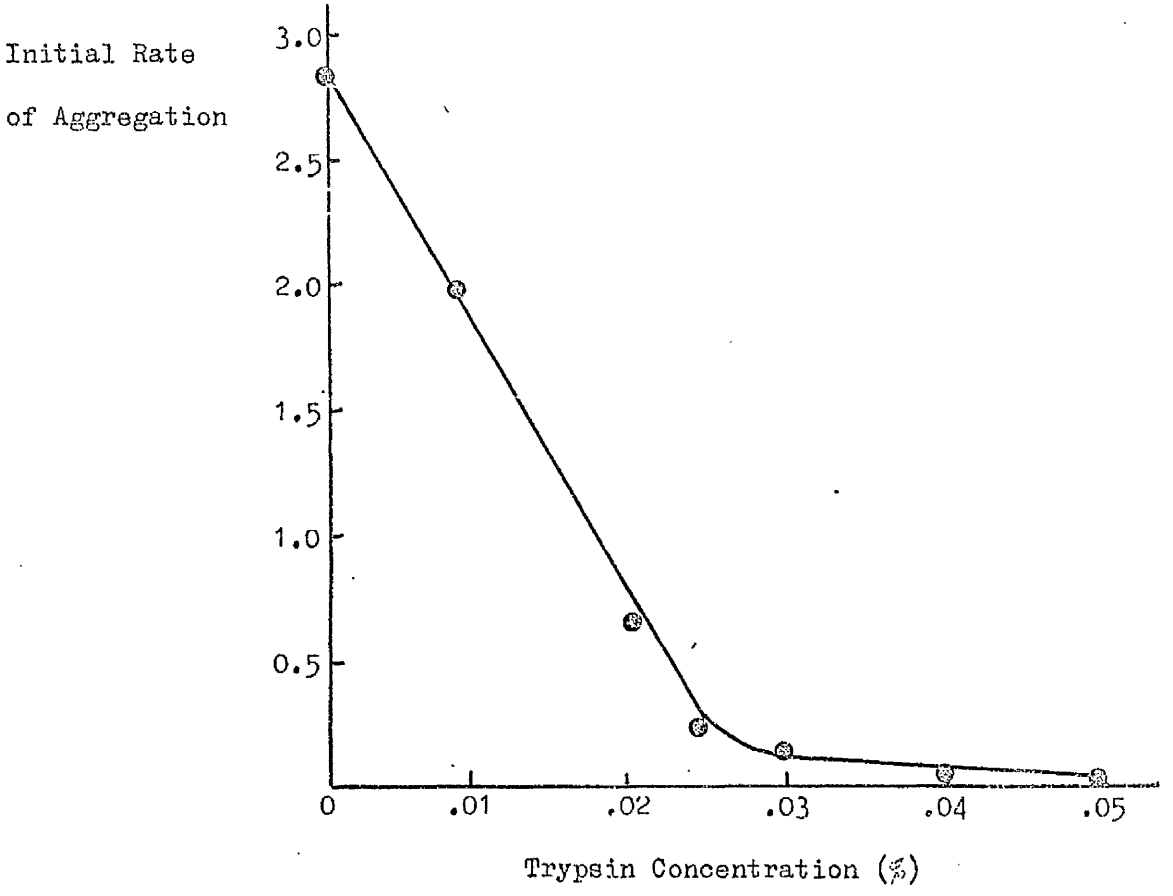
Cells were removed from glass by EDTA ●, or .01% ○, .02% ▲, .025% △, .03% ×, .04% ■, or .05% □ trypsin. The cells were incubated in the specified dispersal media for 5 minutes at 37°C then collected and washed as described in Materials and methods. All suspensions were filtered through 15μ Nytex to obtain good single cell suspensions.

Fig 7B. Effect of trypsin concentration on the extent of aggregation of NAC8 cells after 40 minutes.



Cells were prepared for aggregation as described in Fig 7A.

Fig 7C. Effect of trypsin concentration on the initial rate of aggregation of NAC8 cells.



Cells were prepared for aggregation as in Fig 7A.

The abscissa is the trypsin concentration used to prepare the cell suspensions and the ordinate is the decrease in particle count/minute X 10^4.

Galactosyl Transferase Activity.

Glycosyl transferases have been implicated in cell adhesion (Roseman, 1970) and Bosmann et al (1974) found that trypsinization of cells reduced their galactosyl transferase activity. Could a galactosyl transferase of NAC8 cells have an increased sensitivity to trypsin ? Table 4 shows the results of experiments designed to test this hypothesis. Trypsin reduces the galactosyl transferase activity of all three cell types but reduces the activity of C13 cells by a greater percent (41%) than it does NAC8 (27%) or Py (16%) cells in stationary incubations. Roth and White (1972) have suggested that transformed cells have a higher rate of cis-glycosylation than normal cells. They showed that when 3T3 cells were kept in suspension during a transferase assay they incorporated less radioactivity than when they were allowed to settle and form a loose pellet. Transformed cells did not exhibit this contact dependence. It has been suggested that this is due to the transformed cells having a more fluid membrane. Since trypsin has been reported to increase the fluidity of membranes (Guerin et al, 1974) the transferase activity of trypsinized and untrypsinized cells in suspension was tested. Unlike the results of Roth and White all three cell types showed an increase in activity over that in stationary incubations. This however appeared to be correlated with an increased rate of cell death during the assay of suspended cells as judged by trypan blue exclusion. Trypsinization did not reveal any differences between NAC8 and C13 cells. The possibility exists that all the transferase activity observed is due to a small number of lysed cells and there is no galactosyl transferase at the cell surface. For this reason the activity of isolated membranes

was tested. The results are shown in Table 4B. No difference was found between C13 and NAC8 cells in either plasma membranes or microsomes but the activity of Py cells was lower in both. This of course is not conclusive proof that there is a galactosyl transferase at the cell surface. The activity observed in the plasma membrane fraction could be due to contamination by microsomes. The purity of these membranes is discussed in the next section.

Table 4. Galactosyl transferase activity

(A) Activity of trypsinized(T) or untrypsinized(UT) intact cells with endogenous acceptor

Cell type	stationary * incubation		spinning § incubation	
	T	UT	T	UT
C13	167±97	283±106	253±113	297±143
NAC8	190±60	261±82	258±101	331±111
Py	159±95	188±49	364±264	285±73

Results are expressed as cpm/10^6 cells/2 hours.

* Means and standard deviations of 7 determinations.

§ Means and standard deviations of 4 determinations.

(B) Activity of isolated membranes with exogenous acceptor

Cell type	PM	ER
C13	324±84	1724±148
NAC8	305±165	1760±145
Py	108±63	1588±161

Isolated membranes were incubated with ^{14}C-UDP-galactose and N-acetyl-glucosamine. The reaction product was separated from free galactose on a column of Dowex-2X8. The results are expressed as cpm/mg protein/hour. Values are means and standard deviations of 4 determinations.

Isolation of Plasma Membranes.

The brief exposure to trypsin required to dissociate cells indicates that it is a component of the membrane that is being affected by the enzyme. Thus studies of the chemical composition of the plasma membranes from trypsinized and untrypsinized cells may afford some clues as to the nature of the molecular species affected. Membranes were isolated as described in "Materials and Methods". Table 5A shows the distribution of protein, DNA and RNA in the various fractions. No DNA was found associated with the plasma membrane fraction indicating that it was not contaminated with nuclei. Small amounts of RNA were found in this fraction but whether this is due to contamination or whether RNA is an integral part of the membrane is unknown. Glick and Warren (1969) have suggested that this may be the case. The plasma membrane fraction contained about 1% of the total cell protein. The purity of the membrane was further evaluated by assaying various marker enzymes. There was a five-fold increase in the specific activity of 5' nucleotidase and a twelve-fold increase in the specific activity of the ouabain-sensitive Na^+, K^+-ATPase compared with the cell homogenate. These enzymes are characteristically found in the plasma membrane (Emmelot et al, 1964; Kamat and Wallach, 1965; Coleman et al, 1967; Bingham and Burke, 1972). The specific activities of glucose-6-phosphatase (an endoplasmic reticulum marker) and succinate dehydrogenase (a mitochondrial marker) were low in the plasma membrane fraction, indicating that the plasma membranes isolated were relatively pure. The figures quoted are for C13 cells scraped off glass but the same results were obtained with NAC8 and Py cells.

Table 5. Characterization of fractions from membrane isolation

(A) Chemical composition (as % of homogenate)

	N	S	ER	PM
Protein	45.9±7.2	31.4±5.0	12.8±2.3	1.2±0.3
DNA	72.7±6.1	2.3±0.4	0.9±0.6	0
RNA	10.6±1.3	41.1±4.6	42.8±4.9	0.4±0.3

(B) Distribution of marker enzymes

	H	N	S	ER	PM
5'nucleotidase §	.29±.13	.42±.17	.21±.09	.42±.12	1.4±0.5
Na^+, K^+ATPase §	.60±.27	.46±.31	.07±.05	1.8±.9	7.6±3.2
Glucose-6-phosphatase §	.21±.09	.30±.15	.08±.05	.96±.62	.35±.18
Succinate dehydrogenase*	14.3±3.1	34.9±5.3	.9±.5	10.3±3.3	.47±.27

§ µmoles Pi/mg protein/hour

* µmoles substrate utilised/mg protein/minute

Values quoted are means and standard deviations of 5 determinations

Membrane Composition.

Table 6 gives the composition of the membranes from trypsinized and untrypsinized cells. No major differences were found between the three cell types. An interesting finding was that cells treated with trypsin appeared to lose relatively more lipid than protein but this applied to all three cell types. When the phospholipids were examined Py cells had less of their total lipid as phospholipid but no one phospholipid seemed to be responsible for this (Table 7). Again there were no major differences between C13 and NACB cells.

Table 6. Membrane composition(as % of dry weight)

(A) Cells scraped off glass

	C13	NAC8	Py
Protein	36.3±1.0	37.0±4.6	36.9±4.0
Lipid	55.1±3.9	58.7±7.3	60.2±10.8
Carbohydrate	10.5±2.1	9.8±4.6	11.8±1.1

(B) Cells trypsinized off glass

	C13	NAC8	Py
Protein	43.3±2.3	40.3±5.2	41.3±5.1
Lipid	42.7±5.4	42.6±6.1	55.4±3.7
Carbohydrate	3.6±2.8	2.5±0.3	5.4±2.1

Values quoted are means and standard deviations of 4 determinations.

Table 7. Phospholipids of the plasma membrane

(A) Amount in membranes

	C13	NAC8	Py
µg / mg protein	843±80.2	797±66	685±78
% total lipid	53.0±6.0	54.45±4.9	40.5±6.8

(B) Phospholipid classes (as % of total phospholipid)

	C13	NAC8	Py
Phosphatidyl			
-ethanolamine	18.8±2.2	17.4±3.8	20.2±3.5
-choline	36.7±3.3	37.4±4.1	43.1±4.5
-serine	7.9±0.9	8.6±1.1	6.7±1.0
-inositol	3.3±0.8	3.2±0.9	2.5±0.6
Sphyngomyelin	27.7±3.3	24.3±3.9	24.8±5.8

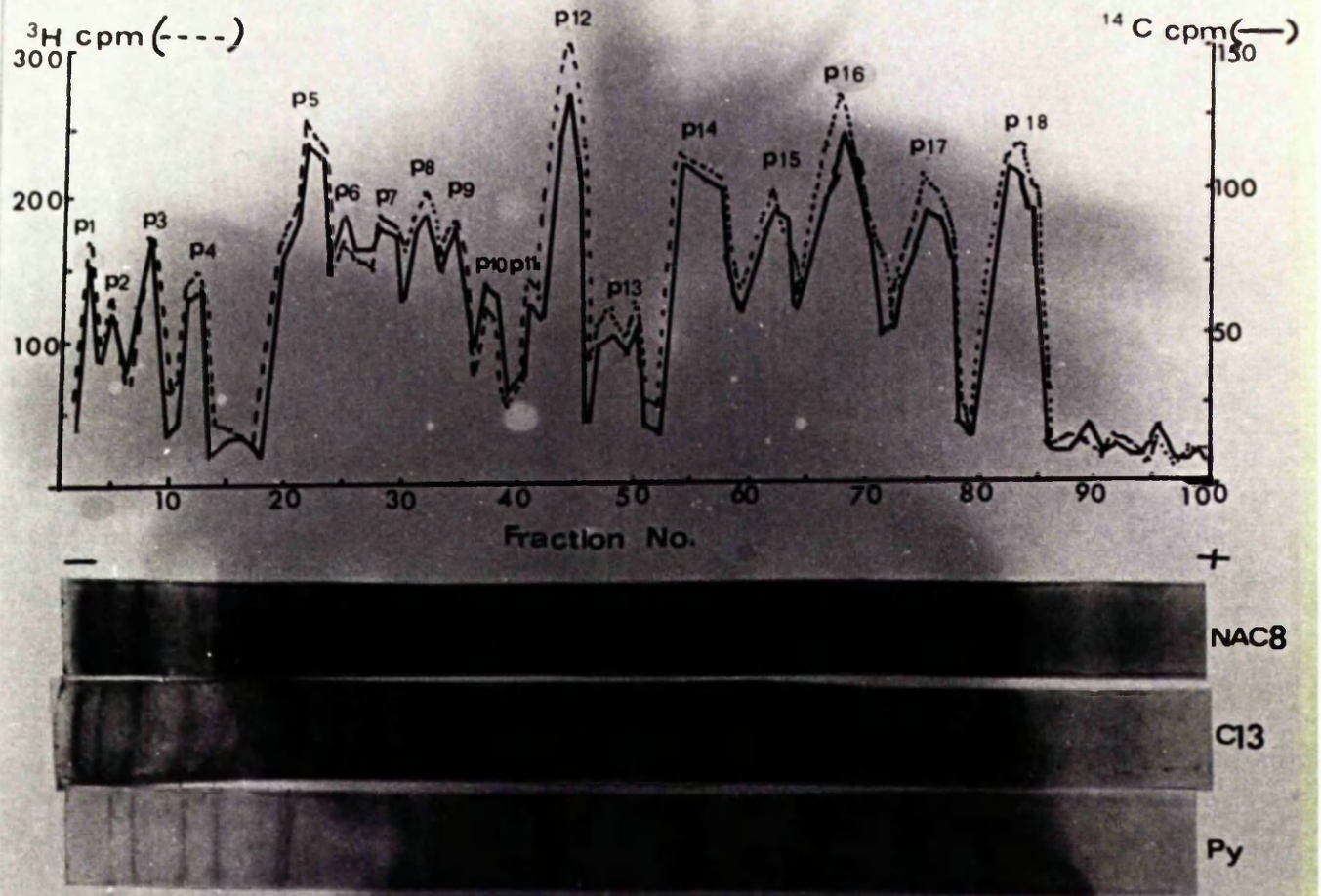
Values quoted are means and standard deviations of 4 determinations.

Gel Electrophoresis.

The proteins of the plasma membrane were analysed by SDS-polyacrylamide gel electrophoresis. Gels were either stained with Coomassie blue or the membranes were isolated from cells grown in the presence of ^{3}H-, or ^{14}C-leucine in which case the gels were cut into 1 mm slices and counted. Fig.8 shows the electrophoretic patterns obtained with proteins from plasma membranes of C13, NAC8 and Py cells. C13 and NAC8 cells appear to have identical proteins in both radioactive and stained gels. The plasma membranes of Py cells however appear to lack two proteins. The peaks labelled p1 and p15 are missing or much reduced in the radioactively labelled gels and the two equivalent bands are missing from the stained gels. When plasma membranes were isolated from cells which had been treated with trypsin the bands which were missing from the Py cells also disappeared from C13 and NAC8 cells. This electrophoretic pattern may be common to all BHK transformed variants as it was found with several polyoma transformed lines and also a Rous Sarcoma virus transformed line (RSV B4). Gels demonstrating these results are shown in Fig.9.

Molecular weight estimates were obtained for the various proteins from a standard curve established with proteins of known molecular weights. The results are shown in Table 8. The proteins that are missing from Py cells and which disappear on trypsinization of C13 and NAC8 cells have apparent molecular weights of 182,000 and 47,500. It is not known if either of these proteins are glycoproteins. Glycoproteins are known to behave differently from standard proteins in detergent gels so that the molecular weights can be no more than rough estimates.

Fig 8 7½% polyacrylamide gel electropheresis patterns of radioactive leucine labelled plasma membrane proteins. Top: C13(——) and NAC8(----). Bottom: C13(——) and Py(---)



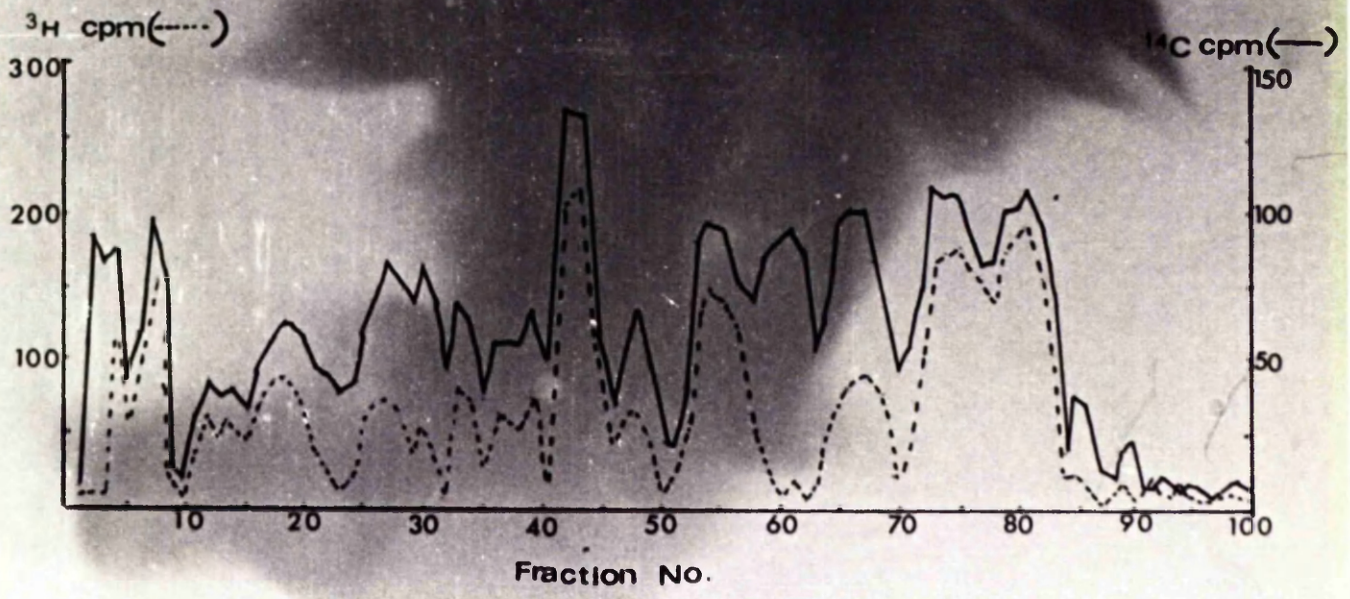
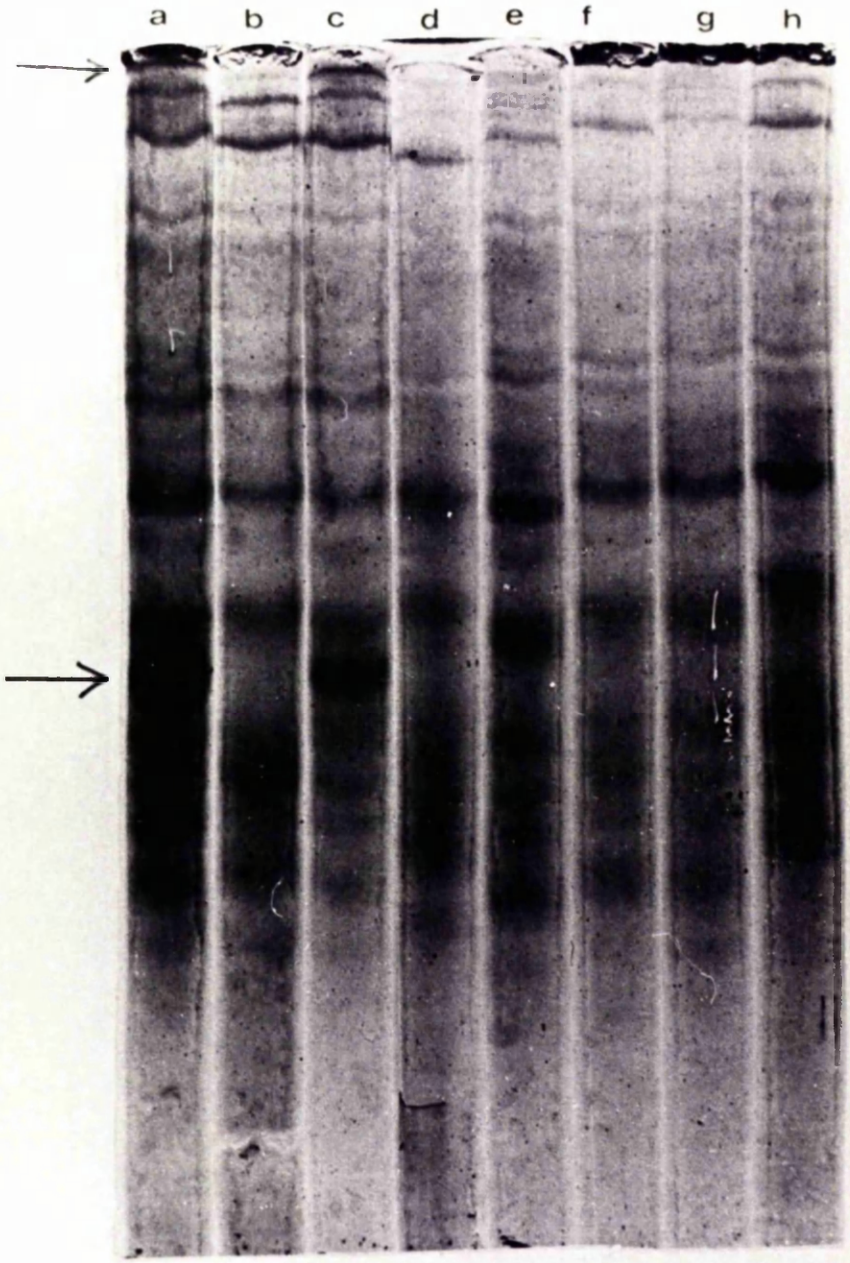


Fig 9 7½ % polyacrylamide gel electrophoresis patterns of Coomassie Blue stained plasma membrane proteins isolated from trypsinized and untrypsinized cells.



a C13 scraped off glass

b C13trypsinized off glass

c MAC8 scraped off

d MAC8 trypsinized

e Py scraped off

f Py trypsinized

g RSV B_4 scraped off

h RSV B_4 trypsinized

Table 8. Apparent molecular weights of plasma membrane proteins

P1	182,000
P2	173,800
P3	162,200
P4	148,000
P5	123,000
P6	106,000
P7	98,000
P8	92,500
P9	87,200
P10	78,200
P11	75,330
P12	67,900
P13	58,100
P14	54,100
P15	47,500
P16	40,300
P17	33,700
P18	25,900

Calculated from 7½% polyacrylamide gels using as standards: γ-globulin(160,000), β-galactosidase(130,000), Bovine serum albumin(68,000), Chymotrypsinogen(25,000), Cytochrome C(12,400). The distance travelled down the gel was a linear function of the log of the molecular weight within the range of the standards used.

DISCUSSION

The adhesive variant described arose spontaneously in cultures of BHK C13 cells. The origin of the variant is not clear. It may be the result of an unsuspected viral infection or a spontaneous mutation of a single cell. If the latter is the case the variant must either have a shorter doubling time or be able to grow to higher cell densities at confluence. Observations of the cells in culture indicate that the second possibility is the more likely. C13 cells generally stop dividing when they have reached a density of about 2×10^5 cells/cm^2 whereas NAC8 cells could achieve densities of nearer 3×10^5 cells/cm^2.

The ability of cells to grow in a semi-solid substrate has generally been considered to be a property of transformed cells and Reich (1973) has proposed that this property requires the presence of an intact fibrinolytic system. Normally with cultured cell lines only transformed cells have the protease which hydrolyses plasminogen to plasmin and these cells are usually associated with an altered morphology. NAC8 cells however have a similar morphology to C13 cells and still grow in soft agar. Whether this is due to a low level of fibrinolytic activity is unknown.

Effect of Neuraminidase

The increased aggregation of C13 cells when treated with neuraminidase can be explained in three different ways. Removal of negatively charged sialyl residues could lower the potential energy barrier thus promoting increased aggregation, or, due to mutual repulsion between the carboxyl charges, sialic acid residues may confer rigidity to the cell surface such that their removal increases the deformability of the surface as suggested by Weiss (1965). This

increased deformability could facilitate the formation of low radius of curvature probes which Bangham and Pethica (1960) have suggested could enable cells to penetrate the potential energy barrier. Neither of these two theories can account for the failure of neuraminidase to increase the adhesiveness of Py and NAC8 cells to the level of C13 cells unless the sialyl residues on Py and NAC8 cells are not accessible to neuraminidase. This seems unlikely since Forrester et al (1964) have shown that although Py cells have a higher electrophoretic mobility than C13 cells the difference can be eliminated by treatment of the cells with neuraminidase. Thirdly, neuraminidase could uncover sites directly involved in intercellular adhesion. This theory has previously been suggested by Vicker and Edwards (1972) and Lloyd and Cook (1974). Both groups propose that neuraminidase increases cellular aggregation by generating acceptor sites for a specific sialyl transferase. Roseman (1970) has postulated that glycosyl transferases can produce adhesion by an interlocking of a substrate on one cell with the enzyme on another cell. This theory would predict that both Py and NAC8 cells lacked the specific transferase. However, the results of the sialyl transferase assay do not support this view. NAC8 cells appear to have as much transferase activity as C13 cells when assayed using exogenous acceptor but less activity towards endogenous acceptor indicating that NAC8 cells have fewer acceptor sites available on their surfaces. The oligosaccharide chains of NAC8 cells may therefore be even shorter than the desialylated product of C13 cells. Hakamori and Murakami (1968) reported that virally transformed cells had lower levels of haematoside and higher levels of lactosylceramide than normal cells and a spontaneous transformant had intermediate levels. However the small decrease in endogenous activity observed (24%) would seem to be too little to account

for the complete abolition of aggregation of NAC8 cells. This could of course be simply a reflection of the fact that the endogenous assay is measuring the activity of several sialyl transferases of which only one may be involved in adhesion. Den et al (1971) have reported that glycosyl transferase activities in polyoma transformed BHK cells were generally reduced to between 36 and 87% of those of normal cells but that the activity of the sialyl transferase catalysing the formation of haematoside from lactosylceramide and CMP-sialic acid was reduced to about 15%.

Effect of Cyclic AMP

The intracellular concentration of 3':5' cyclic AMP seems to be involved in the control of many aspects of behaviour of cultured fibroblasts. It has been reported to regulate growth rate (Otten et al, 1971), saturation density (Johnson and Pastan, 1972b), morphology (Johnson et al, 1971) and motility (Johnson and Pastan, 1972c). Furthermore, several types of transformed fibroblasts have been found to have low levels of cyclic AMP when compared with untransformed cells (Otten et al, 1971; Sheppard, 1971). Several groups of workers have reported that cyclic AMP increases the adhesion of fibroblasts to a substratum. Grinnell et al (1973) and Shields and Pollock (1974) found that the nucleotide reduced the number of cells which could be detached by incubation in EDTA or EGTA. Johnson and Pastan (1972a) discovered that if cells were pre-incubated with cyclic AMP and theophylline it took much longer for the cells to detach from the plastic substrate regardless of whether trypsin or EDTA was used as the dissociating agent. Shields and Pollock (1974) also found that normally Py cells were less adhesive than C13 cells but that cyclic AMP

could increase their adhesiveness to near that of C13 cells. It might therefore be expected that cyclic AMP could increase the intercellular adhesiveness of Py and NAC8 cells and this indeed proved to be the case. However, the extent of stimulation was less for Py and NAC8 cells than for C13 cells. Pre-incubation of the cells in the presence of 1 mM cyclic AMP increased the stimulation slightly but Py and NAC8 cells still did not aggregate even to control levels of C13 cells. It is possible that cell to substrate adhesion operates through a different mechanism from cell to cell adhesion. A number of other workers have suggested that this may be the case. Coman (1961) has shown that some cell types adhere to each other but not to glass whereas other cell types show the reverse behaviour. Berwick and Coman (1962) observed that versene caused a reduction in mutual adhesion but had no effect on adhesion to glass and neuraminidase had the opposite effect. Also, Weston and Roth (1969) found that treatment of embryonic chick heart fibroblasts with 200 mM urea reduced their mutual adhesion but had no effect on their adhesion to glass. Moreover, Py cells will rapidly attach to the glass or plastic surface of culture bottles, yet after twenty-four hours in a gyratory shaker almost no cell-cell adhesions have been formed. These observations may be irrelevant however to the experiments being discussed since the two systems being compared are so different. Johnson and Pastan and Shields and Pollock were looking at the breakdown of cell contacts whereas in the present work their formation was being studied. There is no evidence that the same phenomenon is being examined. The results of Grinnell et al (1973) suggest that it is not. They found that cyclic AMP had no effect on the rate at which either C13 or Py cells attached to a substrate but it made

them more difficult to remove after attachment had occurred. These results suggest that cyclic AMP exerts its effect by strengthening existing adhesions, possibly by some secondary mechanism involving spreading with a concomitant increase in the area of contact. This theory is supported by the observations of Hsie and Puck (1971) and Johnson et al (1971) that the morphological changes induced by cyclic AMP are reversed by agents that disrupt microtubules and those of Shields and Pollock (1974) that colcemid can completely overcome the increased cell adhesion induced by cyclic AMP. Jones and Partridge (1974), however, have shown that limpet haemocytes will attach and spread to cover an increased surface area in a medium containing colchicine and Goldman and Knipe (1972) have shown that BHK cells will attach and spread although the cells appear more epithelial. However, neither of these groups measured the strength of adhesion. Another possibility is that microtubules may stabilise adhesions after they have formed by immobilising adhesion proteins in the membrane. Berlin et al (1974) have suggested that membrane proteins may be connected to an intracellular microtubular network. Cyclic AMP could have its effect by maintaining the assembly of these microtubules possibly through tubulin phosphorylation or changes in calcium fluxes. Calcium is known to dissociate microtubules (Weisenberg, 1972). This theory can be used to interpret the results in the aggregation system as follows: cells with functioning adhesion molecules on their surfaces will aggregate and their adhesions will be consolidated by the attachment of the adhesion molecules to the polymerised microtubular network, thus making them more resistant to breakdown by shear forces, and so a stimulation of adhesion will be effected. Cells that have no functioning adhesion molecules will still be unable to aggregate and so no stimulation

is observed. If these cells have a small number of active molecules a slight stimulation may occur. This would also fit with the results obtained by Waddell et al (1974). They found that the alkaloids colchicine and vinblastine inhibited the aggregation of C13 cells. Inhibition would be due to the breakdown of the microtubular network thus allowing only weak adhesions to form.

Effects of Trypsin

The finding that NAC8 cells would adhere if aggregated over several hours indicates that the cell surface has been damaged during the dissociation procedure. The trypsin treatment could be removing adhesive factors or exposing inhibiting factors or modifying some surface component involved in adhesion.

Trypsin has been shown to remove sialic acid containing glycopeptides from the cell surface (Cook et al, 1960; Winzler, 1967; Snow and Allen, 1970) and the role these molecules may play in adhesion has already been discussed. Several groups have also described a high molecular weight protein located on the cell surface which is sensitive to trypsin (Gahmberg and Hakamori, 1973; Hynes, 1973; Critchley, 1974; Ruoslahti and Vaheri, 1974). It is of interest that this protein disappears when cells are transformed by oncogenic viruses. If this protein is involved in adhesion it would explain why Py cells still do not adhere when recovery from trypsinization is complete. Alternatively the damage caused by trypsin could be non-specific involving molecules required to maintain the membrane in the correct configuration for adhesive interactions to take place or molecules involved in the transport of an intracellular component required for adhesion. Weiss (1966) found that treatment of sarcoma cells with trypsin increased their

deformability. Previously it has been argued that this should result in an increase in adhesion. There may, however, be a range of deformability over which cells can be adhesive. Outside of this range cells would be non-adhesive. Trypsin may take cells out of the adhesive range.

Trypsin has been shown to induce conformational changes in the plasma membrane. The distribution of conA binding sites is altered by proteolytic treatment (Nicolson, 1972) and Phillips (1972) has demonstrated that after trypsin treatment glycopeptide hydrolytic products are no longer in an iodinatable position although when the cells are iodinated before trypsinization the iodine label remains in the membrane after proteolytic digestion.

Finally, Mahadevan and Sauer (1974) found that trypsin inhibited the uptake of palmitate. They did not look at the uptake of any other fatty acids but if it were shown that only certain fatty acids were inhibited this could lead to gross changes in the fatty acid composition of the plasma membrane. Curtis et al (1975) have shown that fatty acid turnover is rapid in the plasma membrane and alteration in the composition can produce substantial changes in adhesion.

Recovery from trypsinization appears to be almost complete after ten hours incubation. This is in agreement with the results of Mastro et al (1974) who found that by ten hours after trypsinization the pattern of iodinated proteins had returned to that of untrypsinized cells. The reduced rate of adhesion of NAC8 cells could be due to an altered metabolic requirement not connected to repair of the cell surface. Some factor may have to be produced to stabilise adhesions. NAC8 cells

may produce this factor at a reduced rate. Many papers have presented evidence that cell adhesion is dependent on metabolism. Aggregation has been shown to be diminished at temperatures below 15°C. Edwards and Campbell (1971) found that trypsinized BHK cells would not aggregate in the cold irrespective of prior incubation at 37°C. Similar results have been reported by Steinberg (1962) and Jones and Morrison (1969), which implies that inhibition is not due to a requirement for repair synthesis. However, low temperature inhibition is not necessarily indicative of a requirement for metabolism. The plasma membrane may be frozen in the cold thus preventing the aggregation of dispersed adhesion components on the cell surface. Frye and Edidin (1970) reported that diffusion of antigenic sites in the membrane was inhibited at 15°C. Probably more indicative of a metabolic requirement was the finding of Oppenheimer et al (1969) that mouse teratoma cells required L-glutamine for aggregation, presumably for the synthesis of hexosamines. However these cells appear to be exceptions as most other cell types examined will aggregate in a basic salts solution.

The general finding is that cells which have been dispersed with proteolytic enzymes show a requirement for metabolism whereas mechanically dissociated cells do not which again emphasises that proteases probably damage the cell surface and that metabolism is required for synthesis rather than adhesion. The results of the EDTA dispersions confirm this view. NAC8 cells dissociated with EDTA will aggregate to the same extent as C13 cells. It was interesting to find that Py cells still do not aggregate. Thus it seems clear that the transformed cells are completely deficient in the molecules required for cell-cell adhesion. This is consistent with the observations that

transformed cells produce elevated levels of proteases such that their surfaces always resemble those of trypsinized normal cells (Bosmann, 1972; Unkeless et al, 1973). Treatment of tumour cells with protease inhibitors has been shown to increase their adhesiveness (Whur et al, 1973) indicating that loss of adhesion is due to degradation of the cell surface rather than an inability to synthesise adhesion molecules or, more precisely, a surface suitable for adhesive interactions to take place.

The observation that using lower concentrations of trypsin to disperse NAC8 cells results in both an increased initial rate of aggregation and a higher final equilibrium value would suggest that aggregation is dependent upon the total number of adhesive sites per cell, if we assume that high trypsin concentrations remove all of the adhesive sites while very low concentrations remove only a small number. If the total number of adhesive sites varies from cell to cell, those cells with a low number would be rendered non-adhesive by lower concentrations of trypsin than those with a high number. Higher trypsin concentrations would increase the number of cells with no adhesive sites. These cells would make up that proportion of the suspension which does not aggregate within forty minutes.

It was mentioned earlier that trypsin causes the release of glycopeptides from the cell surface and a role for surface carbohydrates and glycosyl transferases in cell adhesion has already been discussed. Trypsinization could inhibit these transferases either by removing the substrate or the enzyme itself, or by increasing the fluidity of the plasma membrane. Roth and White (1972) assayed intact 3T3 and 3T12 cells for endogenous galactosyl transferase activity and found that in the transformed cells enzymes and acceptors could interact on a single cell

whereas normal cells could only glycosylate a neighbouring cell. They suggested that this may be due to an increasing mobility of surface components in transformed cells. Cis-glycosylation could lead to a decrease in adhesion by completing the oligosaccharide chains required for intercellular interactions. The results reported here are somewhat at variance with those of Roth and White. Maintaining C13 cells in suspension did not lead to a decrease in transferase activity but in fact led to an increase. This was true for both trypsinized and untrypsinized cells and also for NAC8 and Py cells. This appeared to be correlated with a higher rate of cell death in spinning incubations. Between 14 and 20% of cells stained with trypan blue in stationary incubations as compared to between 30 and 35% in the spinning incubations. However the total loss of cells over the incubation period was difficult to quantitate due to extensive cell aggregation in both systems. These results cast doubt on the validity of the belief that the transferase activity is located on the cell surface. However control assays using the supernatant of pre-incubated cells showed that very little activity seemed to leak out of the cells. This could be due to released enzyme binding to the surface of intact cells and being centrifuged out of suspension or staining with trypan blue may not necessarily mean that cells are leaky to intracellular macromolecules. Galactosyl transferase activity was also estimated in plasma membrane preparations. These membranes appear to be reasonably free from contamination by microsomes and some galactosyl transferase activity was found in these fractions. Several other groups have identified glycosyl transferases in plasma membrane fractions (Warren et al, 1972; Bosmann, 1972; Lloyd and Cook, 1974). The fact that Py cell plasma membranes have only 30% of the

transferase activity of C13 and NAC8 cells as opposed to 90% activity in the microsomes supports the claim that the activity of the plasma membrane is not due to microsomal contamination. No evidence was found that Py membranes were freer from contamination than C13 or NAC8 membranes. If the transferases of the plasma membrane originate from fusion with Golgi vesicles it would seem likely that Py membranes initially have as much enzyme as C13 membranes. The lower activities found could be another reflection of increased proteolysis at their surfaces. In the present investigation however, galactosyl transferases do not seem to be involved in intercellular adhesion or, at least, they are not responsible for the failure of adhesion of NAC8 cells. Alternatively, they may be involved in initial adhesions and NAC8 cells lack some mechanism whereby initial adhesions are stabilised or they may only be involved in recognition or in the synthesis of the surface molecules that control cell interactions and have no direct role in adhesion.

Membrane Composition

If we assume that trypsin has its effect at the cell surface then identification of the molecular species in the membrane affected by trypsin may help to establish the cause of the aberrant adhesive behaviour of NAC8 cells. For this reason membranes were isolated from trypsinized and untrypsinized cells. Worthwhile data can only be obtained from preparations of isolated membranes free from significant contamination and known to include all true components of the membrane. It is easier to check on the first requirement than on the second. Several criteria should be used for assessing the purity of a membrane preparation. The preparation should be free of contaminants recognizable

by light and electron microscopy. It is usually a simple matter to show that the preparation is free of distinctive organelles such as mitochondria, nuclei and rough endoplasmic reticulum. However, very often the plasma membrane is obtained as vesicles which cannot be distinguished from vesicles of smooth endoplasmic reticulum or the membranes of lysosomes. Enzymic analysis of the fractions from a membrane preparation can help in assessing the relative purity of the isolated plasma membranes but the use of marker enzymes does involve complications including the possible adventitious binding of enzymes to subcellular fragments with which they are not ordinarily associated and the danger that enzymes may be inactivated during fractionation. Cation chelating agents such as EDTA are used in many of the procedures for isolating membranes. These agents can inhibit some cation dependent enzymes leading to erroneously high yields. A further problem is that a given enzyme may not be representative of the entire plasma membrane. This is especially true of tissues such as liver which consist of several cell types and have specialised membrane regions, but even with cultured cells Graham et al (1975) have shown that 5' nucleotidase activity, which has been widely held to reside in plasma membranes, can be recovered in a different fraction from the ouabain sensitive Na^+, K^+ activated ATPase which is also considered to be a plasma membrane enzyme. Perhaps the greatest difficulties in the enzyme marker approach derive from two uncertainties. Firstly, an enzyme may be associated with the plasma membrane but may also reside within other membranes. Secondly, we can never be sure that an enzyme associated with a specific organelle is invariably lacking in the plasma membrane. Thus the use of enzyme markers must be approached with caution as regards the interpretation of results. The method can

be made more useful by comparing the specific activity of the enzyme under question in the purified membrane with its specific activity in other fractions so that the percent contamination by protein can be calculated. Using this criterion it can be seen from Table 5 that the plasma membranes isolated are contaminated with 3% microsomal membranes but only 0.03% mitochondria. The chemical analysis shows that the plasma membranes contain some RNA. Glick and Warren (1969) have suggested that RNA may be a component of plasma membranes, however, since the RNA content of the plasma membranes was only 1% of that of the endoplasmic reticulum (See Table 5), it is more likely to be due to contamination by rough endoplasmic reticulum than a reflection of a true membrane component. No DNA could be detected in the plasma membrane fraction indicating that it was free from contamination by nuclei.

A further problem is illustrated in the studies reported by Glick et al (1970). They found that the sialic acid content of the L-cell varies with the rate of growth of the culture. The cells used in the present investigation were therefore always maintained under identical culture conditions and care was taken to ensure that all cultures were harvested at the same degree of confluency.

Finally it should be remembered that after all possible criteria have been employed to measure the extent of contamination there is no way to determine that a preparation contains all the components of the plasma membrane and that loosely bound constituents have not been lost during the isolation.

Within these limitations however the increased specific activity of 5' nucleotidase and the ouabain sensitive Na^+, K^+ ATPase and the appearance of the isolated membranes by phase contrast

microscopy are indications that the preparation under consideration consists mainly of plasma membranes.

The differences in the composition of the membranes from trypsinized and untrypsinized cells are shown in Table 6. The main finding is that the lipid to protein ratio falls from around a value of 1.5 to 1.0 for C13 and NAC8 cells and from 1.5 to 1.3 for Py cells. This is the opposite of what would be expected if trypsin affected only proteins. Thus the implication is that a certain amount of lipid is lost when cells are treated with trypsin. Whether the lipid is attached to protein in a lipoprotein complex or whether it is internalised by the cell due to conformational changes in the membrane induced by trypsin is unknown. That this change in the lipid to protein ratio is involved in the adhesive behaviour of the cells is unlikely however since both C13 cells and NAC8 cells are affected to the same extent. The same reasoning can be used with respect to the carbohydrate lost although it is always possible that if the membranes of C13 and NAC8 cells were in a different configuration prior to trypsinization then different domains of the membrane may be exposed to proteolytic digestion. The finding that NAC8 cells dissociated with EDTA will aggregate in a manner similar to C13 cells argues against this theory.

No significant differences were found between the different phospholipid classes of the three cell types. It should be remembered however that the method used to measure the amount of phospholipid tells us nothing about the fatty acid components. GLC may reveal significant differences between the fatty acid classes of the three cell types. Furthermore the phospholipid to cholesterol ratio was not measured and since cholesterol has been shown to influence the fluidity of membranes this might affect adhesion. Therefore an insufficient

number of experiments have been done to allow us to draw conclusions about the role of lipids in intercellular adhesion.

Isolation of Membrane Proteins

The proteins of the plasma membrane were analysed by polyacrylamide gel electrophoresis in SDS. This technique separates proteins on the basis of molecular weight utilising the sieving properties of the gel, all the proteins having been rendered equally charged by virtue of their interactions with SDS. It is always possible however that a single band contains several proteins of similar molecular weights. Also the amount of carbohydrate attached to a protein can influence its migration in polyacrylamide such that false values for molecular weights are obtained (Segrest et al, 1971).

The plasma membranes of C13 and NAC8 cells appear to be identical in both trypsinized and untrypsinized cells. In contrast to these cells, the plasma membrane of untrypsinized Py cells lacks two proteins of apparent molecular weights 182,000 and 47,500 daltons. These same two proteins are removed from C13 and NAC8 cells by mild trypsinization. Several other workers have reported a high molecular weight, trypsin sensitive protein which is present in reduced amounts or not at all in transformed cells (Hynes, 1973; Hogg, 1973; Gahmberg and Hakamori, 1973; Bussell and Robinson, 1973; Stone et al, 1974; Critchley, 1974). The molecular weights reported for this protein vary from 142,000 to 250,000 daltons. Critchley (1974) has suggested that this variation may be due in part to the lack of use of suitably high molecular weight markers. The log of the molecular weight is only a linear function of the distance travelled down the gel within a certain range of molecular weights. An alternative possibility is that the amount of carbohydrate attached to this protein may vary from cell type

to cell type. Hynes and Humphreys (1974) have shown that this protein co-migrates with a glucosamine labelled polypeptide which indicates that it is a glycoprotein. It also appears to be on the outside of the membrane since it can be labelled by lactoperoxidase catalysed iodination and also by galactose oxidase, tritiated borohydride. Graham et al (1975) found that this protein was present at a low level in isolated plasma membranes and heavily concentrated in a high density fraction which contained predominantly carbohydrate and they speculated that this fraction consisted of surface coat. However other workers have found this protein in the membrane fraction (Hogg, 1973; Stone et al, 1974; Russell and Robinson, 1973). This could be due to the different methods of homogenization used. Graham et al used nitrogen cavitation whereas the others used Potter-Elvejhem or Dounce type homogenizers. Alternatively all of these workers are looking at a different protein, but this seems unlikely since Hogg has detected it in isolated membranes by Coomassie blue staining and also in whole cells labelled by the lactoperoxidase method.

Stone et al (1974) also found that a polypeptide of molecular weight 39,000 daltons was present in decreased amounts in the isolated membranes of transformed cells. This protein has not been reported by those workers who label the outside of the membrane either by the lactoperoxidase method, which iodinates available tyrosine residues, or by the galactose oxidase method which labels available galactose residues. This strongly suggests that this protein is either buried in the membrane or is on the cytoplasmic face. Pertinent to this discussion are the findings of Ruoslahti and Vaheri (1974). They described a fibroblast-specific cell surface antigen which could be

released from the surface by mild trypsinization. This antigen was also present in homologous serum and was lost in transformed cells. They reported that the protein consisted of two polypeptides of molecular weights 210,000 and 145,000 daltons. Wartiovaara et al (1974) demonstrated that this antigen was confined to membrane processes and surface ridges. They observed that after trypsin treatment the antigen first reappeared at the periphery of rounded cells at the sites of the first new cell processes. It was first detected about ten hours after trypsinization and appeared to correlate with bundles of microfilaments seen by electron microscopy. Furthermore a polypeptide of molecular weight 45,000 daltons, that co-migrated with purified fibroblast actin in acrylamide gels, was associated with immuno-precipitates of the antigen.

Are these proteins involved in cell adhesion ? Experiments by Edwards and Campbell (1971) showed that the aggregation of C13 cells was dependent upon the density of the cultures from which the suspensions were derived; it is greatest in dense cultures and hardly evident at all in cells from sparse cultures. O'Neill (1973) found that cells rendered quiescent by serum starvation would aggregate. Induction of growth by addition of serum resulted in a rapid loss of aggregation. These results correlate well with the findings of Hynes and Bye (1974) that the high molecular weight, trypsin sensitive protein was present at a high level when cells were in G O, that is when they were either density inhibited or serum starved. The level fell when growth was stimulated and was almost indetectable in mitotic cells. NAC8 cells may fail to aggregate by virtue of the fact that they do not enter G O until they reach higher cell densities than C13 cells. This argument falls down however when we consider that cells are prepared for

aggregation by trypsinization. The concentration of trypsin used to prepare cell suspensions is sufficient to release the high molecular weight protein from the cell surface. O'Neill also showed that medium which had been conditioned by exposure to cell cultures promoted the aggregation of low density cultures. The factor in this conditioned medium could be the high molecular weight protein. As mentioned earlier Ruoslahti and Vaheri (1974) found evidence that their surface antigen was also present in homologous serum. It is therefore possible that although the protein is removed by trypsinization some of it remains bound to the cell surface and is not removed by washing the cells when they are prepared for aggregation. The more rigorous washing procedures used during membrane preparation may be sufficient to remove all traces of the protein. An alternative possibility is that this protein is not involved in the initial aggregation but acts to stabilise adhesions after early contacts have formed.

One other question that must be asked is; what is the nature of the polypeptide of molecular weight 47,500 daltons ? Several reports have appeared of actin associated with cell membrane (Wickus et al, 1975; Spudich, 1974; Pollard and Korn, 1973; Clark et al, 1975). Wickus et al also reported that the amount of actin associated with the membrane decreased by 30-50% after viral transformation. Other workers have found microfilaments which bind heavy meromyosin associated with cell contacts (Goldman, 1975; Perdue, 1973; Heaysman and Pegrum, 1973). Following trypsinization the sub-membranous bundles of microfilaments disappear but quickly re-assemble when cells make contact(Goldman, 1975). Without a more rigorous examination of the 47,500 protein it is impossible to say whether or not it is actin. It should really be isolated and fingerprinted but until these experiments are done it is

tempting to speculate that it is actin and that the coincidental loss of it with the high molecular weight protein by transformation or trypsinization and the reduced adhesion induced by these agents is more than just a casual relationship. It is difficult however to reconcile this theory with the fact that both C13 and NAC8 cells have these proteins, both lose them after treatment with trypsin and it would appear to take at least ten hours for the high molecular weight protein to reappear, if this protein is the equivalent of the antigen described by Wartiovaara et al (1974). An alternative explanation is that initial adhesions occur by some, as yet undefined, method. These could involve glycosyl transferases, Van der Waal's forces or electrostatic forces of attraction. The formation of these adhesions may depolarise the plasma membrane at the adhesion sites causing a release of calcium ions from an intracellular site of sequestration. The localised increase in calcium ion concentration could then trigger the polymerisation of actin in the immediate vicinity. As the high molecular weight protein is synthesised it would be inserted into the membrane close to bundles of microfilaments. If the protein were attached in some way to the microfilaments this would prevent its lateral movement in the membrane. The role of this protein would therefore be the stabilisation of the initial weak adhesions. The protein may be transported to the surface by a system of microtubules which are stabilised by cyclic AMP. NAC8 cells have a lower level of adenyl cyclase activity and so their microtubular system may be less well organised than that of C13 cells. Thus the slower aggregation of NAC8 cells would be due to a slower rate of replacement of the high molecular weight protein into the membrane.

A further possibility is that a certain amount of the high molecular weight protein survives trypsinization and this is responsible for the initial adhesion. The assumption then has to be made that less survives in NAC8 cells than in C13 cells. This however would explain a number of observations such as the increased aggregation of NAC8 cells when lower concentrations of trypsin are used and the initial rapid rate of aggregation followed by a slower rate exhibited by C13 cells. Alternatively the high molecular weight protein in NAC8 cells is not more sensitive to trypsin but fewer molecules of it are present on the surface. This could occur if NAC8 cells had an increased proteolytic activity at their surfaces, which could also possibly account for their ability to grow in soft agar, or if fewer cells were in the G O stage of the cell cycle. Finally, C13 cells may have a larger intracellular pool of the high molecular weight protein than NAC8 cells such that the protein removed by trypsinization could be more quickly replaced in C13 cells than in NAC8 cells. Wartiovaara et al (1974) found that their antigen was detectable within one hour of trypsinization if the cells were fixed with acetone which apparently destroyed the plasma membrane. The antigen detected at this time was possibly part of an intracellular pool. Further information is needed before a choice can be made between these various alternatives. However this adhesive variant, and others like it, should be useful tools in the analysis of the events involved in intercellular adhesion.

BIBLIOGRAPHY

Abercrombie, M. & E.J.Ambrose (1962)

The surface properties of cancer cells. Cancer Res. 22 pp. 525 - 548.

Abercrombie, M. & J.E.M.Heaysman (1954)

Social behaviour of cells in tissue culture: II Monolayering of fibroblasts. Exptl. Cell Res. 6 pp. 293 - 306.

Allen, A. & S.M.Minnikin (1975)

The binding of the mucoprotein from gastric mucus to cells in tissue culture and the inhibition of cell adhesion. J. Cell Sci. 17 pp. 617 - 631.

Allison, A.C. (1973)

Microfilaments and microtubules in cell movement. In "Locomotion of Tissue Cells", CIBA Foundation Symposium. Associated Scientific Publishers. pp. 108 - 148.

Angello, J.C. & Hauschka, S.D. (1974)

Glucosamine binding to serum proteins: its possible relevance to cell surface and conditioned medium studies. Biochim. biophys. Acta. 367 pp. 148 - 164.

Armstrong, P.B. (1966)

On the role of metal cations in cellular adhesion: effect on cell surface charge. J. Exptl. Zool. 163 pp. 99 - 109.

Armstrong, P.B. & D.F.Jones (1968)

On the role of metal cations in cellular adhesion: cation specificity. J. Exptl. Zool. 167 pp. 275 - 282.

Ashwell, G. (1957)

Colorimetric analysis of sugars.

In "Methods in Enzymology, vol.3", Eds. Colowick, S.P. & N.O.Kaplan.

Academic Press, London. pp. 73 - 105.

Ashworth, L.A.E. & C.Green (1966)

Plasma membranes: phospholipid and sterol content.

Science. 151 pp. 210 - 211.

Balsamo, J. & J.Lilien (1974a)

Functional identification of three components which mediate tissue type specific embryonic cell adhesion.

Nature. 251 pp. 522 - 524.

Balsamo, J. & J.Lilien (1974b)

Embryonic cell aggregation: kinetics and specificity of binding of enhancing factors. Proc. natn. Acad. Sci. U.S.A. 71 pp. 727 - 731.

Bangham, A.D. & B.A.Pethica (1960)

The adhesiveness of cells and the nature of chemical groups at their surfaces. Proc. Roy. Phys. Soc. Edin. 28 pp. 43 - 52.

Bar, R.S., D.W.Deamer & D.G.Cornwell (1966)

Surface area of human erythrocyte lipids: re-investigation of experiments on plasma membranes. Science. 153 pp. 1010 - 1012.

Berlin, R.D., J.M.Oliver, T.E.Ukena & H.H.Yin (1974)

Control of cell surface topography. Nature. 247 pp. 45 - 46.

Berlin, R.D. & T.E.Ukena (1972)

Effect of colchicine and vinblastine on the agglutination of polymorphonuclear leucocytes by concanavalin A. Nature New Biol. 238 pp.120 - 121.

Bernhard, W. & S.Avrameas (1971)

Ultrastructural visualisation of cellular carbohydrate components by means of concanavalin A. Exptl. Cell Res. 64 pp. 232 - 236.

Berwick, L. & D.R.Coman (1962)

Some chemical factors in cellular adhesion and stickiness. Cancer Res. 22 pp. 982 - 986.

Bingham, R.W. & D.C.Burke (1972)

Isolation of plasma membrane and endoplasmic reticulum fragments from chick embryo fibroblasts. Biochim. Biophys. Acta. 274 pp. 348 - 352.

Bodansky, O. & M.K.Schwartz (1963)

Comparative effects of L-histidine on the activities of 5' nucleotidase and alkaline phosphatase. J. Biol. Chem. 238 pp. 3420 - 3427.

Born, G.V.R. (1962)

Aggregation of blood platelets by ADP and its reversal. Nature. 194 pp. 927 - 929.

Bosmann, H.B. (1972a)

Elevated glycosidases and proteolytic enzymes in cells transformed by RNA tumour viruses. Biochim. Biophys. Acta. 264 pp. 339 - 343.

Bosmann, H.B. (1972 b)

Platelet adhesiveness and aggregation. Biochim. Biophys. Acta. 279 pp. 456 - 474.

Bosmann, H.B., K.R.Case & H.R.Morgan (1974)

Surface biochemical changes accompanying primary infection with Rous Sarcoma Virus. Exptl. Cell Res. 83 pp. 15 - 24.

Bosmann, H.B., A.Hagopian & E.H.Eylar (1968)

Cellular membranes: the isolation and characterisation of the plasma and smooth membranes of HeLa cells. Arch. Biochem. Biophys. 128 pp. 51 - 69.

Branton, D. (1969)

Membrane structure. Ann. Rev. Plant Physiol. 20 pp. 209 - 238.

Bretscher, M.S. (1971)

A major protein which spans the human erythrocyte membrane. J. Mol. Biol. 59 pp. 351 - 357.

Bretscher, M.S. (1972)

Asymmetrical lipid bilayer structure for biological membranes. Nature New Biol. 236 pp. 11 - 12.

Brightman, M.W. (1965)

The distribution within the brain of ferritin injected into cerebrospinal fluid compartments: I Ependymal distribution. J. Cell Biol. 26 pp. 99 - 123.

Burk, R.R. (1968)

Reduced adenyl cyclase activity in a polyoma virus transformed cell line. Nature. 219 pp. 1272 - 1275.

Burger, M.M. & A.R.Goldberg (1967)

Identification of a tumour specific determinant on neoplastic cell surfaces. Proc. natl. Acad. Sci. U.S.A. 57 pp. 359 - 366.

Burton, K. (1956)

A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of DNA. Biochem. J. 62 pp. 315 - 323.

Bussell, R.H. & W.S.Robinson (1973)

Membrane proteins of uninfected and Rous Sarcoma Virus transformed avian cells. J. Virol. 12 pp. 320 - 324.

Carter, S.B. (1965)

Principles of cell motility: the direction of cell movement and cancer invasion. Nature. 206 pp. 1183 - 1187.

Clarke, M., G.Schatten, D.Mazia & J.A.Spudich (1975)

Visualisation of actin fibres associated with the cell membrane in amoebae of Dictyostelium discoideum .

Proc. natl. Acad. Sci. U.S.A. 72 pp. 1758 - 1762.

Coleman, R., R.H.Michell, J.B.Finean & J.N.Hawthorne (1967)

A purified plasma membrane fraction isolated from rat liver under isotonic conditions. Biochim. Biophys. Acta. 135 pp. 573 - 579.

Collins, M. (1966)

Electrokinetic properties of dissociated chick embryo cells. J. Exptl. Zool. 163 pp. 23 - 47.

Coman, D.R. (1944)

Decreased mutual adhesiveness, a property of cells from squamous cell carcinomas. Cancer Res. 4 pp. 625 - 629.

Coman, D.R. (1961)

Adhesiveness and stickiness: Two independent properties of the cell surface. Cancer Res. 21 pp. 1436 - 1438.

Coman, D.R. & T.F.Anderson (1955)

A structural difference between the surfaces of normal and of carcinomatous epidermal cells. Cancer Res. 15 pp. 541 - 543.

Cook, G.M.W., D.H.Heard & G.V.F.Seaman (1960)

A sialomucopeptide liberated by trypsin from the human erythrocyte. Nature. 188 pp. 1011 - 1012.

Cook, G.M.W., G.V.F.Seaman & L.Weiss (1963)

Physicochemical differences between ascitic and solid forms of Sarcoma 37 cells. Cancer Res. 23 pp. 1813 - 1818.

Cox, R.P. & B.M.Gesner (1965)

Effect of simple sugars on the morphology and growth pattern of mammalian cell cultures. Proc. natl. Acad. Sci. U.S.A. 54 pp. 1571 - 1578.

Crandall, M.A. & T.D.Brock (1968)

Molecular aspects of specific cell contact. Science. 161 pp. 473 - 475.

Critchley, D.R. (1974)

Cell surface proteins of NIL 1 hamster fibroblasts labelled by a galactose oxidase tritiated borohydride method. Cell. 3 pp. 121 - 125.

Culp, L.A. & P.H.Black (1972)

Release of macromolecules from BALB/c mouse cell lines treated with chelating agents. Biochemistry. 11 pp. 2161 - 2172.

Cumar, F.A., R.O.Brady, E.H.Kolodny, V.W.McFarland & P.T.Mora (1970)
Enzymatic block in the synthesis of gangliosides in DNA virus transformed tumorigenic mouse cell lines. Proc. natl. Acad. Sci. U.S.A. 67 pp. 757 - 764.

Curtis, A.S.G. (1960)
Cell contacts: some physical considerations. Am. Naturalist. 94 pp. 37 - 56.

Curtis, A.S.G. (1962)
Cell contact and adhesion. Biol. Rev. 37 pp. 82 - 129.

Curtis, A.S.G. (1963)
The effect of pH and temperature on cell re-aggregation. Nature. 200 pp. 1235 - 1236.

Curtis, A.S.G. (1964)
The adhesion of cells to glass: a study by interference reflection microscopy. J. Cell Biol. 19 pp. 199 - 215.

Curtis, A.S.G. (1966)
Cell adhesion. Sci. Prog. Oxf. 54 pp. 61 - 86.

Curtis, A.S.G. (1967)
The Cell Surface: Its Molecular Role in Morphogenesis, Logos Press, London, Academic Press, London.

Curtis, A.S.G. (1969)
The measurement of cell adhesiveness by an absolute method. J. Embryol. Exp. Morph. 22 pp. 305 - 325.

Curtis, A.S.G. (1973)
Cell adhesion. Progress in Biophys. and Mol. Biol. 27 pp. 315 - 386.

Curtis, A.S.G., J.Campbell & F.M.Shaw (1975)
Cell surface lipids and adhesion. I. The effects of lysophosphatidyl compounds, phospholipase A_2 and aggregation-inhibiting protein. J. Cell Sci. 18 pp. 347 - 356.

Curtis, A.S.G., F.M.Shaw & V.M.C.Spires (1975)
Cell surface lipids and adhesion. II. The turnover of lipid components of the plasmalemma in relation to cell adhesion. J. Cell Sci. 18 pp. 357 - 373.

Curtis, A.S.G., C.Chandler & N.Picton (1975)
Cell surface lipids and adhesion. III. The effects on cell adhesion of changes in plasmalemmal lipids. J. Cell Sci. 18 pp. 375 - 384.

Curtis, A.S.G. & M. De Sousa (1973)
Factors influencing adhesion of lymphoid cells. Nature New Biol. 244 pp. 45 - 47.

Curtis, A.S.G. & M.F.Greaves (1965)
The inhibition of cell aggregation by a pure serum protein. J. Embryol. Exp. Morph. 13 pp. 309 - 326.

Curtis, A.S.G. & G.Van De Vyver (1971)
The control of cell adhesion in a morphogenetic system. J. Embryol. Exp. Morph. 26 pp. 295 - 312.

Dan, K. (1936)
Electrokinetic studies of marine ova. III. Physiol. Zool. 9 pp. 43 - 57.

Dan, K. (1947)
Electrokinetic studies of marine ova. V, VI & VII. Biol. Bull. 93 pp. 259 - 286.

Danielli, J.F. & H.Davson (1935)

A contribution to the theory of permeability of thin films. J. Cellular Comp. Physiol. 5 pp. 495 - 508.

Deman, J.J. & E.A.Bruyneel (1975)

Intercellular adhesiveness and neuraminidase effect following release from density inhibition of growth. Biochem. Biophys. Res. Commun. 62 pp. 895 - 900.

Deman, J.J., E.A.Bruyneel & M.M.Mareel (1974)

A study on the mechanism of intercellular adhesion. J. Cell Biol. 60 pp. 641 - 652.

Den, H., A.M.Schultz, M.Basu & S.Roseman (1971)

Glycosyl transferase activities in normal and polyoma transformed BHK cells. J. Biol. Chem. 246 pp. 2721 - 2733.

Deppert, W., H.Werchau & G.Walter (1974)

Differentiation between intracellular and cell surface glycosyl transferases: galactosyl transferase activity in intact cells and in cell homogenates. Proc. natl. Acad. Sci. U.S.A. 71 pp. 3068 - 3072.

Dubois, I.M., K.A.Gellès, J.K.Hamilton, P.A.Rebers & F.Smith (1956)

Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 pp. 350 - 356.

Dulbecco, R. (1970)

Topoinhibition and serum requirement of transformed and untransformed cells. Nature. 227 pp. 802 - 804.

Edelman, G.M., B.A.Cunningham, G.N.Reeke, J.W.Becker, M.J.Waxdal & J.L.Wang (1972)

The covalent and three dimensional structure of concanavalin A. Proc. natl. Acad. Sci. U.S.A. 69 pp. 2580 - 2584.

Ediidin, M. & A.Weiss (1972)

Antigen cap formation in cultured fibroblasts: a reflection of membrane fluidity and of cell motility. Proc. natl. Acad. Sci. U.S.A. 69 pp. 2456 - 2459.

Edwards, J.G. (1973)

Intercellular adhesion. In "New Techniques in Biophysics and Cell Biology, Vol. 1", Eds. Pain, R.H. & B.J.Smith. John Wiley. pp. 1 - 27.

Edwards, J.G. & J.A.Campbell (1971)

The aggregation of trypsinized BHK 21 cells. J. Cell Sci. 8 pp. 53 - 71.

Edwards, J.G., J.A.Campbell & J.F.Williams (1971)

Transformation by polyoma virus affects adhesion of fibroblasts. Nature New Biol. 231 pp. 147 - 148.

Emmelot, P. & C.J.Bos (1962)

Adenosine triphosphatase in the cell membrane fraction from rat liver. Biochim. Biophys. Acta. 58 pp. 374 - 375.

Emmelot, P., C.J.Bos, E.Benedetti & P.Rumke (1964)

Studies on plasma membranes: I. Chemical composition and enzyme content of plasma membranes isolated from rat liver. Biochim. Biophys. Acta. 90 pp. 126 - 145.

Engelman, D.M. (1970)

X-ray diffraction studies of phase transitions in the membrane of Mycoplasma laidlawii . J. Mol. Biol. 47 pp. 115 - 117.

Evans, W.H. (1974)

Nucleotide pyrophosphatase, a sialo glycoprotein located on the hepatocyte surface. Nature. 250 pp. 391 - 394.

Evans, P.M. & B.M.Jones (1974)

Studies on cellular adhesion-aggregation. Exptl. Cell Res. 88 pp. 56 - 62.

Farquar, M.G. & G.Palade (1963)

Junctional complexes in various epithelia. J. Cell Biol. 17 pp. 375 - 412.

Finean, J.B. (1962)

The nature and stability of the plasma membrane. Circulation. 26 pp. 1151 - 1162

Fleischer, B., S.Fleischer & H.Ozawa (1969)

Isolation and characterisation of Golgi membranes from bovine liver. J. Cell Biol. 43 pp. 59 - 79.

Fleischer, S. & G.Rouser (1965)

Lipids of subcellular particles. J. Amer. Oil Chem. Soc. 42 pp. 588 - 607.

Folch, J., M.Lees & G.Sloane Stanley (1957)

A simple method for the isolation and purification of total lipids from animal tissues. J. Biol Chem. 226 pp. 497 - 509.

Forrester, J.A., E.J.Ambrose & J.A.MacPherson (1962)

Electrophoretic investigations of a clone of hamster fibroblasts and polyoma transformed cells from the same population. Nature. 196 pp. 1068 - 1070.

Forrester, J.A., E.J.Ambrose & M.G.P.Stoker (1964)

Microelectrophoresis of normal and transformed clones of hamster kidney fibroblasts. Nature. 201 pp. 945 - 946.

Franke, W.W., B.Deumling, B.Ermen, E.D.Jarasch & H.Kleinig (1970)
Nuclear membranes from mammalian liver: isolation procedures and general characterisation. J. Cell Biol. 46 pp. 379 - 395.

Frye, L.D. & M.Edidin (1970)
The rapid intermixing of cell surface antigens after formation of mouse human heterokaryons. J. Cell Sci. 7 pp. 319 - 335.

Gahmberg, C.G. (1971)
Proteins and glycoproteins of hamster kidney fibroblast plasma membranes and endoplasmic reticulum. Ph.D. Thesis, Univ. of Helsinki.

Gahmberg, C.G. & S.Hakamori (1973)
Altered growth behaviour of malignant cells associated with changes in externally labelled glycoprotein and glycolipid. Proc. natl. Acad. Sci. U.S.A. 70 pp. 3329 - 3333.

Gahmberg, C.G. & K.Simons (1970)
Isolation of plasma membrane fragments from BHK 21 cells. Acta Pathol. Microbiol. Scand. B78 pp. 176 - 182.

Gail, M.H. & C.W.Boone (1972)
Cell-substrate adhesivity: a determinant of cell motility. Exptl. Cell. Res. 70 pp. 33 - 40.

Garber, B. (1963)
Inhibition by glucosamine of aggregation of dissociated embryonic cells. Dev. Biol. 7 pp. 630 - 641.

Garrod, D.R. & D.Gingell (1970)
A progressive change in the electrophoretic mobility of pre-aggregation cells of the slime mould Dictyostelium discoideum . J. Cell Sci. 6 pp. 277 - 284.

Gasic, G.J. & N.L.Galanti (1966)

Proteins and disulphide groups in the aggregation of dissociated cells of sea sponges. Science. 151 pp.203 - 205.

Gasic, G.J. & T.Gasic (1962)

Removal of the sialic acid from the cell coat in tumour cells and vascular endothelium and its effect on metastasis. Proc. natl. Acad. Sci. U.S.A. 48 pp. 1172 - 1177.

Gasic, G.J. & T.Gasic (1963)

Removal of PAS positive surface sugars in tumour cells by glycosidases. Proc. Soc. Exptl. Biol. Med. 114 pp. 660 - 663.

George, J.V. & K.V.Rao (1975)

The role of sulphydryl groups in cellular adhesiveness. J. Cell Physiol. 85 pp. 547 - 556.

Gershman, H. (1970)

On the measurement of cell adhesiveness. J. Exptl. Zool. 174 pp. 391 - 406.

Giudice, G. (1965)

The mechanism of aggregation of embryonic sea urchin cells: a biochemical approach. Dev. Biol. 12 pp. 233 - 247.

Glaesar, R.M., J.E.Richmond & P.W.Todd (1968)

Histotypic self organisation by trypsin dissociated and EDTA dissociated chick embryo cells. Exptl. Cell Res. 52 pp. 71 - 85.

Glick, M.C., C.Comstock & L. Warren (1970)

Membranes of animal cells: VII. Carbohydrates of surface membranes and whole cells. Biochim. Biophys. Acta. 219 pp. 290 - 300.

Glick, M.C. & L.Warren (1969)

Membranes of animal cells: III. Amino acid incorporation by isolated surface membranes. Proc. natl. Acad. Sci. U.S.A. 63 pp. 563 - 570.

Goldman, R.D.& E.A.C.Follett (1969)

The structure of the major cell processes of isolated BHK 21 fibroblasts. Exptl. Cell Res. 57 pp. 263 - 276.

Goldman, R.D. & D.M.Knipe (1972)

Functions of cytoplasmic fibres in non-muscle cell motility. Symp. Quant. Biol. 37 pp. 523 - 534.

Goldman, R.D., E.Lazarides, R.Pollack & K.Weber (1975)

The distribution of actin in non-muscle cells. Exptl. Cell Res. 90 pp. 333 - 344.

Goodenough, D.A. & J.P.Revel (1970)

A fine structural analysis of intercellular junctions in the mouse liver. J. Cell Biol. 45 pp. 272 - 290.

Gorter, E. & R.Grendel (1925)

On bimolecular layers of lipoids on the chromocytes of the blood. J. Exptl. Med. 41 pp. 439 - 443.

Gottlieb, D.I., R.Merrell & L.Glaser (1974)

Temporal changes in embryonal cell surface recognition. Proc. natl. Acad. Sci. U.S.A. 71 pp. 1800 - 1802.

Graham, J.M., R.O.Hynes, E.A.Davidson & D.F.Bainton (1975)

The location of proteins labelled by the ^{125}I-lactoperoxidase system in the NIL 8 hamster fibroblast. Cell. 4 pp. 353-365.

Green, D.E., S.Mii & P.M.Kohout (1955)

Studies on the terminal electron transport system.

J. Biol. Chem. 217 pp. 551 - 567.

Green, D.E., H.D.Tisdale, R.S.Criddle & R.M.Bock (1961)

The structural protein and mitochondrial organisation.

Biochem. Biophys. Res. Commun. 5 pp. 81 - 84.

Greenberg, C.S. & M.C.Glick (1972)

Electrophoretic study of the polypeptides from surface membranes of mammalian cells. Biochemistry. 11 pp. 3680-3685.

Grimes, W.J. (1970)

Sialic acid transferases and sialic acid levels in normal and transformed cells. Biochemistry. 9 pp. 5083 - 5092.

Grinnell, F. (1973)

Con A increases the strength of BHK Cell attachment to a substratum. J. Cell Biol. 58 pp. 602 - 607.

Grinnell, F. (1974)

Studies on the mechanism of cell attachment to a substratum with serum in the medium: further evidence supporting a requirement for two biochemically distinct processes.

Arch. Biochem. Biophys. 165 pp. 524 - 530.

Grinnell, F., M.Milam & P.A.Srere (1973)

Adhesion of baby hamster kidney cells. J. Cell Biol.

56 pp. 659 - 665.

Grinnell, F., M.Milam & P.A.Srere (1973)

Cyclic AMP does not affect the rate at which cells attach to a substratum. Nature New Biol. 241 pp. 82 - 83.

Guerin, C., A.Zachowski, B.Prigent, A.Parof, I.Dunia, M.Diawara & E.Benedetti (1974)
Correlation between the mobility of inner plasma membrane structure and agglutination by con A in two cell lines of MOPC 173 plasmacytoma cells. Proc. natl. Acad. Sci. U.S.A. 71 pp. 114 - 117.

Guidotti, G. (1972)
Membrane proteins. Ann. Rev. Biochem. 41 pp. 731 - 752.

Gunther, G.R., J.L.Wang, I.Yahara, B.A.Cunningham & G.M.Edelman (1973)
Concanavalin A derivatives with altered biological activities. Proc. natl. Acad. Sci. U.S.A. 70 pp. 1012 - 1016.

Gwynn, I. , R.B.Kemp, B.M.Jones & U.Groschel-Stewart (1974)
Ultrastructural evidence for myosin of the smooth muscle type at the surface of trypsin dissociated embryonic chick cells. J. Cell Sci. 15 pp. 279 - 289.

Hakamori, S. & W.T.Murakami (1968)
Glycolipids of hamster fibroblasts and derived malignant transformed cell lines. Proc. natl. Acad. Sci. U.S.A. 59 pp. 254 - 261.

Hakamori, S., T.Saito & P.K.Vogt (1971)
Transformation by Rous Sarcoma Virus: effects on cellular glycolipids. Virology. 44 pp. 609 - 621.

Haslam, R.J. (1972)
Inhibition of blood platelet function by cytochalasins: effects on thrombosthenin and glucose metabolism. Biochem. J. 127 pp. 34P.

Haydon, D.A. & J.M.Taylor (1963)
The stability and properties of bimolecular lipid leaflets in aqueous solutions. J. Theor. Biol. 4 pp. 281 - 296.

Hax, W.M.A., R.A.Demel, F.Spies, B.J.Vossenberg & W.A.M.Linnemans (1974)
Increased phospholipase A activity and formation of communicative contacts between Acanthamoeba castellanii cells. Exptl. Cell Res. 89 pp. 311 - 319.

Heaysman, J.E.M. & S.M.Pegrum (1973)
Early contacts between fibroblasts: an ultrastructural study. Exptl. Cell Res. 78 pp. 71 - 78.

Hogg, N.M. (1974)
A comparison of membrane proteins of normal and transformed cells by lactoperoxidase labelling. Proc. natl. Acad. Sci. U.S.A. 71 pp. 489 - 492.

Hoover, R.L. (1974)
Surface characterisation of two amoebae relative to cell adhesion. Exptl. Cell Res. 87 pp. 265 - 276.

Hsie, A.W. & T.T.Puck (1971)
Morphological transformation of Chinese hamster cells by dibutyryl adenosine cyclic 3':5'-monophosphate and testosterone. Proc. natl. Acad. Sci. U.S.A. 68 pp. 358 - 361.

Hubbard, A.L. & Z.A.Cohn (1975)
Externally disposed plasma membrane proteins: metabolic fate of iodinated polypeptides of mouse L-cells. J. Cell Biol. 64 pp. 461 - 479.

Humphreys, T. (1963)
Chemical dissolution and in vitro reconstruction of sponge cell adhesion. Dev. Biol. 8 pp. 27 - 47.

Humphreys, T. (1965)

Cell surface components participating in aggregation: evidence for a new cell particulate. Exptl. Cell Res. 40 pp. 539 - 543.

Hynes, R.O. (1973)

Alteration of cell surface proteins by viral transformation and by proteolysis. Proc. natl. Acad. Sci. U.S.A. 70 pp. 3170 - 3174.

Hynes, R.O. & J.M.Bye (1974)

Density and cell cycle dependence of cell surface proteins in hamster fibroblasts. Cell. 3 pp. 113 - 120.

Hynes, R.O. & K.C.Humphreys (1974)

Characterisation of the surface proteins of hamster fibroblasts. J. Cell Biol. 62 pp. 438 - 448.

Inbar, M. & L.Sachs (1969)

Interaction of the carbohydrate binding protein concanavalin A with normal and transformed cells. Proc. natl. Acad. Sci. U.S.A. 63 pp. 1418 - 1425.

Jamieson, G.A., C.L.Urban & A.J.Barber (1971)

Enzymatic basis for platelet-collagen adhesion as the primary step in haemostasis. Nature New Biol. 234 pp. 5 - 7.

Johnson, G.S., R.M.Friedman & I.Pastan (1971)

Restoration of several morphological characteristics of normal fibroblasts in sarcoma cells treated with adenosine-3': 5'-cyclic monophosphate and its derivatives. Proc. natl. Acad. Sci. U.S.A. 68 pp. 425 - 429.

Johnson, G.S. & I.Pastan (1972a)

Cyclic AMP increases the adhesion of fibroblasts to a substratum. Nature New Biol. 236 pp. 247 - 249.

Johnson, G.S. & I.Pastan (1972b)

Role of 3':5'-adenosine monophosphate in regulation of morphology and growth of transformed and normal fibroblasts. J. Natl. Cancer Inst. 48 pp. 1377 - 1387.

Johnson, G.S. & I.Pastan (1972c)

Regulation of cell motility by cyclic AMP. Nature. 235 pp. 54 - 56.

Jones, B.M. (1966)

A unifying hypothesis of cell adhesion. Nature. 212 pp. 362 - 365.

Jones, B.M. & G.A.Morrison (1969)

A molecular basis for indescriminate and selective cell adhesion. J. Cell Sci. 4 pp. 799 - 813.

Jones, G.E. & T.Partridge (1974)

Effects of cytochalasin B and colchicine on cell spreading and aggregation. J. Cell Sci. 16 pp. 385 - 399.

Jones, P.C.T. (1966)

A contractile protein model for cell adhesion. Nature. 212 pp. 365 - 369.

Kamat, V.B. & D.F.H.Wallach (1965)

Separation and partial purification of plasma membrane fragments from Ehrlich ascites carcinoma microsomes. Science. 148 pp. 1343 - 1345.

Kapeller, M., R.Gal-Oz, N.B.Grover & F.Doljanski (1973)
Natural shedding of carbohydrate containing macromolecules from cell surfaces. Exptl. Cell Res. 79 pp. 152 - 158.

Kaplan, D.M. & R.S.Griddle (1971)
Membrane structural proteins. Physiol. Revs. 51 pp.249-272.

Kay, M.M.B. & H.H.Fudenberg (1973)
Inhibition and reversal of platelet activation by cytochalasin B or colcemid. Nature. 244 pp. 288 - 289.

Kemp, R.B. (1968)
Effect of the removal of cell surface sialic acids on cell aggregation in vitro. Nature. 218 pp. 1255 - 1256.

Klenk, H.D. & P.W.Choppin (1969)
Lipids of plasma membranes of monkey and hamster kidney cells and of parainfluenza virions grown in these cells. Virology. 38 pp. 255 - 268.

Kojima, K. & A.Maekawa (1972)
A difference in the architecture of surface membranes between two cell types of rat ascites hepatoma. Cancer Res. 32 pp. 847 - 852.

Kuroda, Y. (1968)
Preparation of an aggregating promoting supernatant from embryonic chick liver cells. Exptl. Cell Res. 49 pp. 626 - 637.

Langley, O.K. & E.J.Ambrose (1967)
The linkage of sialic acid in the Ehrlich ascites carcinoma cell surface membrane. Biochem. J. 102 pp. 367 - 372.

Lee, K.C. (1972)

Cell electrophoresis of the cellular slime mould Dictyostelium discoideum. J. Cell Sci. 10 pp. 229 - 265.

Lenard, J. & S.J.Singer (1966)

Protein conformation in cell membrane preparations as studied by ORD and CD. Proc. natl. Acad. Sci. U.S.A. 56 pp. 1828 - 1835.

Lenaz, G., A.M.Sechi, L.Masotti & G.P.Castelli (1969)

Non ionic interaction between proteins and lipids in the mitochondrial membranes. Biochem. Biophys. Res. Commun. 34 pp. 392 - 397.

Lesseps, R.J. (1963)

Cell surface projections: their role in the aggregation of embryonic chick cells as revealed by electron microscopy. J. Exptl. Zool. 153 pp. 171 - 186.

Lilien, J.E. (1968)

Specific enhancement of cell aggregation in vitro. Dev. Biol. 17 pp. 657 - 678.

Lloyd, C.W. & G.M.W.Cook (1974)

On the mechanism of the increased aggregation by neuraminidase of 16C malignant rat dermal fibroblasts in vitro. J. Cell Sci. 15 pp. 575 - 590.

Lloyd, C.W. & R.B.Kemp (1971)

A biochemical investigation into the inhibitory effects of glucosamine and N-acetyl glucosamine on the aggregation in vitro of embryonic chick muscle cells. J. Cell Sci. 9 pp. 85 - 102.

Lowenstein, W.R. (1968)

Communication through cell junctions. Implications in growth control and differentiation. In "The Emergence of Order in Developing Systems. 27th Symp. Soc. for Dev. Biol., Dev. Biol. Suppl. 2." Ed. Locke, M. pp. 151 - 183.

Lowry, O.H., N.J.Rosebrough, A.L.Farr & R.J.Randall (1951)

Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 pp. 265 - 275.

Luduena, M.A. & N.K.Wessells (1973)

Cell locomotion, nerve elongation and microfilaments. Dev. Biol. 30 pp. 427 - 440.

McDonough, J. & J.Lilien (1975)

Inhibition of mobility of cell surface receptors by factors which mediate specific cell-cell interactions. Nature. 256 pp. 416 - 417.

McLean, J.R. & H.B.Bosmann (1975)

Cell-cell interactions: enhancement of glycosyl transferase ectoenzyme systems during Chlamydomonas gametic contact. Proc. natl. Acad. Sci. U.S.A. 72 pp. 310 - 313.

McNutt, N.S., L.A.Culp & P.H.Black (1973)

Contact inhibited revertant cell lines isolated from SV40 transformed cells. IV. Microfilament distribution and cell shape in untransformed, transformed and revertant Balb/c 3T3 cells. J.Cell Biol. 56 pp. 412 - 428.

McQuiddy, P. & J.Lilien (1971)

Sialic acid and cell aggregation. J. Cell Sci. 9 pp.823-833.

Mahadevan, S. & F.Sauer (1974)

Effect of trypsin, phospholipases and membrane impermeable reagents on the uptake of palmitic acid by isolated rat liver cells. Arch. Biochem. Biophys. 164 pp. 185 - 193.

Margoliash, E., J.R.Schenk, M.P.Margie, S.Burokas, W.R.Richter, G.M.Barlow & A.A.Moscona (1965)

Characterisation of specific cell aggregating materials from sponge cells. Biochem. Biophys. Res. Commun. 20 pp. 383 - 388.

Maslow, D.E. & L.Weiss (1972)

Cell exudation and cell adhesion. Exptl. Cell Res. 71 pp. 204 - 208.

Mastro, A.M., C.T.Beer & G.C.Mueller (1974)

Iodination of plasma membrane proteins of BHK cells in different growth states. Biochim. Biophys. Acta. 352 pp. 38 - 51.

Moores, G.R. (1970)

A study of the flocculation of phospholipids as a model of membrane interactions. Ph.D. Thesis, Univ. of Glasgow.

Moscona, A.A. (1961a)

Rotation mediated histogenic aggregation of dissociated cells.Exptl. Cell Res. 22 pp. 455 - 475.

Moscona, A.A. (1961b)

Effect of temperature on adhesion to glass and histogenic cohesion of dissociated cells. Nature. 190 pp. 408 - 409.

Moscona, A.A. (1963)

Studies on cell aggregation: demonstration of materials with selective cell binding activity. Proc. natl. Acad. Sci. U.S.A. 49 pp. 742 - 747.

Moscona, A.A. & H.Moscona (1952)

The dissociation and aggregation of cells from organ rudiments of the early chick embryo. J. Anat. 86 pp. 287 - 301.

Moscona, M.H. & A.A.Moscona (1963)

Inhibition of adhesiveness and aggregation of dissociated cells by inhibitors of protein and RNA synthesis. Science. 142 pp. 1070 - 1071.

Moscona, M.H. & A.A.Moscona (1966)

Inhibition of cell aggregation in vitro by puromycin. Exptl. Cell Res. 41 pp. 703 - 706.

Neville, D.M. & C.R.Kahn (1974)

Isolation of plasma membranes for cell surface membrane receptor studies. In "Methods in Molecular Biology, Vol.5" Eds. Laskin, A.I. & J.A.Last. Dekker, New York. pp. 58 - 85.

Nicolson, G.L. (1972)

Topography of membrane con A sites modified by proteolysis. Nature New Biol. 239 pp. 193 - 196.

Nicolson, G.L., R.Hyman & S.J.Singer (1971)

The two-dimensional topographic distribution of H-2 histocompatibility alloantigens on mouse red blood cell membranes. J. Cell Biol. 50 pp. 905 - 910.

Nicolson, G.L. & M.Lacorbiere (1973)

Cell contact dependent increase in membrane D-galactopyranosyl like residues on normal but not virus or spontaneously transformed fibroblasts. Proc. natl. Acad. Sci. U.S.A. 70 pp. 1672 - 1676.

Nicolson, G.L., S.P.Masouredis & S.J.Singer (1971)

Quantitative two-dimensional ultrastructural distribution of $Rh_o(D)$ antigenic sites on human erythrocyte membranes. Proc. natl. Acad. Sci. U.S.A. 68 pp. 1416 - 1420.

Nicolson, G.L. & S.J.Singer (1971)

Ferritin conjugated plant agglutinins as specific saccharide stains for electron microscopy: application to saccharides bound to cell membranes. Proc. natl. Acad. Sci. U.S.A. 68 pp. 942 - 945.

Nicolson, G.L. & J.L.Winkelhake (1975)

Organ specificity of blood-borne tumour metastasis determined by cell adhesion. Nature. 255 pp. 230 - 232.

O'Neill, C.H. (1973)

Growth induction by serum or polyoma virus inhibits the aggregation of trypsinized suspensions of BHK 21 tissue culture fibroblasts. Exptl. Cell Res. 81 pp. 31 - 39.

O'Neill, C.H. & E.A.C.Follett (1970)

An inverse relation between cell density and the number of microvilli in cultures of BHK 21 hamster fibroblasts. J.Cell Sci. 7 pp. 695 - 709.

Oppenheimer, S.B., M.Edidin, C.W.Orr & S.Roseman (1969)
An L-glutamine requirement for intercellular adhesion. Proc. natl. Acad. Sci. U.S.A. 63 pp. 1395 - 1402.

Orr, C.W. & S.Roseman (1969)
The purification and properties of a horse serum protein that promotes neural retina cell aggregation. J. Membrane Biol. 1 pp. 125 - 143.

Otten, J., G.S.Johnson & I.Pastan (1971)
Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. Biochem. Biophys. Res. Commun. 44 pp. 1192 - 1198.

Perdue, J.F. (1973)
The distribution, ultrastructure and chemistry of microfilaments in cultured chick embryo fibroblasts. J. Cell Biol. 58 pp.265-283.

Pessac, B. & V.Defendi (1972a)
Cell aggregation: role of acid mucopolysaccharides. Science. 175 pp. 898 - 900.

Pessac, B. & V.Defendi (1972b)
Evidence for distinct aggregation factors and receptors in cells. Nature New Biol. 238 pp. 13 -15.

Pethica, B.A. (1961)
The physical chemistry of cell adhesion. Exptl. Cell Res. Suppl. 8. pp. 123 - 140.

Phillips, D.R. (1972)
Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. Biochemistry. 11 pp. 4582 - 4588.

Pollard, T.D. & E.D.Korn (1973)
Electron microscopic identification of actin associated with isolated amoeba plasma membranes. J. Biol. Chem. 248 pp. 448 - 450.

Porter, C.W. & R.J.Bernacki (1975)
Ultrastructural evidence for ectoglycosyl transferase systems. Nature. 256 pp. 648 - 650.

Porter, K., D.Prescott & J.Frye (1973)
Changes in surface morphology of CHO cells during the cell cycle. J. Cell Biol. 57 pp. 815 - 836.

Pricer, W.E. & G.Ashwell (1971)
The binding of desialylated glycoproteins by plasma membranes of rat liver. J. Biol. Chem. 246 pp. 4825 - 4833.

Rabinovitch, M. & M.DeStefano (1974)
Cell to substrate adhesion and spreading: inhibition by cationic anesthetics. J. Cell Physiol. 85 pp. 189 - 194.

Rajaraman, R., D.E.Rounds, S.P.S.Yen & A.Rembaum (1974)
A scanning electron microscope study of cell adhesion and spreading in vitro. Exptl. Cell Res. 88 pp. 327 - 339.

Rambourg, A. & C.P.Leblond (1967)
Electron microscope observations on the carbohydrate rich cell coat present at the surface of cells in the rat. J. Cell Biol. 32 pp. 27 - 53.

Rambourg, A., M.Neutra & C.P.Leblond (1966)
Presence of a cell coat rich in carbohydrate at the surface of cells in the rat. Anat. Record. 154 pp. 41 - 71.

Reich, E. (1973)

Tumour associated fibrinolysis. Fed. Proc. 32 pp. 2174-2175.

Rizki, M.T.M. (1961)

The influence of glucosamine hydrochloride on cellular adhesiveness in Drosophila melanogaster. Exptl. Cell Res. 24 pp. 111 - 119.

Robbins, P.W. & I.MacPherson (1971)

Control of glycolipid synthesis in a cultured hamster cell line. Nature. 229 pp. 569 - 570.

Robertson, J.D. (1957)

New observations on the ultrastructure of the membranes of frog peripheral nerve fibres. J. Biochem. Biophys. Cytol. 3 pp. 1043 - 1047.

Rogers, J.C. & S.Kornfield (1971)

Hepatic uptake of proteins coupled to fetuin glycopeptide. Biochem. Biophys. Res. Commun. 45 pp. 622 - 629.

Roseman, S. (1970)

The synthesis of complex carbohydrates by multi-glycosyl transferase systems and their potential function in intercellular adhesion. Chem. Phys. Lipids. 5 pp. 270-297.

Rosen, S.D., D.L.Simpson, J.E.Rose & S.H.Barondes (1974)

Carbohydrate-binding protein from Polysphondylium pallidum implicated in intercellular adhesion. Nature. 252 pp.128-150.

Rosenberg, M.D., K.Aufderheide & J.Christianson (1969)

In vitro enhancement of cell clumping by surface region fractions. Exptl. Cell Res. 57 pp. 449 - 454.

Rosenberg, S.A. & G.Guidotti (1969)

Fractionation of the protein components of human erythrocyte membranes. J. Biol. Chem. 244 pp. 5118 - 5124.

Rossomondo, E.F. & M.Sussman (1972)

Adenyl cyclase in Dictyostelium discoideum: a possible control element of the chemotactic system. Biochem. Biophys. Res. Commun. 47 pp. 604 - 610.

Roth, S., E.J.McGuire & S.Roseman (1971)

Evidence for cell surface glycosyl transferases. J. Cell Biol. 51 pp. 536 - 547.

Roth, S. & D.White (1972)

Intercellular contact and cell surface galactosyl transferase activity. Proc. natl. Acad. Sci. U.S.A. 69 pp. 485 - 489.

Ruoslahti, E. & A.Vaheri (1974)

Novel human serum protein from fibroblast plasma membranes. Nature. 248 pp. 789 - 791.

Ruoslahti, E., A.Vaheri, P.Kuusela & E.Linder (1973)

Fibroblast surface antigen: a new serum protein. Biochim. Biophys. Acta. 322 pp. 352 - 358.

Russell, T. & I.Pastan (1973)

Plasma membrane cyclic adenosine 3':5' monophosphate phosphodiesterase of cultured cells and its modification after trypsin treatment of intact cells. J. Biol. Chem. 248 pp. 5835 - 5840.

Ryan, G.B., E.R.Unanue & M.J.Karnovsky (1974)
Inhibition of surface capping of macromolecules by local anaesthetics and tranquilisers. Nature. 250 pp. 56 - 57.

Sanger, J.W. & H.Holtzer (1972)
Cytochalasin B: effects on cell morphology, cell adhesion and mucopolysaccharide synthesis. Proc. natl. Acad. Sci. U.S.A. 69 pp. 253 - 257.

Segrest, J., R.Jackson, E.Andrews & V.Marchesi (1971)
Human erythrocyte membrane glycoprotein: a re-evaluation of the molecular weight as determined by SDS polyacrylamide gel electrophoresis. Biochem. Biophys. Res. Commun. 44 pp. 390 - 395.

Sheppard, J.R. (1972)
Difference in the cyclic AMP levels in normal and transformed cells. Nature New Biol. 236 pp. 14 - 16.

Shields, R. & K.Pollock (1974)
The adhesion of BHK and PyBHK cells to the substratum. Cell. 3 pp. 31 - 38.

Shoham, J. & L.Sachs (1972)
Differences in the binding of fluorescent con A to the surface membrane of normal and transformed cells. Proc. natl. Acad. Sci. U.S.A. 69 pp. 2479 - 2482.

Simmons, J.L., P.H.Fishman, E.Freese & R.O.Brady (1975)
Morphological alterations and ganglioside sialyl transferase activity induced by small fatty acids in HeLa cells. J. Cell Biol. 66 pp. 414 - 424.

Singer, S.J. & G.L.Nicolson (1972)

The fluid mosaic model of the structure of cell membranes. Science. 175 pp. 720 - 731.

Smart, J.E. & R.O.Hynes (1974)

Developmentally regulated cell surface alterations in Dictyostelium discoideum. Nature. 251 pp. 319 - 321.

Smith, S.B. & J.P.Revel (1972)

Mapping of concanavalin A binding sites on the surface of several cell types. Dev. Biol. 27 pp. 434 - 441.

Snow, C. & A.Allen (1970)

The release of radioactive nucleic acids and mucoproteins by trypsin and EDTA treatment of baby hamster cells in tissue culture. Biochem. J. 119 pp. 707 - 714.

Spring-Mills, E. & J.J.Elias (1975)

Cell surface differences in ducts from cancerous and non-cancerous human breasts. Science. 188 pp. 947 - 949.

Spudich, J.A. (1974)

Biochemical and structural studies of actomyosin like proteins from non-muscle cells. J. Biol. Chem. 249 pp. 6013 - 6020.

Steck, T.L. (1972)

The organisation of proteins in human erythrocyte membranes. In "Membrane Research. ICN-UCLA Symposium on Molecular Biology." Ed. Fox, F.

Academic Press, New York, London. pp. 71 - 93.

Steinberg, M.S. (1958)

On the chemical bonds between animal cells: a mechanism for type specific association. Amer. Nat. 92 pp. 65 - 81.

Steinberg, M.S. (1962)

The role of temperature in the control of aggregation of dissociated embryonic cells. Exptl. Cell Res. 28 pp. 1 - 10.

Steinberg, M.S. (1964)

The problem of adhesive selectivity in cellular interactions. In "Cellular Membranes in Development." Ed. Locke, M. Academic Press, New York. pp. 321 - 366.

Steinberg, M.S. (1970)

Does differential adhesion govern the self-assembly of tissue structure ? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. J. Exptl. Zool. 173 pp. 395 - 434.

Steinberg, M.S. & I.A.Gepner (1973)

Are con A receptor sites mediators of cell-cell adhesion ? Nature New Biol. 241 pp. 249 - 251.

Steiner, S. & J.L.Melnick (1974)

Altered fucolipid patterns in cultured human cancer cells. Nature. 251 pp. 717 - 718.

Stoker, M. & I.MacPherson (1964)

The Syrian hamster fibroblast cell line BHK21 and its derivatives. Nature, 203 pp. 1355 - 1357.

Stone, K.R., R.E.Smith & W.K.Joklik (1974)

Changes in membrane polypeptides that occur when chick embryo fibroblasts and NRK cells are transformed with avian sarcoma virus. Virology. 58 pp. 86 - 100.

Takeichi, M. (1971)

Changes in the properties of cell-substrate adhesion during cultivation of chicken fibroblasts *in vitro* in a serum free medium. Exptl. Cell Res. 68 pp. 88 - 96.

Taylor, A.C. (1961)

Attachment and spreading of cells in culture. Exptl. Cell Res. Suppl. 8. pp. 154 - 173.

Taylor, R.B., W.P.H.Duffus, M.C.Raff & S.DePetris (1971)

Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. Nature New Biol. 233 pp. 225 - 229.

Toole, B.P., G.Jackson & G.Gross (1972)

Hyaluronate in morphogenesis: inhibition of chondrogenesis *in vitro*. Proc. natl. Acad. Sci. U.S.A. 69 pp. 1384 - 1386.

Touster, O., N.N.Aronson, J.T.Dulaney & H.Hendrickson (1970)

Use of nucleotide pyrophosphatase and phosphodiesterase 1 as marker enzymes. J. Cell Biol. 47 pp. 604 - 618.

Townes, P.L. & J.Holtfreter (1955)

Directed movements and selective adhesion of embryonic amphibian cells. J. Exptl. Zool. 128 pp. 53 - 120.

Trinkaus, J.P., T.Betchaku & S.Krulikowski (1971)

Local inhibition of ruffling during contact inhibition of cell movement. Exptl. Cell Res. 64 pp. 291 - 300.

Tyler, A. (1946)

An auto-antibody concept of cell structure, growth and differentiation. Growth. 10 Symposium 6. pp. 7 - 19.

Unkeless, J.C., A.Tobia, L.Ossowski, J.P.Quigley, D.B.Rifkin & E.Reich (1973)
An enzymatic function associated with transformation of fibroblasts by oncogenic viruses. J. Exptl. Med. 137 pp. 85 - 111.

Vasiliev, J.M., I.M.Gelfand, L.V.Domnina, O.Y.Ivanova, S.G.Komm & L.V.Olsherskaja (1970)
Effect of colchicine on the locomotory behaviour of fibroblasts. J. Embryol. Exptl. Morphol. 24 pp. 625 - 640.

Vicker, M.G. & J.G.Edwards (1972)
The effect of neuraminidase on the aggregation of BHK21 cells and BHK21 cells transformed by polyoma virus. J. Cell Sci. 10 pp. 759 - 768.

Waddell, A.W., R.T.Robson & J.G.Edwards (1974)
Colchicine and vinblastine inhibit fibroblast aggregation. Nature. 248 pp. 239 - 241.

Wallach, D.F.H. & M.V. de Perez Esandi (1964)
Sialic acid and the electrophoretic mobility of three tumour cell types. Biochim. Biophys. Acta. 83 pp. 363 - 366.

Wallach, D.F.H. & P.S.Lin (1973)
A critical evaluation of plasma membrane fractionation. Biochim. Biophys. Acta. 300 pp. 211 - 254.

Wallach, D.F.H. & P.H.Zahler (1966)
Protein conformations in cellular membranes. Proc. natl. Acad. Sci. U.S.A. 56 pp. 1552 - 1559.

Walther, B.T., R.Ohman & S.Roseman (1973)
A quantitative assay for intercellular adhesion. Proc. natl. Acad. Sci. U.S.A. 70 pp. 1569 - 1573.

Ward, P.D. & E.J.Ambrose (1969)
Electrophoretic and chemical characterisation of the charged groups at the surface of murine CL3 ascites leukaemic cells. J. Cell Sci. 4 pp. 289 - 298.

Warren, L., D.Critchley & I.MacPherson (1972)
Surface glycoproteins and glycolipids of chicken embryo cells transformed by a temperature sensitive mutant of Rous Sarcoma virus. Nature. 235 pp. 275 - 278.

Warren, L., J.P.Fuhrer & C.A.Buck (1972)
Surface glycoproteins of normal and transformed cells: a difference determined by sialic acid and a growth dependent sialyl transferase. Proc. natl. Acad. Sci. U.S.A. 69 pp. 1838 - 1842.

Warren, L. & M.C.Glick (1968)
The metabolism and turnover of the surface membrane. J. Cell Biol. 37 pp. 729 - 746.

Warren, L. & M.C.Glick (1971)
The isolation of the surface membranes of animal cells: a survey. Biomembranes. 1 pp. 257 - 288.

Wartiovaara, J., E.Linder, E.Ruoslahti & A.Vaheri (1974)
Distribution of fibroblast surface antigen: association with fibrillar structures of normal cells and loss upon viral transformation. J. Exptl. Med. 140 pp. 1522 - 1533.

Weinstein, D.B., J.B.Marsh, M.C.Glick & L.Warren (1969)
Membranes of animal cells. IV. Lipids of the L-cell and its surface membrane. J. Biol. Chem. 244 pp. 4103 - 4111.

Weisenberg, R.C. (1972)

Changes in the organisation of tubulin during meiosis in the eggs of the surf clam Spisula solidissima. J. Cell Biol. 54 pp. 266 - 278.

Weiss, L. (1961)

The measurement of cell adhesion. Exptl. Cell Res. Suppl. 8. pp. 141 - 153.

Weiss, L. (1965)

Studies on cell deformability. I. Effect of surface charge. J. Cell Biol. 26 pp. 735 - 739.

Weiss, L. (1966)

Studies on cell deformability. II. Effects of some proteolytic enzymes. J. Cell Biol. 30 pp. 39 - 43.

Weiss, L. & M.K.Chang (1973)

Some effects of actinomycin D, cycloheximide and puromycin on cell adhesion. J. Cell Sci. 12 pp. 655 - 664.

Weiss, L. & D.E.Maslow (1972)

Some effects of trypsin dissociation on the inhibition of reaggregation among embryonic chicken neural retina cells by cycloheximide. Dev. Biol. 29 pp. 482 - 485.

Weiss, P. (1947)

The problem of specificity in growth and development. Yale J. Biol. Med. 19 pp. 235 - 278.

Wessells, N.K., B.S.Spooner, J.F.Ash, M.O.Bradley, M.A.Luduena, E.L.Taylor, J.T.Wrenn & K.M.Yamada (1971)

Microfilaments in cellular and developmental processes. Science. 171 pp. 135 - 143.

Westerman, M.P., L.E.Pierce & W.N.Jensen (1961)
A direct method for the quantitative measurement of red cell dimensions. J. Lab. Clin. Med. 57 pp. 819 - 824.

Weston, J.A. & S.A.Roth (1969)
Contact inhibition: behavioural manifestations of cellular adhesive properties in vitro. In "Cellular Recognition." Eds. Smith, R.T. & R.A.Good.
Appleton-Century-Crofts, New York. pp. 29 - 34.

White, J.G. (1971)
Platelet microtubules and microfilaments: effects of cytochalasin B on structure and function. In "Platelet Aggregation." Ed. Caen, J.
Masson et Cie, Paris. pp. 15 - 52.

Whur, P., R.T.Robson & N.E.Payne (1973)
Effect of a protease inhibitor on the adhesion of Ehrlich ascites cells to host cells in vivo.
Brit. J. Cancer. 28 pp. 417 - 428.

Wickus, G., E.Gruenstein, P.W.Robbins & A.Rich (1975)
Decrease in membrane associated actin of fibroblasts after transformation by Rous Sarcoma virus. Proc. natl. Acad. Sci. U.S.A. 72 pp. 746 - 749.

Willingham, M.C., R.E.Ostlund & I.Pastan (1974)
Myosin is a component of the cell surface of cultured cells. Proc. natl. Acad. Sci. U.S.A. 71 pp. 4144 - 4148.

Winzler, R.J. (1970)
Carbohydrates in cell surfaces. Int. Rev. Cytol. 29 pp. 77 - 125.

Winzler, R.J., E.D.Harris, D.J.Pekas, C.A.Johnson & P.Weber (1967)
Studies on glycopeptides released by trypsin from intact human erythrocytes. Biochemistry. 6 pp. 2195 - 2207.

Woodruff, J. & B.M.Gesner (1968)
Lymphocytes: circulation altered by trypsin. Science. 161 pp. 176 - 178.

Zigmond, S.H. & J.G.Hirsch (1972)
Cytochalasin B: inhibition of D-2-deoxyglucose transport into leucocytes and fibroblasts. Science. 176 pp. 1432 - 1434.