Selective and Non-Selective Adhesion of Neural, Glial and Fibroblastic Cells

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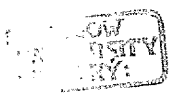

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SUMMARY

A study was made of the selective and non-selective adhesive properties of neural, glial and fibroblastic cells. Cell adhesion was measured by the attachment of radioactively labelled cells onto preformed cell sheets.

Embryonic chick skin, heart, limb and meningeal fibroblasts showed no specific adhesion with each other, but appeared to differ quantitatively in adhesiveness. Distinct morphological differences between cultures of the different fibroblastic cell types were noted.

The adhesive properties of several normal and transformed fibroblastic cell lines were examined, and a relationship was found between adhesiveness and cell morphology. Transformed fibroblasts forming cultures with a criss-crossed appearance were highly adhesive, whereas transformed fibroblasts deficient in lamellar cytoplasm showed a marked reduction in adhesiveness. Dibutyryl cyclic AMP treatment of CHO cell sheets partially restored the lamellar cytoplasm of these cells and also increased their adhesiveness by 2.0-2.5 times. An epithelial cell line, also lacking lamellar cytoplasm, was found to form very non-adhesive cell sheets. Evidence for the relationship of adhesiveness to lamellar cytoplasm and for the presence of two different types of transformation alteration in these cell types is discussed.

Five normal and transformed cell lines could be arranged in a hierarchy of adhesiveness, in each case showing quantitative adhesive interactions when different

combinations of the cell types were tested.

In contrast to this, HEF (Hamster Embryo Fibroblast) cells showed a two-fold specificity of adhesion with neural retina cells. No specificity was found between neural retina cells and Cl3 fibroblasts or skin fibroblasts, and two possibilities are discussed for the apparent restriction of the capacity to show specific adhesion with neural cells to certain fibroblasts.

Extensive studies were made to determine the most suitable conditions for the culturing of embryonic chick glial cells, and for the preparation of single cell suspensions of these cells. Glial cells obtained from neural retina, cerebrum and optic lobe had very similar morphological and growth properties. Evidence was provided by time lapse filming and by alteration of the culture conditions for the presence of small numbers of macrophage-like cells in these cultures. Neural cells spread well on glial cells and appeared to show a higher adhesive affinity for glial cell outgrowths than for other neural cells.

Preliminary experiments showed that glial cells attached poorly to glial cell sheets, and that neural cells appeared to attach approximately two-fold better to glial cell sheets than did embryonic chick fibroblasts. These results are discussed in relation to the possible roles of adhesive interactions between neural, glial and fibroblastic cells in the organogenesis of the nervous system.

Neural, glial and fibroblastic cells showed similar requirements for attachment to serum-coated plastic, with the exception of cerebrum cells. The latter attached very

poorly to plastic, but showed no reduction in the ability to rapidly aggregate. An approximate correlation was found between the rates of attachment of these cell types and the extents to which they spread on the substrate.

Hemagglutinating activity was found in homogenates of neural retina, cerebrum and optic lobe tissues and in cultures of glial cells. Similar yields of activity were obtained from each tissue or cell type, and the optimal activity appeared to be in the crude membrane fraction. Tentative evidence was obtained for the inhibition of the cerebrum and optic lobe hemagglutinating activities by specific sugars. The possible role of hemagglutinins in cell adhesion is discussed.

INTRODUCTION

The early stages of morphogenesis of multicellular animals are brought about by a highly ordered sequence of cellular movements, both of individual cells and of cell masses. Much interest has been shown in the possibility that cellular adhesion, whether specific (Tyler, 1946; Weiss, 1947; Moscona, 1962; Roth and Weston, 1967) or non-specific (Curtis, 1960; Steinberg, 1963), could play a role in the regulation of these movements. Adhesive interactions between cells are also believed to be important in homeostatic processes, for example in the positioning of lymphocytes (Curtis and De Sousa, 1973; De Bono, 1976), as well as in the aberrant behaviour of malignant cells (Coman, 1944; Nicholson and Winkelhake, 1975).

1. Cell Adhesion in Morphogenetic Movements

Several studies, made using both intact embryos and disaggregated cells, have pointed to the possible involvement of adhesive interactions between cells in cell positioning (for a review see Marchase et al, 1976). The justification for using disaggregated cells in such studies has been provided by the finding that reaggregates of isolated cells appear to contain at least some of the information required for the assembly, or reconstitution,of tissues. Wilson (1907) showed that cellular aggregates of the sponge Microciona prolifera, prepared by passing whole sponges through fine silk cloth and allowing the dispersed cells to reaggregate on the bottom of a dish, were able to reconstitute whole sponges having a normal morphology after 2-3 weeks in culture.

Wilson observed what appeared to be transitional forms between some cell types, and suggested that the reconstitution could be best explained by the redifferentiation of the cells according to their positions in the aggregates. However, Huxley (1921) and Galtsoff (1923,1925) found very little evidence for cellular dedifferentiation in studies of cells dispersed from Sycon sp. and Microciona prolifera respectively, and the former concluded that the histogenetic assembly of sponges was accomplished by the selective movements of differentiated cells.

The capacity of dispersed cells to reconstruct normal tissue morphology was initially thought to be restricted to cells of lower invertebrates. However, the development of techniques for the dispersion and reaggregation of amphibian (Holtfreter, 1943) and mammalian (Moscona, 1952) cells made it possible to show that the process of reconstruction, although occurring to a more limited extent with vertebrate cells, appeared to be a general phenomenon (see Armstrong, 1971). Evidence has also been provided in some cases for the lack of cellular dedifferentiation in reaggregates of vertebrate cells (Trinkaus and Gross, 1961; Okada, 1965).

Some interest in the possible role of specific cell adhesion in tissue reconstruction was brought about following the observations that when cells from different tissues were mixed together they would often segregate, or sort out, according to tissue-type, or in other cases, species-type. Wilson (1907) first described species-specific segregation of sponge cells. Galtsoff (1925) showed that segregation of cells

from the sponges Microciona and Cliona occurred at the aggregation stage and proposed that this was caused by species-specific adhesion between the cells.

Holtfreter (1939) showed that when pieces taken from the three primary germ layers of an amphibian gastrula were fused, they would sort out such that the mesoderm ended up as a layer in between the inner endoderm and the outer ectoderm. He similarly attributed this to selective "affinities" between these cell types.

A large number of subsequent studies on sorting out between combinations of different embryonic vertebrate tissues have shown that, as a general rule, neural, epithelial, myoblastic and fibroblastic cell types will segregate from each other, both in mixed aggregates and when fused as tissue fragments (see Steinberg, 1970). However, less is known about the extent to which the properties determining the capacity to sort out are shared by cells of the same type but from different parts of the embryo.

Fibroblasts from different tissues have been shown to readily intermingle when pieces of the tissues are fused (Wolff, 1954; Wolff and Weniger, 1954; Bresch, 1955), in contrast to the parenchymal cells of such tissues. It is not known whether different types of fibroblasts will also mix randomly in aggregates.

Epithelial cells from different tissues have in most cases been found to sort out from each other. Using fused tissue fragments, Bresch (1955) reported that five embryonic chick epithelial tissues sorted out from each

other, although the patterns obtained were variable, depending partly on the relative sizes of the fragments used. Similar results were obtained by Bermann (1960).

Townes and Holtfreter (1955) noticed that in tripartite combinations of cells dispersed from the three amphibian primary germ layers, the endoderm in some cases segregated internally but in other cases formed a mixed outer layer with the ectoderm. It was also found that the medullary plate and endoderm would sort out in mixed aggregates but not when fused as tissue fragments. Therefore, different epithelial tissues may have a limited capacity to segregate from each other. This is supported by the finding of Chiakulas (1952) that in urodeles, grafts of ectodermal and oral, but not of endodermal, epithelia were able to fuse with the host skin epithelium. A similar selectivity was found when various combinations of epithelial tissues were cultured as explants. Embryonic chick epidermal, liver and pigmented retinal epithelial cells sort out in mixed aggregates (see Steinberg, 1970), but less is known about more closely related types of epithelia.

Garber and Moscona (1972a) found that the ability of different embryonic chick neural tissues to sort out in mixed aggregates appeared to vary in accordance with the proximity of the tissues being tested. This may reflect the presence of a gradient of a property involved in sorting out. Zwilling (1968) showed that embryonic chick chondrocytes from different organs did not appear to sort out.

Species-specific sorting out has been found between both like and unlike tissues. Moscona (1957, 1961) obtained results which suggested that the segregation of embryonic chick and mouse cells showed tissue - but not species - specificity. However, more recently, Burdick and Steinberg (1969) and Burdick (1972) have described sorting out between chick and mouse myocardial cells and limb mesoblast cells. Sorting out of liver cells did not occur in mixed aggregates but was found when pieces of liver tissues were fused.

The demonstration of the segregation of tissues was initially thought to be evidence for the existence of specific cell adhesion (Moscona, 1957; 1962; Steinberg, 1958). However, it was later pointed out (Curtis, 1962; Steinberg, 1963) that specific cell adhesion could not by itself account for the patterns that were found when different combinations of tissues were tested.

Three types of pattern have been described in mixed aggregates: complete enclosure of one cell type by another; partial enclosure; and small islands of one cell type (the discontinuous phase) scattered throughout the other (the continuous phase). Any hypothesis of sorting out must explain both the segregation of tissues and the patterns that can be formed, although any model which can account for the latter will also explain the former. Therefore, the process of sorting out is not a useful test for the involvement of specific cell adhesion in cell patterning, but at the same time it cannot be used as evidence against this.

There are a number of experimental findings which are relevant to the mechanism of sorting out. Steinberg (1970) showed that, for 7 embryonic chick tissues, the same patterns were formed in mixed aggregates and after the fusion of tissue fragments, and also that the tissues could be arranged in a hierarchy with respect to their abilities to segregate internally. These two findings suggest that the patterns formed during sorting out represent equilibrium configurations, and that different cell types are likely to sort out by the same mechanism.

It is also significant that under certain conditions it is possible to reverse or alter the patterns of sorting out, for example by adjusting the ratio of the two tissues used (the phase ratio) or the dissociation procedure (Bresch, 1955; Armstrong and Niederman, 1972; Wiseman et al, 1972), or by allowing one of the cell types to age in culture prior to the experiment (Curtis, 1962; Phillips et al, 1977).

It is not known whether cell motility is required for sorting out. In some cases (Abercrombie and Weston, 1969), very little or no evidence has been found for the intermingling of cells from different tissues, however, Armstrong and Armstrong (1973) and Gershmann and Drumm (1975) have described the penetration of cells into aggregates, indicating that at least some degree of movement of individual cells is possible in solid tissues.

There have been several reports of the inhibition of sorting out by cytochalasin B (Steinberg and Wiseman, 1972; Maslow and Mayhew, 1972; Sanger and Holtzer, 1972),

although Armstrong and Armstrong (1973) and Overton and Kapmarski (1975) found that this drug had little or no effect when other combinations of cells were used. However, the site of action of cytochalasin B is not known, and it is not certain that it would inhibit the locomotion of cells in aggregates. Garrod and Steinberg (1973) observed membrane ruffling activity during the sorting out of embryonic chick heart and liver cells on a culture substrate, suggesting that active cell locomotion may be required for two dimensional sorting out.

Adler (1970) and Elton and Tickle (1971) have made quantitative studies of sorting out. Adler found that chick neural tube cells appeared to be randomly positioned in 1½ hour aggregates, but no attempt to measure segregation was made. Elton and Tickle introduced a statistical method for the measure of the degree of segregation and found evidence for significant amounts of segregation in 2 and 4 hour aggregates of chick heart and limb bud cells. Tentative evidence was also found for early positioning of these cell types, suggesting that the segregation may have resulted from early sorting out rather than from specific adhesion during the initial stages of aggregation. Armstrong (1971) reported a lack of segregation in early aggregates of chick neural retinal and pigmented retinal epithelial cells, however, no statistical assessment of this process was made.

Four main hypotheses have been put forward to explain sorting out. Townes and Holtfreter (1955) and Stefanelli and Zacchei (1958) proposed that cells sorted out in response to chemotactic gradients. However, this may be

inconsistent with some of the subsequent findings which have been made, including the demonstration of a hierarchy of sorting out (Steinberg, 1970) and also the observation of Steinberg (1962) that if heart cells were mixed in a very low (1%) phase ratio with neural retinal cells, to which they normally segregate internally, they did not accumulate at the center of the aggregate, but at intermediate equilibrium positions. More recently, Edelstein (1970) has proposed on theoretical grounds that a chemotactic explanation of sorting out could be possible.

Curtis (1961,1962a) suggested that sorting out could be caused by temporal changes in some cellular property, for example, adhesiveness or susceptibility to contact inhibition. In the first case, the cell type which is most adhesive initially would aggregate first and therefore end up internally to the second cell type. In support of this hypothesis, Curtis (1961) showed that preincubation of amphibian endodermal cells for 4-6 hours interfered with their abilities to sort out from mesodermal and ectodermal cells.

However, more recently, Curtis (1974) has proposed that sorting out is accomplished by the secretion of soluble factors, or morphogens, which decrease the adhesiveness of heterotypic cells. Differences in either the production or in the rates of diffusion of morphogens by different cell types could lead to the formation of the patterns found both in sorting out and during normal histogenesis. Such factors have been shown to be secreted by sponges, mammalian lymphocytes and chick neural retinal and liver cells (see Curtis, 1974). The addition of neural retinal or liver cell morphogens to newly formed mixed aggregates of

these two cell types was found to reverse and randomize, respectively, the normal pattern of sorting out. In order to account for the wide variety of cell types which sort out, it would be necessary to show that morphogens could act on a wide range of heterotypic cell types. This possibility is supported by the finding that the diffusible factors obtained from lymphocytes decreased the adhesiveness of a large variety of cell types, with only one exception (Curtis and De Sousa, 1975).

Steinberg (1963, 1964, 1970) proposed that sorting out is acheived by the passive movements of cells and that the patterns which result are determined by differences in their interfacial energies of adhesion, analogous to the separation of immiscible liquids with different surface tensions. By this mechanism, the different patterns which result are determined by the relative strengths of heterotypic and homotypic adhesions (Steinberg, 1964). In the most common instance of the complete enclosure of one cell type by another, this model requires that the energy of heterotypic adhesion be intermediate to that of homotypic adhesion, and is therefore incompatible with any mechanism involving specific cell adhesion. Phillips and Steinberg (1969) were able to show that the degrees of flattening of embryonic chick limb bud, heart and liver cell aggregates in a centrifugal field correlated with their positions in the hierarchy, the most internally segregating cells being the most resistant to flattening, and also that this was independent of the initial shapes of the aggregates.

The differential adhesion hypothesis has been

criticized by Harris (1975), mainly in that it does not allow for a) the formation of focal adhesions, the result of which would be that the energy of adhesion would not correspond with the amount of intercellular contact area, b) the modification of initial adhesive contacts so that the formation and breakage of adhesions would not necessarily involve the same amounts of work, and c) changes in cell shape made possible by metabolic activity. Harris proposed three alternative but similar models of sorting out, also based on the analogy of this process with the separation of immiscible liquids. The first of these, the differential contraction hypothesis, explains sorting out by differences in contractility of the free surfaces of different cell types, and predicts that the most strongly contractile cells will segregate internally.

In an extension of this model, Harris (1977) suggested that the control by contractile microfilaments, both of changes in the shapes of cell sheets and of the lateral movements of adhesive sites in the cell membrane, could provide a similar mechanism, at the mechanical level, of controlling the four main types of morphogenetic movement: epithelial folding, cellular locomotion, ingression and cavitation. This hypothesis was made as an attempt to explain the interconvertability of these four processes, both during evolution and after experimental manipulation, for example in the formation of the neural tube or in the sorting out of the three primary germ layers. This is explained in the model by basing three processes, the locomotion of single cells, the

contraction of epithelial sheets and the formation and breakage of junctional contacts, on the same mechanism, mentioned above. However, there is no experimental evidence in support of this model, other than the likelihood (eg. Wessels et al, 1971) that epithelial folding is generally accomplished by the contraction of microfilaments. The interconvertibility of morphogenetic movements could be controlled at other levels than the strictly mechanical one.

The relevance of sorting out to histogenesis is not clear, however, the cellular properties which are responsible for sorting out must also contribute to the patterns formed during morphogenesis. It is possible that normal histogenesis is exclusively determined by these properties, but it also possible that the more complex patterning found in this process involve additional factors specific for cell types which normally interact during development. The radial patterning of the nervous system is suggestive of a sorting out process. However, other cellular properties which may be important in this patterning have been described, for example, the orientation of adhesive sites on the surfaces of neural retinal cells and the apparent segregation of two classes of rosette-forming cells during the reconstruction of neural retinal tissue in cell reaggregates (see General Discussion). The relationship of these processes to that of sorting out is not known.

Some evidence for the possible role of cell adhesion in morphogenetic movements has also come from

studies of the behaviour of cells on substrates, in particular in the phenomena of contact guidance and contact inhibition of locomotion. The contact guidance of cell locomotion on substrates has been shown to be determined by the physical topography of the surface (Weiss, 1941), possibly as a result of the limited ability of microfilament bundles to function when bent (Dunn and Heath, 1976), and by differential adhesiveness of the surface (Carter, 1965; Harris, 1973a). Very little evidence has been found for chemotactic migration of embryonic vertebrate cells, although Weiss and Scott (1963) showed that embryonic chick heart and liver cells would move up a steep pH gradient. Contact inhibition of locomotion (Abercrombie and Heaysman, 1954) may also contribute to tissue patterning, and has been shown to occur between corneal fibroblasts in situ (Bard and Hay, 1975).

2. Cell-to-Substrate and Cell-to-Cell Adhesion

The study of cell-to-substrate adhesion provides a useful means of examining some of the basic properties of cell adhesion. There is a considerable amount of evidence which suggests that the adhesions of cells to substrates and to other cells are to a large extent similar processes, but that the latter differs from the former mainly in the involvement of specific recognition processes between cells. However, one difficulty in making comparisons between cell-to-substrate and cell-to-cell adhesions is that almost all of the studies of the former have been made using fibroblastic cells, and it is not clear to what extent the results obtained will also apply to other cell types.

(i) Attachment of cells to substrates

The ability of cells to attach to substrates appears to be dependent on two properties of the substrate: wettability, or surface tension, and mechanical rigidity. Wettability is believed to be measurable by the critical contact angles formed by liquid droplets spreding on a surface, and is thought to reflect the adhesiveness of the surface for the droplets (see Baier, 1970). Carter (1965) and Grinnell et al (1972) have shown that both the rates of attachment and the strenghts of adhesion of fibroblasts to various metallic and organic surfaces were correlated with the wettabilities of these surfaces. Carter (1965) and Maroudas (1973) also found a similar relationship between surface wettability and cell spreading.

The importance of a minimal degree of mechanical rigidity of the substrate for cell attachment to occur has been stressed by Maroudas (1973, 1975). He was able to show that by increasing the concentration of a PVP (sauflon polyvinylpyrrolidine) gel from 46% to 96%, this surface was rendered adhesive for fibroblasts (Maroudas, 1975). Maroudas attributed the requirement of cells for some degree of mechanical rigidity to the need for the substrate to withstand the tensile forces exerted by the cells.

It is likely that alterations in the wettability or in the mechanical rigidity of the cell surface could be important in the control of adhesive interactions between cells. For example, the lack of cell spreading and movement which has been observed in some cases when cells are seeded onto cell monolayers (Bell, 1972; Buultjens and Edwards, 1977), as well as the low adhesiveness of some epithelial cell monolayers (DiPasquale and Bell, 1974; Vasiliev et al, 1975) and of the apical surfaces of limiting epithelia in situ (DeRidder et al, 1974) could be due to the lack of sufficient mechanical support of these surfaces.

The attachment of cells to substrates depends on membrane spreading movements, the organization of adhesive sites and the formation of adhesive contacts. It may be possible to some extent to determine separately the requirements of each of these processes for cell-to-substrate adhesion. It is also likely that these processes are involved in cell-to-cell adhesion, although perhaps to

different extents.

It has been known for some time (Taylor, 1961) that the properties of cell-to-substrate attachment are considerably modified by the presence of serum in the medium. Although only a few comparative studies have been made, there is some evidence that the requirements of cell attachment for divalent cations and an energy source, and its inhibition by low temperatures, cytochalasin B, tranquilizers and formaldehyde, may only be found in the presence of serum, when normal culture substrates are used (Taylor, 1961; Nordling, 1967; Takeichi and Okada, 1972; Rabinowitch and DeStefano, 1973b; Grinnell, 1974a). The attachment of cells to highly wettable surfaces, such as acid-washed glass, may be independent of these conditions.

Grinnell (1974a,b) showed that when BHK fibroblasts were allowed to attach to serum-coated coverslips in a centrifugal field, the requirements for divalent cations, and inhibition by cytochalasin B and low temperatures were abolished, but the attachment was still inhibited by sulfhydryl-binding agents and tranquilizers. Grinnell suggested that the first three treatments were affecting cell spreading movements but that the latter two were acting directly on the formation of adhesive contacts.

However, it is difficult to rule out the possibilities of the inhibition of glycolysis by the sulfhydryl-binding reagents and general effects on the membrane by the tranquilizers. For example, cationic anaesthetics have been shown to inhibit capping in lymphocytes (Ryan et al, 1974), possibly by decreasing the

fluidity of the membrane. Schaeffer and Curtis (1977) found that the attachment of L929 cells to serum-coated plastic was increased by the addition of saturated fatty acids and decreased by that of unsaturated fatty acids. They suggested that the effects of the fatty acids might be to alter the aggregability of adhesive sites in the membrane by decreasing or increasing, respectively, the fluidity of the membrane.

The requirement of cell-to-substrate attachment for the lateral clustering of adhesive molecules in the cell membrane has been proposed by Shields and Pollock (1974) and by Rees et al (1977). Rees et al showed that the treatment of rat dermal fibroblasts with concanavalin A made the cells resistant to detachment with trypsin, but that the treated cells were detached by the addition of hapten at 37 °C. If the hapten was added to the cells at 2 °C and subsequently removed, the cells detached when the medium was heated to 37 °C, indicating that a moderate temperature appeared to be required for the detachment of the cells after the removal of the lectin. The authors postulated that concanavalin A stabilized cell adhesion by cross-linking adhesive glycoproteins.

The requirements of cell-to-cell adhesion appear to be quite similar to those of "serum-dependent" cell-to-substrate adhesion. Cell aggregation has been shown to be inhibited by sulfhydryl-binding reagents (George and Rao, 1975), cytochalasin B (Jones and Partridge, 1974), low temperatures

Edwards et al, 1971), and alterations in the lipid composition of the membrane (Hax et al, 1971; Curtis and Buultjens,1973; Curtis et al, 1975). It is possible that the effects of these treatments on cell-to-cell adhesion could also be mainly on membrane spreading movements and on the organization of adhesive sites, rather than on the formation of adhesive contacts, as has been proposed for cell-to-substrate adhesion.

Very little is known about the sites of action of the inhibitors listed above on cell-to-cell adhesion. Edwards et al (1975) found that BHK fibroblasts would aggregate in the presence of metabolic inhibitors but not at low temperatures, and attributed this to the effect of the latter on membrane fluidity. Edwards and Campbell (1971) also showed that trypsinized BHK cells which had been preincubated at 37 °C were still unable to aggregate in the cold, suggesting that the inhibition of aggregation of these cells at low temperatures was not likely to be due to a requirement for recovery from trypsinization. However, Curtis (1973) pointed out the inabilities of different cell types to aggregate at low temperatures appears to only occur when the cell types have been previously trypsinized, suggesting that the requirement of cell aggregation for a moderate temperature, and perhaps also for metabolic activity, could generally be artefacts of trypsinization. It is not known whether the same would be true for cell-to-substrate adhesion.

Some differences between cell-to-substrate and cell-to-cell adhesion have been reported. Weston and Roth

(1969) claimed that the treatment of chick heart fibroblasts with urea reduced their mutual adhesion but not their abilitiy to attach to substrates. Coman (1961) found that rabbit V2 carcinoma cells were less adhesive to glass but more adhesive to homologous cells than normal rabbit epidermal cells. However, cell-to-cell adhesion was measured by the resistances of the cells to detachment by micromanipulation, and may have been influenced by several properties not directly related to adhesion. Berwick and Coman(1962) found that both EDTA and neuraminidase treatment of cells had opposite effects on cell-to-substrate and cell-to-cell adhesion, although their results have been contradicted by those of many other workers. There generally appears to be a poor correlation between the extents to which cells aggregate and attach to substrates, but this may be in part due to the different assays which are used to measure thes two types of adhesion.

(ii) Effect of serum on attachment

It is generally accepted that serum exerts its effect on cell-to-substrate attachment by forming a coat which covers the substrate. In serum-containing media, a coat of approximately 100 nm in thickness has been shown to form rapidly on the substrate (Baier and Dutton, 1969; Revel and Wolken, 1973). Most likely the cells are attached directly to this coat and do not contact the substrate. Maroudas (1975) has argued that steric exclusion of the cells by the serum coat would be expected on physical grounds, as this is a general property of polymers. Some evidence for this was provided by the finding of Revel et al

(1974) that the treatment of attached cells with amyl acetate immediately after fixation in ethanol made it possible to remove the serum coat, together with the attached cells, cleanly off the substrate. It is of interest that cells can respond to differences in the adhesiveness of substrates through a serum coat (Carter, 1965), as well as through a film, several hundred nanometers in thickness, of stearate-stearic acid (Rosenberg, 1963), although this is most likely caused by an effect of the substrate on some property of the serum coat or lipid film, rather than directly on the cells.

The effect of the serum coat on cell attachment could be due to: a decreased wettability of the surface in the presence of serum; the presence of specific serum factors; the non-specific physicochemical properties of a glycoprotein coat; or, possibly, the selective release of a cellular microexudate bound to the serum coat. There is some evidence which suggests that the serum coat acts in more than one of these ways.

Maroudas (1977) showed that the divalent cation requirement of cell attachment could be induced in a quantitative manner by decreasing the wettability of the substrate in the absence of serum, suggesting that the principal effect of the serum coat might be a to reduce the wettability of the substrate.

However, a more specific effect of the serum coat is suggested by the observation of Pegrum and Maroudas (1975) that the formation of electron-dense

plaques at the sites of adhesion to the substrate appeared only to occur in the presence of a serum coat. The addition of manganese ions, which otherwise altered the morphology of cell spreading to that found on highly wettable substrates, did not alter the serum-dependence of plaque formation.

The effect of the serum coat may be caused by specific serum components attached to the substrate. Revel and Wolken (1973), Unhjem and Prydz (1973) and Culp (1976) have analysed the serum components adsorbed to the substrate by SDS electrophoresis, and in each case detected a major band corresponding to α-1 globulin. Takeichi and Okada (1972) also showed that α-1 globulin could be substituted for serum in its effect on cell attachment. Culp(1976) found a total of 8 bands which were detectable by Coomassie-blue staining and which persisted during the attachment and growth of cells. These components were not removable by treatment with EDTA. However, one argument against the requirement for specific serum components is the finding that other glycoprotein coats, for example gelatin (Rabinowitch and DeStefano,1973b) can be substituted for serum.

The presence of a serum coat may either be required for cell spreading or may retard spreading, depending on the cell type. Grinnell (1976b) isolated a serum factor which appeared to be required for the spreading of several fibroblast lines: BHK, CHO, HeLa and L cells. The factor bound to the substrate but was inactive if the substrate had been precoated with protein.

The activity of the factor appeared to have a specific requirement for calcium ions.

The retardation of spreading in the presence of serum was first shown by Taylor (1961) for human conjunctiva cells, and has subsequently been observed in a number of cell types. Rajaraman et al (1974) and Witkowski and Brighton (1972) found that the presence of serum both reduced the rates and altered the morphologies of spreading of W138 and MCR-5 cells on glass. In the absence of serum, spreading was characterized by a thin layer of cytoplasm moving over randomly oriented and branched filipodia, and appeared to be indicative of a passive process. If serum was present, the filipodia were oriented radially, were unbranched and relatively few in number, and the cytoplasmic processes showed more diversity in appearance. Rabinowitch and DeStefano (1973) and Pegrum and Maroudas (1975) obtained similar results with Sarcoma-1 and embryonic rat fibroblastic cells, and showed that the addition of manganese ions reversed the effects of the serum coat on both the rate and the morphology of spreading.

The observation of the poorly oriented spreading of cells on highly wettable surfaces, indicating a loss of cellular control of spreading under these conditions, suggest that it is likely that under physiological conditions adhesive interactions between cells are of the "serum-dependent" type. This is also suggested by the sensitivity of cell aggregation to various inhibitors, by the general presence of glycoprotein coats on cell surfaces

(Rambourgh and Leblond, 1967), and by the likelihood that within a certain range of adhesiveness, a high degree of control over cellular behaviour could be achieved by relatively small differentials in adhesivity (Harris, 1973a; Curtis and Boultjens, 1973).

(iii) Morphology of Adhesion

Several workers have suggested that microextensions, or filipodia, may play a role in the initial attachment and early spreading of cells, both in cell-to-substrate and in cell-to-cell adhesion. However, their function is not clear. Bangham and Pethica (1960) suggested that the small radius of curvature of such processes would enable them to more readily penetrate the electrostatic barrier between cells, after which broader areas of the cell surfaces could follow. Curtis (1973) pointed out that these authors may have overestimated the ability of microextensions to act in this capacity, but that they could enhance the abilities of cells to form adhesions by facilitating the drainage of the gap between the cells.

Witkowski and Brighton (1971) suggested that microextensions might act primarily as sensors, guiding the broader lamellar processes to areas of high adhesivity, since the authors considered that the former would be too small to form effective adhesions. Albrecht-Buehler (1976) found that the microextensions of cultured 3T3 cells showed a scanning motion prior to attaching to the substrate, and proposed that these were exploring the surface for the most favourable areas.

Witkowski and Brighton further speculated that microextensions might differ, mainly in their ability to rapidly elongate (from 1 to 10 in BHK cells), from the shorter microvilli which cover the surfaces of suspended BHK cells and which may be required for nutrient uptake (Witkowski and Brighton, 1971) or for storing surface

available for spreading (Follett and Goldman, 1970).

There have been some reports of the participation of microextensions in cell-to-cell adhesion. Lesseps (1963) and Spring-Mills and Elias (1975) claimed, using electron microscopy, to have seen contacts between such processes. Howard et al (1975) examined the attachment of fibroblasts to monolayers of homologous cells by scanning electron microscopy. They found evidence for contacts between microextensions of either the suspended or the monolayer cells and broader surface areas of the other cells, but few contacts between two such processes.

Fibroblasts may adhere to normal culture substrates by two types of adhesive structure: small and apparently quite specialized "focal contacts", and broader areas of "close contact" (terminology of Izzard and Lochner, 1976). These are thought to be separated from the substrate by gaps of about 10-20 nm and 30 nm, respectively. Very little is known about the relative contributions of these two types of structure to the behaviour of fibroblasts on substrates, as they have usually been found to occur together in the same cells. In one study, it was mentioned that only the focal contacts persisted during the formation of confluent cultures of chick heart fibroblasts (Izzard and Lochner, 1976). It is possible that these two types of contact correspond to some of the

surface specializations found at regions of adhesion between cells, for example at the zonula adherens and the zonula occludens.

Abercrombie et al (1971), in an electron microscopic study of chick heart fibroblasts, found that 25% of the area under the leading lamella was comprised of discrete adhesive sites, each of about 1-2μ in diameter, closely attached (30 nm) to the substrate. It was calculated that each cell might have 10-100 such sites, most of which were likely to be under the leading lamella. These focal contacts were associated with small electron-dense plaques, present on the cytoplasmic side of the membrane, and with bundles of microfilaments which appeared to be inserted into the plaques.

Similar focal contacts have been described by Cornell (1968), Flaxman et al (1968) and Pegrum and Maroudas (1975) in fibroblasts, and by Brunk et al (1971) in glial cells. Brunk et al and Pegrum and Maroudas noted the presence of electron-dense plaques and microfilament bundles at the sites of these contacts. Similar plaques have also been shown to be formed during the early stages of intercellular adhesion, for example at the leading edges of cultured chick heart fibroblasts (Heaysman and Pegrum, 1973) and during the early aggregation of 16C rat fibroblasts (Lloyd et al, 1976).

The ability of cells to form focal adhesions appears to be restricted to certain parts of the cell surface, both in contacts between cells and between cells and substrates. Bragnina et al (1975) found that bundles of microfilaments

were only found in the microextensions and lamellar areas of newly attached mouse embryo fibroblasts, even when other regions of the surface were in close contact with the substrate. The formation of electron-dense plaques, not necessarily present in all cases when microfilament bundles are formed, may be restricted even further to only the lamellar regions. Heaysman and Pegrum (1973) mentioned that plaques were seen in contacts between two lamellas but not between a lamella and a lamellipodium. They attributed this to the transient nature of the latter.

Revel and Wolken (1973) and Revel et al (1974) found some evidence for the simultaneous presence of both focal and broad contacts in a number of fibroblast lines. Revel and Wolken used the surface replica technique to examine the undersurfaces of normal and transformed BHK cells. By this method, cells attached to the substrate are fixed, peeled off the substrate and inverted, and examined by scanning electron microscopy.

The undersides of the cells were found to be smooth and quite flat, and both points of focal contact and broad areas, corresponding to "close contacts" could be distinguished. These areas were considered to be in close contact with the substrate, judged by their inpenetrability to Thorotrast (diameter of 15-30 nm). Bundles of microfilaments could be seen running through both of these structures in normal BHK cells, but were reported to be seldom seen in the transformed cells. This finding may be related to the reduced ability of the latter to form aggregates (Edwards et al, 1971).

Revel et al (1974) obtained similar results by examining the patterns of cellular deposits remaining on the substrate after attached cells had been removed. They described the presence of both pointlike material, suggested to correspond to the footpads seen at the bases of retraction fibrils of these cells after trypsinization, and large deposits which were thought to be derived from sole plates (close contacts). The latter were lined with what were presumed to be bundles of microfilaments.

The authors pointed out the possibility that the sole plates could be attached to the surface at discrete points and be held together as a unit structure by a system of microfilaments. The opposite is also possible, that adhesive contacts are made by large regions of the surface but are linked to microfilament bundles only at small points. It was mentioned that the small sizes of the footpads could have been artefacts of trypsinization.

Culp (1975) labelled cells with (^{14}C)-glucosamine and examined the radioactive substrate-attached material (SAM) which remained after the cells had been detached. He found that the distribution of the SAM coincided with that of the previously attached cells, indicating that it had been deposited directly onto the substrate by the cells and not adsorbed uniformly after being released into the surrounding medium. However, no attempt was made to distinguish between materials which had been secreted and footprints which had been left behind during the detachment of the cells.

Parts of the SAM were condensed into "focal droplets" of about 1-2 diameter, similar in size to both

the footpads observed by Revel et al (1974) and to the electron-dense plaques described by Abercrombie et al (1971). The total numbers of these contact sites found to be present in each cell were comparable in all three studies, however the relatively uniform distributions of the SAM, and also of the footpads, underneath the cells are not consistent with some of the findings of other workers.

Similar results have been obtained from studies made on living cells. Ambrose (1961) observed the locomotion of fibroblasts on a substrate using the surface contact microscope. He reported seeing lines of close contact to the substrate under the leading lamellas of the cells, running parallel to the long axes of the cells. However, no comparable finding has been made using any other technique.

Curtis (1964) developed the method of interference reflection microscopy as a means of measuring the gap between the undersides of living cells and the substrate. Using chick heart fibroblasts after 24 hours in culture, he found large areas of approximately uniform separation from the substrate, and suggested that these were acting as one adhesive unit. The closest contacts to the substrate, about 10-20 nm, were found mainly at the cell periphery and under the leading lamella. It was found that this gap could be increased or decreased by altering the pH or the ionic composition of the medium, suggesting that microexudates were not likely to be mediating the adhesion of the cells across this gap.

Izzard and Lochner (1976), also using this technique, described both focal and close contacts, separated from the substrate by distances of approximately 10-15 nm and 30 nm, respectively. These were suggested to correspond to the two types of adhesion previously described by Curtis (1964). The focal contacts appeared as thin streaks, 2-10μ long. which may have resulted from the formation of retraction fibrils caused by the movement of the cells. These contacts were seen mainly under the leading lameelae, and lasted about four minutes in rapidly moving cells. The numbers of focal contacts per cell were estimated to be similar to those calculated by Abercrombie et al (1971). Presumed bundles of microfilaments were also seen running through these contacts.

By using a combination of two techniques, micromanipulation and time lapse cinematography, Harris (1973b) showed that a variety of normal and transformed fibroblasts were attached to the substrate only at narrow margins at the cell periphery and near regions of ruffling activity. Vaughan and Trinkaus (1966) similarly found that cultured sheets of chick epidermal cells appeared to be held to the substrate only at the peripheral margins of the sheet.

3. Evidence for Specific Cell Adhesion

The demonstration that cells can show type-specific adhesion would be of considerable interest to an understanding of the possible role of cell adhesion in cell positioning. Although many early workers speculated that specific adhesive interactions between cells could provide a basis for morphogenetic movements (Galtsoff, 1925; Weiss, 1947; Tyler, 1946; Townes and Holtfreter, 1955; Sperry, 1958), assays which could be used to detect specific adhesion were not developed until quite recently.

Roth and Weston (1967) introduced the collecting aggregate assay, in which a tritiated thymidine-labelled suspension of one cell type is incubated in a gyratory shaker with 24 hour aggregates of both isotypic and heterotypic cells. At given time intervals the aggregates are removed by pipette and the numbers of cells attached to each aggregate estimated by autoradiography. This technique was later modified by Roth et al (1971a), mainly in the use of a (^{32}P)-orthophosphate label and liquid scintillation counting for more accurate and rapid results, and also by McGuire and Burdick (1976), who were able to greatly improve both the degree of specificity shown and the extent of collection by using more irregularly-shaped 1-2 hour aggregates and by carefully controlling the flow conditions.

Walther et al (1973) described an analogous assay, based on the attachment of radioactively-labelled cells to preformed monolayers or multilayers of different cell types. The weakly- or non- adherent cells are washed off and the remaining cells counted by the liquid scintillation method.

This assay has the advantages of being both rapid and visually observable, but it does require that the cells can form stable cell sheets, usually after a period of 12-48 hours. Gottlieb and Glaser (1975) were able to obtain monolayers of embryonic chick neural cells which were firmly attached after only one hour by derivitizing the glass substrates with γ-aminopropyl-triethoxysilane followed by treatment with glutaraldehyde, but it is not known what effect this highly adhesive surface would have on the adhesive properties of the cells.

Other methods have also been used to test for the occurrence of specific cell adhesion. Barbera et al (1973) measured the attachment of single cells to whole optic tectal halves pinned onto an agar substratum in petri dishes. A similar method was used by De Ridder et al (1975) in a study of the adhesiveness of various epithelial tissues. Curtis (1970a) showed that the kinetics of initial cell aggregation could be used to detect the presence of specific adhesion by comparing the adhesive rates when different ratios of two cell types were coaggregated.

Almost all of the evidence for tissue-type specific adhesion has come from work on embryonic chick cells, mainly neural, myoblastic and liver epithelial cells. Roth and Weston (1967) and Roth (1968), using the collecting aggregate assay, found that isotypic adhesions were preferred to heterotypic ones in almost every case in a number of different combinations of cells: NR (neural retina) and liver; liver and heart ventricle; and liver, pectoral muscle and cartilage. Mouse NR, liver and heart ventricle cells shared some of the tissue-

type specificity with the embryonic chick cells, although there was a considerable variation in the degrees of specificity shown in different cases. The collections of NR and liver cells by isotypic aggregates were found to have lags of 30 minutes and 2 hours respectively, after which time the specific collection of isotypic cells increased gradually up to at least 5 hours in each case. Roth et al (1971a) confirmed some of these results using (^{32}p)-labelled cells and also found specific adhesion between NR and forebrain and between NR and heart ventricle using NR aggregates, but the reciprocal experiments (using forebrain and heart aggregates) were not carried out.

McClay and Baker (1975) obtained quite different results using a similar assay but with a high aggregate: single cell ratio to ensure the collection of most of the labelled cells. They found that cells from four embryonic chick neural tissues: NR, pigmented retinal epithelium, cerebrum and optic tectum, and also liver cells, showed a very small degree of specific adhesion when cerebrum aggregates were used, but that there was no specificity between NR and cerebrum when NR aggregates were used. It was also found that subpopulations of cerebrum cells fractionated by Ficoll gradients attached at slightly different rates to isotypic aggregates, in a way that appeared to correlate with cell size.

On the other hand, McGuire and Burdick (1976) obtained results which suggested that the specificity reported by Roth and Weston (1967) could be representative of the majority of the cells. They found that NR and liver

cells showed a high degree of specific adhesion under conditions in which about 25-70% of the labelled cells were collected by the aggregates. They also found a considerable degree of specificity between NR and mesencephalon cells, although this was less pronounced when the former were used as the labelled cells.

Using the monolayer collection assay, Walther et al (1973) found that liver and heart ventricle cells showed adhesive specificity with NR cells but not with each other, in contrast to the results of Roth et al (1971a). The degree of specificity obtained was also considerably lower than that found between the same cells using the collecting aggregate technique (Roth et al, 1971; McGuire and Burdick, 1976). Walther et al also found specific adhesion between mouse teratoma and secondary culture mouse kidney cells, but not between a variety of other cell types.

Gottlieb and Glaser (1975), using a modified version of this assay, obtained the unexpected result of reverse specificity between NR and mesencephalon cells, in direct contrast to the results of McGuire and Burdick. It was later suggested (Gottlieb et al, 1976) that this could be due to the sites for isotypic adhesion being used up in lateral contacts between cells in the monolayers, but this is not borne out by the results of Walther et al (1973), although it is possible that the unusually adhesive substrates used by Gottlieb and Glaser may have exaggerated the process of the lateral clustering of adhesive sites.

Very few studies of specific cell adhesion have used cell types which normally interact <u>in vivo</u>. Barbera

et al (1973) found that cells from the dorsal or ventral NR preferentially adhered to the opposite tectal halves, mimicking the selectivity of innervation during development. The same results were obtained using de-innervated embryos, indicating that the capacities to show this specific adhesion developed indepently in each organ. Pigmented retinal epithelial cells shared the ability to show specificity with NR cells, but cells from several other neural tissues, including the optic tectum, did not.

Barbera (1975) was able to increase the ratio of specificity from about twofold to sixfold by performing the assay in a reciprocating shaker. He also showed that the abilities of the dorsal and ventral NR cells to show specificity had developed by 3 days and 6 days of development, respectively, and were found in tectal cells between 8-14 days of development. Barbera proposed that the results could be best explained by the existence of gradients of two types of adhesive molecules running across the dorsoventral axes of the NR and the optic tectum. This would be consistent with the requirement of the ventral but not of the dorsal NR cells for recovery from trypsinization before showing specificity.

In support of the possibility of such gradients, Gottlieb et al (1976) reported that dorsal and ventral NR cells showed specific affinities for monolayers prepared from cells from the opposite halves of the NR. Assays in which the adhesive interactions between combinations of cells obtained from six adjacent bands along the dorsoventral axis were compared showed that the gradient of specificity appeared to be continuous and was shared by cells

taken from nine and twelve day embryos. However, Barbera et al (1973) did not find any specificity between dorsal and ventral tectal cells (see above) as might be expected from the gradient model.

Buultjens and Edwards (1977) found a moderate degree of adhesive specificity between NR and choroid cells, using the monolayer collection assay. They suggested that the poor adhesive association of these two cell types could contribute to the development and to the maintenance of the tripartite arrangement of NR, pigmented retinal epithelium and choroid tissues in the normal retina. It was also found that the pigmented retinal epithelial cells formed relatively poorly adhesive monolayers.

A number of examples of species-specific cell adhesion have been reported. The aggregation of sponge cells has in some cases been shown to be highly species-specific (Galtsoff, 1925; Humphreys, 1963) or strain-specific (Curtis and Van de Vyver, 1971), leading to the formation of unmixed aggregates, although in other cases mixed aggregates have been found (Sara et al, 1966; Curtis, 1962b,1970b; Humphreys, 1970). The formation of unmixed aggregates has only been observed between cells taken from different species, and has also been shown to occur between juvenile chick and rat liver cells (Obrink et al, 1977).

Using the collecting aggregate assay, Grady and McGuire (1976) found species-specific adhesion between embryonic chick and mouse liver cells, but not between the corresponding mesencephalon cells. This suggests that the ability to show species-specific adhesion may be confined

to certain cell types. Guidice and Mutolo (1970) also using this assay, found adhesive specificity between two species of sea urchin. McClay and Hausman (1975) confirmed this finding and also showed that cells from hybrid sea urchins attached to aggregates at an intermediate rate. The capacity of these cells to show specific adhesion appeared to be dependent on the expression of the paternal genome.

The existence of specific cell adhesion can be explained in several ways. Curtis (1970a; 1974) has suggested that the occurrence of adhesive specificity could be accounted for by changes in the relative adhesivities of the different cell types being tested either with time, for example during recovery from trypsinization, or by factors secreted by the cells which are capable of modifying the adhesiveness of selected cell types. Both of these processes could also be operative in vivo. Curtis (1970a) found that the relative adhesivities of embryonic chick NR and liver cells reversed over a period of six hours, and mentioned that this could explain the specific adhesion between these cells reported by Roth and Weston (1967).

It is difficult to explain the large discrepancies between the results obtained by different workers using the same cell types, although there are a number of variables which could affect these results. The strength of the trypsinization procedure used and the extent to which the cells have recovered from trypsinization appear to be very important in this respect. The initial

adhesions formed between cells from different tissues, shortly after the disaggregation of the tissues, have been shown in some cases to be apparently non-specific. Curtis (1974) found, by using immunological markers for cell identification, that embryonic chick NR and liver cells, and also mouse B and T lymphocytes, formed apparently random adhesions during the first hour of aggregation. Sheffield (1970) and Fujisawa (1971) similarly provided evidence for a lack of adhesive specificity between different cell types of the chick NR in the early stages of reaggregation. The formation of mixed aggregates between different types of tissue cells indicates that if there is any adhesive specificity between these cells during their initial aggregation it cannot be complete. However, a significant amount of sorting out has been described in early mixed aggregates of chick heart and limb bud cells (Elton and Tickle, 1971), suggesting that a considerable amount of tissue-specific adhesion may take place in some cases during the initial formation of aggregates.

Roth (1968) showed that the addition of unlabelled NR cells decreased the collection of liver cells by liver aggregates but increased their collection by NR aggregates. If the unlabelled NR cells were preincubated for 4-6 hours before being added, these effects were not observed. Roth interpreted these results as meaning that the NR cells adhered non-specifically to the liver cells following trypsinization until the molecular structures conferring specificity had been restored. He also suggested that this would account for the lag periods found in the collections of isotypic cells, since the

aggregates which had already recovered might only collect cells with matching adhesive sites.

Grady and McGuire (1976) were able to observe a high degree of specific adhesion between the same cells, with no detectable lag periods, when 1-2 hour aggregates were used. They suggested that the differences between their results and those of Roth (1968) could be due to the heavier trypsinization procedure used by the latter. Steinberg et al (1973) also found that the aggregation of NR cells showed a 30 minute lag period only if the trypsinization of the cells had been carried out in the absence of protein or calcium ions.

The large differences in the degrees of specificity obtained when different assay methods are used appear to depend to a large extent on the flow conditions and on the organization of the collecting surfaces. McGuire and Burdick (1976) found that better results could be obtained when more irregularly-shaped aggregates were used. The relatively low levels of specificity found when the monolayer collection assay is used could be caused by the sparsity of adhesive sites on the dorsal surfaces of the monolayer cells. It is also possible that some degree of intercellular organization is required before specific adhesion can occur and that isolated tissue cells do not have this capacity even after recovery from trypsinization.

4. Models of Cell Adhesion

The mechanism of cell adhesion has recently been reviewed by Curtis (1967, 1973), Edwards (1973, 1977), Roth (1973) and Marchase et al (1976).

Three models have been proposed to explain cell adhesion. These attribute adhesion to: 1) physical forces of low specificity, determined by the bulk properties of the cell surface (Curtis, 1960), 2) direct molecular bonding between integral membrane or surface components (eg. Roseman, 1970), and 3) indirect bonding between cells mediated by extracellular bridging agents (Moscona, 1965).

The distinction between the second and third of these models is not always clear, both because it is possible that substances which appear to act as bridging agents may normally be integral components of the membrane or surface, and because some components of the cell surface may be analogous to bridging agents if they are not directly bound to the cytoskeletal system.

These three models can account for both cell-to-cell and cell-to-substrate adhesion. In the latter case, the second and third models would be applicable to the bonding between cells and a serum coat component or a deposited microexudate, respectively.

(i) Physical forces

Curtis (1960) proposed that both cell-to-cell and cell-to-substrate adhesions were determined by the interactions between electrostatic forces of repulsion and attractive London dispersion forces, analogous to those that had been postulated in the DLVO theory (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948) to account for the flocculation of lyophobic colloidal particles. The DLVO

theory predicts that two types of stable adhesion can form: one at a very close range (1nm) and a weaker, more readily reversible one at about 10-20 nm. Curtis (1960) drew attention to the similarities between these separation distances and those formed at the sites of adhesion between cells, for example at the zonula occludens and the zonula adherens, and also suggested that the effects of divalent cations on cell adhesion could be due to their decreasing the electrostatic forces of repulsion. The DLVO model of cell adhesion has been further supported by the finding that one of the separation distances found between cells and substrates could also result from adhesions in the secondary minimum (Curtis, 1964; Izzard and Lochnar, 1976).

Parsegian and Gingell (1972) suggested that this model could account for the occurrence of specific cell adhesion if there were specific London forces, however, Curtis (1973) has pointed out that the range of molecular species found at cell surfaces is too small for this to be likely.

Although there is no definitive evidence available in favour of either of the different models of adhesion, it is clear that the bulk electrostatic and London dispersion forces of cell surfaces must play a major role in determining the ability of cells to form adhesions, and perhaps also the types of adhesive contacts which will form.

(ii) Molecular components of adhesion

Most of the evidence for the involvement of specific molecular compounds in cell adhesion has come from direct attempts to isolate such substances. These have been

obtained from several sources: disaggregation and conditioned media; sera and ascitic fluids; cellular microexudates; and cell membrane- or surface- derived fractions.

Most of the substances which have been isolated belong to one of four groups: 1) acidic mucopolysaccharides and collagen, 2) glycosyl transferases, 3) lectins, 4) possible components of the cytoskeletal system, including LETS protein (see below). In some cell types, several different types of tentative adhesive substance have been studied, although in other cell types only one of these groups of compounds has been proposed to mediate adhesion. It is also possible that these substances could exert their effects on adhesion indirectly, for example by modifying the bulk adhesive properties of the cell surface.

Cell types

Sponges. The species-specific aggregation of sponge cells has been proposed to be mediated by specific adhesive factors. Moscona (1963) and Humphreys (1963) found that cells of Microciona prolifera and Haliclona occluda which were dispersed in calcium- and magnesium- free media did not reaggregate at 4 °C, but that the disaggregation media contained factors which would promote the aggregation at 4 °C of cells from homologous species. The factors

obtained from these two sponges were partially purified by Margoliash et al (1965) and the Microciona factor also by Humphreys (1965). In both studies the factors were shown to be acidic proteoglycans, although the two reports differed in their estimates of the molecular weight of the Microciona factor.

Henkart et al (1973) characterized the Microciona parthena factor as a proteoglycan with a molecular weight of several million daltons, and as having approximately equal amounts of protein and carbohydrate. The purified factor appeared in electron micrographs as an inner ring with radiating arms. Both the proposed size and shape of the factor were consistent with its physical properties, examined in a separate study (Cauldwell et al, 1973). The Microciona factor was proposed to act as a bridging agent between cells, bound to the cell surfaces with calcium ions.

An alternative explanation of the species-specific aggregation of sponge cells was provided by Curtis and Van De Vyver (1971). They showed that two strains of the freshwater sponge Ephydatia fluviata produced soluble factors which promoted strain-specific aggregation by decreasing the adhesiveness of heterologous cells. In the absence of these factors, no strain-specificity of aggregation was observed.

Hemagglutinins have also been isolated from sponge cells, although there is little evidence for their involvement in cell adhesion.(see Van De Vyver, 1975). Moscona (1968) found that rabbit antisera to the Microciona factor agglutinated cells from Microciona but not from

Haliclona. MacLennan (1969) found a similar specificity using cells taken from six species of sponge, and also found that this specificity was retained by antisera made to glycopeptide extracts prepared from whole sponges.

The above results are suggestive that specific factors may participate in species- or strain- specific interactions between sponges, for example in non-confluence or in graft rejection, however, it is not known whether these may also be involved in morphogenetic interactions between sponge cells.

Cellular slime moulds. The highly species-specific aggregation of cellular slime moulds during the onset of their cohesive phase may to some extent be mediated by developmentally-regulated lectins and lectin-receptors, present on the surfaces of these cells. Rosen et al (1973) isolated a glycoprotein from cohesive-phase cells of Dictyostelium discoideum which agglutinated sheep erythrocytes. The agglutinin was found to be absent in vegetative cells, increasing about 400-fold between 3-9 hours after the starvation of the cells, the period during which these cells gain their cohesive properties. Intact cohesive cells could be rosetted by erythrocytes, indicating that the agglutinin was present on the cell surface.

Frazier et al (1975) showed that this agglutinin could be separated into two active components with quite distinct properties and which appeared at different times during development. Reitherman et al (1975) found that both of these lectins would agglutinate glutaraldehyde-fixed Dictyostelium cells, and that the numbers of receptors for

the agglutinins present on the surfaces of the cells increased markedly during the onset of cohesiveness, paralleling the appearances of the agglutinins. The agglutinins of Dictyostelium and Polysphondylium were also found to show higher affinities for the receptors of homologous cells, suggesting that this lectin-receptor system could possibly be involved in the species-specific aggregation.

Beug et al (1973) found that a class of univalent antibodies specific for the cohesive-phase cells of Dictyostelium discoideum inhibited aggregation completely when only bound to 2% of the surface, whereas non-specific univalent antibodies which covered 80% of the surface did not affect aggregation. The binding sites for these antibodies were completely absent in vegetative cells, suggesting a similarity with the lectins isolated by Rosen et al, however, there is no evidence that these compounds are related. In another study, Sussman and Boschwitz (1975a) showed that membrane ghosts prepared from vegetative- and cohesive- phase Dictyostelium cells can retain the aggregative properties of the cells from which they are isolated, although it was not tested that this is also true of species-specific cell aggregation.

Embryonic chick neural cells. Most of the attempts to isolate adhesive factors from vertebrate cells capable of showing specific adhesion have been made using embryonic chick neural cells. Moscona (1962) obtained a factor from the conditioned media of NR (neural retina) cells which specifically enhanced the aggregation of homologous cells. Lilien and Moscona (1967) were able to obtain larger yields of this factor by collecting the medium from monolayers of NR cells which had been incubated for 24 hours in serum-free medium, and obtained similar results. Garber and Moscona (1972b) also isolated a factor from cerebrum cells, using the same method, which specifically increased the diameter of cerebrum aggregates. A small degree of species-specific activity of the factor was found when chick and mouse cerebral cell aggregates were compared.

The NR factor was purified by Hausman and Moscona (1973,1975) and found to be a glycoprotein of about 50,000 daltons and containing about 10-15% carbohydrate. The factor was inactivated by trypsin, but not by hyaluronidase, neuraminidase, galactose oxidase or periodate, suggesting that the protein but not the carbohydrate portion of the molecule is required for activity. One criticism of this work would be that the size of 24 hour aggregates is not a direct reflection of cell adhesiveness, and it is difficult to know what cellular property is being affected by the factor. Another problem is that the incubation of the cells in serum-free medium during the preparation of the factor is likely to cause cell leakage, as well as other cell damage, and to increase the extent of proteolytic activity.

Roth (1968) found that conditioned media obtained from cultures of chick NR, liver and heart ventricle cells increased the collection rates of cells by homologous aggregates, but did so both when homologous and heterologous conditioned media were used. Curtis (1974) prepared conditioned media from monolayers of chick NR and liver cells, incubated in the presence of serum . He found that these cells released soluble factors into the media which decreased the adhesiveness of the other cell type. Adhesiveness was measured using the cell lawn (monolayer) collection assay. However, the factors present in the conditioned media were not characterized in either of these studies.

Rutishauser et al (1976) prepared antibodies to proteins purified from the conditioned media obtained from both monolayer cultures and intact tissues of chick neural retina. Using immunofluorescence microscopy to test the binding of the antibodies, they found that two proteins, with molecular weights of 140,000 (F1) and 110,000 (F2, a dimer), were present on the cell surface. These appeared to be proteolytic cleavage products of cell surface precursor molecules having molecular weights of 240,000 and 150,000, respectively. The former appeared to be converted to the latter in normal tissues and in suspension, but not in monolayer,culture. Antibodies to F2, but not to F1,inhibited cell-to-cell adhesion, both when neural retinal and when cerebral cells were tested. Antibodies to F1 and to F2 also stained embryonic chick fibroblasts, but not thymocytes or erythrocytes. The authors suggested that adhesion might be mediated by identical F2 monomers on adjacent cells and be controlled by proteolytic cleavage of the precursor molecules.

Lilien (1968) isolated aggregation promoting materials from the growth media of NR monolayer cultures. These were reported to bind specifically to and to specifically enhance the aggregation of homologous cells. Balsamo and Lilien (1975) found that the abilities of the NR and cerebral factors to bind to homologous cells were relatively insensitive to treatment with pronase, but were reduced by treatment with β-N-Acetylhexosaminidase or α-mannosidase, respectively. Pronase digestion of the two factors showed that these contained N-Acetylgalactosaminyl and mannosaminyl residues. The factor-induced aggregation of homologous cells appeared to be more complex and to require the participation of a third, more labile component (Balsamo and Lilien, 1974).

Merrell and Glaser (1973) found that plasma membranes isolated from NR and cerebellar tissues specifically bound to and inhibited the initial rates of aggregation of homologous cells. Gottlieb et al (1974) obtained similar results with membranes prepared from NR and optic lobe tissues, and also found an age specificity between membranes from tissues of 7,8, and 9 day embryos. Merrell et al (1975) were able to solubilize these membranes in lithium diiodosalicyclate, and obtained protein fractions showing the same tissue and age specificity as the membranes from which they were isolated. However, it is possible that these factors are altering the surfaces in an indirect way, for example by steric hindrance. Preliminary attempts to determine the molecular weights of the solubilized proteins gave contradictory results.

There is some evidence for the involvement of galactosyl transferases in the adhesion of NR cells. Roseman (1970) proposed that the formation and breakage of bonds between glycosyltransferases and their substrates on the surfaces of adjacent cells could provide a general means of controlling cell adhesion. Roth et al (1971b) showed that NR cells catalysed the transfer of labelled UDP-galactose to both exogenous and endogenous acceptors, indicating the presence of externally exposed galactosyl transferases on these cells. Roth et al (1971a) found that treatment of NR aggregates with a glycosidase-rich extract of Diplococcus pneumoniae abolished the 25 minute lag period found in the collection of homologous cells by these aggregates. Treatment with two partially purified β-galactosidases increased the non-specific collection of both NR and liver cells. These results pointed to a possible role of terminal β-galactoside groups in the specific adhesion of NR cells. A similar treatment of liver cell aggregates did not have this effect.

The effects shown by the transferases could have resulted primarily from their biosynthetic activity, rather than from a direct interference with a specific adhesive process. Roth and Shur (1973) showed by autoradiography that the activities of several glycosyl transferases in early chick embryos had different patterns of distribution which could in some cases be related to known morphogenetic events. However, the same criticism could apply to this finding.

A number of adhesive factors have been isolated from other types of non-fibroblastic cells, but very few of these have been characterized in any detail. Two examples will be mentioned here.

Mouse teratoma cells. Oppenheimer and Humphries (1971) isolated an aggregation factor from mouse teratoma cells which was capable of binding the cells together without a requirement for cellular metabolism and which showed a preference for homologous cells. Oppenheimer (1972) showed that there appeared to be a specific carbohydrate requirement for the factor-mediated adhesion. A similar factor was partially purified by Meyer and Oppenheimer (1976) who found that at least two components of the factor were responsible for the aggregation-promoting activity, one of which showed some activity by itself and another which enhanced the activity of the first. Mouse teratoma cells have been reported to show specific adhesion with secondary culture mouse kidney adult cells (Walther et al, 1973).

Mammalian lymphocytes. Curtis and De Sousa (1973) showed that mouse B and T lymphocytes maintained in short term cultures released factors into the medium which decreased the adhesiveness of the heterotypic cell type. These were found to have molecular weights of 3000 and 9000 daltons respectively (Curtis and De Sousa, 1975), and their dose-response curves of binding to the cells suggested that they were acting indirectly by modifying the adhesive properties of the cell surfaces. No species-specificity was found between the factors prepared from a variety of cell types. It was also found that these factors could redirect lymphocyte positioning when administered *in vivo*.

Fibroblasts. Several different types of substance have been proposed to play a role in the adhesion of fibroblasts. One of these has been termed LETS (large external transformation sensitive) protein by Hynes (1973). Hynes exposed NIL-8 hamster fibroblasts to (^{125}I)-lactoperoxidase, which selectively labels the tyrosine residues of externally exposed proteins. He found that one strongly labelled band, corresponding to the LETS protein, was absent in sarcoma virus-transformed NIL cells and was also very sensitive to small doses of trypsin. It was later found that LETS protein decreased in normal NIL cells during mitosis, increased as the cells approached confluence and could be reduced by serum stimulation of the confluent cultures (Hynes and Bye, 1974).

Large glycoproteins which may be identical with or very similar to LETS protein have been isolated from the membranes or surfaces of a number of fibroblast cell lines and also embryonic fibroblasts. These appear in each case to be lost after viral transformation and range in molecular weights from about 210,000-270,000 (reviewed by Hynes, 1976). Chen et al (1977) also found a correlation between the tumorigenicities of several adenovirus-transformed cell lines and their reductions in LETS protein, although this correlation has not been found in some other cases (Hogg, 1974; Pearlstein et al, 1976).

There is some evidence that LETS proteins may be involved, either directly or indirectly, in cell-to-substrate and cell-to-cell adhesion. Yamada et al (1976a; 1976b) and Ali et al (1977) showed that the addition

of LETS protein to transformed fibroblasts both increased the adhesiveness of the cells to the substrate and restored their morphologies to those of their untransformed counterparts. Yamada et al (1976a) suggested that the CSP (cell surface protein) acted primarily on the adhesiveness of the cells and that the changes in cell morphology were a consequence of this.

Wartiovaara et al (1974) found that the binding of antibodies made to the SF (surface antigen) human fibroblasts was restricted to membrane processes and ridges and appeared to be associated with sites of adhesion of the cells to the substrate. The binding sites for the antibodies were removed by trypsinization of the cells, but reappeared one hour after the reattachment of the cells, in correspondance with the appearance of new membrane processes.

Mautner and Hynes (1977) presented some evidence for the presence of LETS protein on the ventral surfaces of attached fibroblasts, at sites of close apposition to the substrate. The LETS protein could be observed underneath the cells as early as 4-5 hours after trypsinization and replating of the cells. Attached cells which were removed with EDTA left behind footprints containing LETS protein, but only if the cells had been cultured in low levels of serum (0.3%) as opposed to in 5% serum, possibly due to the higher adhesiveness of the cells to the substrate in the first case. The footprints were similar in size to the footpads described by Revel et al (1974; see Introduction 2).

Albrecht-Buehler and Chen (1977) showed that in cultures of 3T3 cells, LETS protein appeared to accumulate at sites of intercellular contact. Chen et al (1977) reported that the small amounts of LETS protein that could be detected in several highly tumorigenic cells were found mainly in fibrils interconnecting the cells. Mautner and Hynes (1977) mentioned that in some cases continuous fibers of LETS protein could be seen running through two cells. The numbers of fibers in these cells increased with time during culturing until the fibers completely surrounded the cells in confluent cultures.

These findings and also the correlation often found between the appearances of LETS protein and of microfilament bundles, as well as the detection of actin in immuno-precipitates of LETS protein (Wartivaara et al, 1974), suggest that this protein may be involved in the coupling of adhesive contacts to the cytoskeletal system. However, there is very little evidence that LETS protein contributes to the adhesive contacts themselves.

The embryonic chick fibroblast LETS protein (CSP) has been shown to agglutinate sheep erythrocytes, and also several types of tissue cells. The hemagglutination required divalent cations and was inhibited by L-lysine but not by simple sugars, and is therefore quite distinct from lectin-mediated hemagglutination. (Yamada et al, 1975). Pearlstein (1976) showed that the cell adhesion factor (CAF) of BHK fibroblasts was secreted into the medium by both the normal and virus-transformed cells, but that the latter were unable to retain CAF on their surfaces.

The addition of CAF to cultures of the transformed cells restored the abilities of these cells to attach to and spread on collagen. The CAF-mediated attachment of the cells was inhibited by L-lysine and required divalent cations, similar to the hemagglutination properties of the embryonic chick CSP, and was therefore quite distinct from the galactosyl transferase-mediated attachment of platelets to collagen (Jamieson et al, 1971). It is therefore possible that LETS protein may be required for the attachment of fibroblasts to collagen, and possibly also to the surface-bound collagen of other cells.

It is not clear to what extent the LETS proteins of different cell types share the same properties. There have been three reports of the isolation of LETS protein from membranes (Hogg, 1973; Bussell and Robinson, 1973; Stone et al, 1974). However, Graham et al (1975) showed that the LETS protein of NIL cells sedimented in a high density fraction, distinct from the plasma membrane fraction, during differential centrifugation. This fraction was enriched in carbohydrates and appeared in electron micrographs as an amorphous meshwork, and was suggested to have been derived from the surface coat. The findings that LETS protein is not solubilized in non-ionic detergents (Hynes et al, 1976) and that it does not patch following the binding of antibodies (Mautner and Hynes, 1977) also indicate that it is unlikely to be an integral membrane component. No studies have been made of LETS proteins in cell types which show specific adhesion. Rutishauser et al (1976) found that the chick neural retinal cell adhesion molecule (CAM) differed from LETS protein in the sizes of

its cleavage products, and also found no immunological cross-reaction between the two molecules, but did not rule out the possibility that these were related.

Dysart and Edwards (1977) isolated a lectin (hemagglutinin) from BHK fibroblasts. The lectin was present in both endoplasmic reticulum and plasma membranes, the latter having the highest activity. Hemagglutination was not affected by solubilization of the membranes in 1% deoxycholate, but was inhibited by two derivatives of D-galactose (D-fucose and N-acetylgalactosamine), although D-galactose itself showed very little inhibition.

Some evidence has been found for the possible involvement of galactosyl or N-acetylgalactosaminyl groups in BHK cell adhesion. Vicker and Edwards (1972) showed that treatment with neuraminidase stimulated the aggregation of these cells but not of the poorly aggregating polyoma virus-transformed derivatives, and suggested that this could be explained by the exposure of the above sugar groups on the cell surface following treatment. Vicker (1976) further showed that BHK cell aggregation was inhibited by galactose oxidase-sensitive tryptic glycopeptides prepared from the same cells. Edwards et al (1976) also found that a series of ricin-resistant mutants of these cells showed a reduced adhesiveness to substrates and a decreased rate of aggregation. This was only found in mutants which were deficient in ricin binding. Ricin binds to galactosyl and N-acetylgalactosaminyl groups.

The evidence that animal cell lectins are involved in cell adhesion is only indirect, with the possible exception of the slime mould lectins (see earlier). Hemagglutinins have been isolated from L6 myoblasts (Gartner and Podleski, 1975) and embryonic chick myoblasts (Den et al, 1976). In both of these studies, hemagglutination was found to be inhibited by lactose and thiodigalactoside, and in the first case, but not in the second, thiodigalactoside was shown to inhibit myoblast fusion, apparently by preventing the formations of adhesions between the myoblasts. The lack of agreement between these two reports could be due to the different cell types used.

The possible role of these hemagglutinins in normal myoblast fusion is suggested by the findings (Nowak et al, 1976; Den et al, 1976) that the appearance of hemagglutinin activity in embryonic chick myoblasts paralleled the cessation of mitosis and the onset of fusion of these cells. Teichberg et al (1975) also showed that lactose- and thiodigalactoside- sensitive lectins were present in a number of tissues and cell lines, but appeared to show the highest activity in rapidly differentiating neural and muscular tissues.

It is likely that lectins in different cell types and also different lectins in the same cells may have diverse functions. The characteristic presence of lectins in cell membranes (eg. Stockert et al,1974; Teichberg et al, 1975; Dysart and Edwards, 1977) suggests that some of these functions may be associated with interactions

between cells. Stockert et al (1974) described a rabbit hepatic lectin which appeared likely to play a role in the recognition of liver cells by serum asialoglycoproteins. Teichberg et al (1975) showed that the binding of the electric eel lectin to erythrocytes was rapidly reversed by the addition of lactose, indicating the possibility of the control of cell adhesion by lectins in an analogous manner to that proposed by Roseman (1970) for glycosyltransferase-mediated adhesion. In some cases, lectins may act primarily as glycosyltransferases or glycosidases, although Teichberg et al mention that the former is unlikely to be the case for the lectins described in their study.

Lectins differ from LETS protein in several respects. Their sensitivities to inhibitors of hemagglutination are quite distinct (see above). The BHK cell lectin was found not to be decreased after viral transformation or after treatment with trypsin (Dysart and Edwards, 1976), in contrast to LETS protein. These two molecules are also likely to have very different molecular weights. Teichberg et al (1975) estimated the molecular weight of the electric eel lectin to be about 33,000 daltons.

Acidic mucopolysaccharides (AMP'S) may also play a role in the adhesions of fibroblasts. Culp and Black (1972) showed that 3T3 cells and flat revertants of sarcoma virus-transformed 3T3 cells deposited 3-6 times more microexudate than did the transformed cells. Terry and Culp (1974) found that the microexudates of the

normal and transformed cells were similar in composition, in both cases consisting mainly of small amounts of protein linked to hyaluronic acid chains of high molecular weight.

Moore (1976) similarly reported that Rous sarcoma virus-transformed rat fibroblasts produced 3-6 times less substrate-coating material than the normal fibroblasts, but that this amount was increased 7-fold by the addition of dibutyryl cyclic AMP, which also increased the adhesiveness of the cells to the substrate. Kraemer (1976) found that variants of CHO cells which had been selected for increased resistance to detachment from the substrate also produced higher amounts of hyaluronic acid.

Pessac and Defendi (1972a) showed that mouse lymphoblasts were aggregated by MPS factors present in serum and in the conditioned media obtained from the cultures of a number of different cell types. The factors had hyaluronic acid as a major component and were inactivated by hyaluronidase. Hyaluronic acid was also effective in stimulating aggregation. Pessac and Defendi also suggested that some of the previously described aggregation factors, for example the sea urchin factor described by Kondo and Sakai (1971) might also be hyaluronic acid. However, Edwards et al (1975) mentioned that 12.5 g/ml bovine testicular hyaluronidase did not inhibit the aggregation of BHK cells.

With the exception of the sponge factors, there is no evidence that AMP's are involved in specific cell adhesion. However, the selective release or degradation of AMP's could contribute to the control of cell

movements, for example in morphogenesis (Toole, 1973) and in the invasiveness of metastatic tumour cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

Media

The most frequently used media were:

ECT, EFT. Eagles minimal essential medium (Glasgow modification), supplemented with 10% bovine serum (ECT) or 10% foetal bovine serum and 10% tryptose phosphate broth.

Hanks BSS, Hanks-HEPES. Hanks balanced salt solution, pH 7.4, buffered with Tris (BSS) or with 10 mM HEPES (Hanks-HEPES). In some adhesion assays the latter was supplemented with 10% bovine serum or 10% foetal bovine serum.

CMF-HEPES. Calcium- and magnesium- free Hanks-HEPES. Contained per litre: NaCl 8.0 gm, KCl 0.4 gm, Glucose 1.0gm, KH_2PO_4 0.5 gm, HEPES 2.38 gm, Phenol red (0.5%) 2.0 ml, pH 7.4.

PBS. Phosphate-buffered saline. Contained per litre: NaCl 8.0 gm, KCl 0.2 gm, KH_2PO_4 0.2 gm, $Na_2HPO_4.2H_2O$ 1.15 gm, pH 7.2.

Cell lines

The following cell lines were used:

Hamster. BHK-C13 (Baby Hamster Kidney), PyC1 (Polyoma virus-transformed C13 cells, recloned), PyAA (a subclone of PyC1 cells, selected for resistance to aza-adenine). CHO-K1 (Chinese Hamster Ovary cells, proline auxotrophs, kindly provided by Dr. R. Wilson), HEF (Hamster Embryo Fibroblasts), and HAK (Hamster Adult Kidney epithelial cells; S. Afr. J. Med. Sci. (1963) 28, 81)). Primary Hamster Embryo Fibroblasts (HEF) were also used.

Mouse. S-180, L929 and 3T3 (Swiss mouse).

Culture of Cell Lines

The cell lines were routinely cultured in ECT in glass culture bottles (area 60 cm^2 or 120 cm^2). A 500 mM L-proline supplement was required for the CHO-K1 cells. Cells from confluent or nearly confluent cultures were removed with 0.05% Trypsin (Difco 1:250) in Tris-buffered saline and collected in ECT. In order to obtain confluent cultures of the cells after two days the appropriate numbers of cells seeded were (per 120 cm^2): for C13, HEF, and HAK cells, 5×10^6 cells and for the other cell types, 3×10^6 cells. The cells were subcultured at 2-4 day intervals and were discarded after 4-5 weeks. Cell stocks were stored at -70°C or in liquid nitrogen in a glycerol-based freezing medium (Eagles 65%, Foetal Calf Serum 30%, Glycerol 5%). The stocks were thawed at 37°C and plated in EFT for one day, or until enough cells had attached, after which the cells were cultured by the above method.

Embryonic Chick Cells

Dissections were performed under aseptic conditions in a laminar flow hood. The tissues were placed in Hanks-HEPES at room temperature and were collected in ice-cold EFT after the dissociation of the tissues. Tissues which did not require tryptic dissociation were placed directly in cold EFT. Fibroblasts, glial and neural cells were isolated from 10 day, 10-11 day and 7 day embryos, respectively.

Fibroblasts.

Fibroblasts were prepared skin, heart, limbs and

meninges. A similar procedure was used in each case, with the exception of the meningeal cells which were isolated without tryptic dissociation.

Skin. Skin was peeled from the anterior head region starting at the periocular end, taking care to avoid contamination by the underlying tissues. The most heavily vascularized areas were also removed. The tissues were cut into small pieces (2-3 mm across) and were incubated in 0.25% Trypsin (Difco 1:250) in Tris-buffered saline, containing 0.1 µg/ml DNAase for 25 mins at 37°C. The tissues were then aspirated 100 times using a Pasteur pipette, and were spun at 300 g for 5 mins. Most of the undissociated tissue, including much of the epithelium, formed a fluffy layer which floated on the medium and could be removed. The pellet was dispersed in 20 mls EFT, collected in a 5 ml disposable syringe and passed gently through a strip of 50µ-mesh Nitex (nylon bolting) placed inside a Millipore filter assembly. A 100µ mesh Nitex prefilter was often necessary. The cells obtained were plated in plastic tissue culture flasks (area 25 cm^2) or in glass bottles and checked microscopically for the absence of epithelial cells.

Heart. Heart ventricles were removed, parts of the pericardia were peeled off and the blood was squeezed out. The ventricles were cut into 1 mm^2 pieces and were trypsinized for 30 mins at 37°C. All other aspects of the dissociation and plating procedures were the same as for skin fibroblasts. The cultures were washed twice at about 60-90 mins after being plated in order to remove most of the

myoblasts, and were subcultured at least twice before being used.

Limbs. Upper thighs were cleaned of bones and skin and cut into small pieces (1-2 mm across). The same procedure was followed as for the heart cells except that the limb tissue often became sticky and had to be diluted before being passed through the Nitex cloth.

Meninges. Meninges were removed from the cerebral lobes and cleaned of surrounding tissues. Fibroblastic cells were obtained by gentle aspiration of the whole tissues using a Pasteur pipette. Treatment of the tissues with the standard dose of trypsin appeared to damage the cells.

Neural cells. Neural retinas were trypsinized (0.25% Trypsin, Difco 1:250, in Tris-buffered saline) for 30 mins at 37 °C. The dissociated tissues were diluted with EFT, aspirated 40 times using a Pasteur pipette, spun at 300 g for 5 mins and collected in 20 mls EFT. Optic and cerebral lobes were dispersed by aspirating the tissues using a Pasteur pipette and removing the clumps of cells either by sedimentation at 1 g or by filtration through a 50μ mesh Nitex cloth. Predominantly single cell suspensions were obtained using this method.

Glial cells. Glial cells were prepared from neural retinal, optic lobe and cerebral tissues, using the same method in each case. Three or four embryos were usually sufficient for the preparation of all three tissues. Meningeal and other surrounding tissues, including vascular areas, were carefully removed. The neural tissues were aspirated

gently using a Pasteur pipette: 40, 25 and 20 times, respectively, for neural retinal, cerebral and optic lobe tissues, in order of their increasing brittleness. The suspensions were passed through a 50μ mesh Nitex cloth and examined by phase contrast microscopy. Depending on the degree of tissue disruption, approximately 6, 8and 8 $\times 10^6$ cells were plated in plastic tissue culture flasks (area 75 cm^2) in EFT. A larger number of cells was required if the suspensions consisted mainly of single cells. The flasks were shaken inside the incubator to prevent the cell aggregates from clustering in the centers of the flasks.

Monolayers of glial cells formed after 3-6 days and were detached by adding 4.5 mls of 0.05% Trypsin (Difco 1:250) for one minute and collecting the cells in EFT after a further 3-5 minutes. The monolayers came off as large sheets of cells which were then aspirated using a Pasteur pipette and replated in smaller plastic tissue culture flasks (area 25 cm^2) to total about 1-3 times the area from which they were taken, the amount depending on the sizes of the cell sheets. A larger area was required if the sheets could not be well dispersed. The cells were subcultured 2-3 times over a 6-10 day period before being used in experiments. Predominantly single cell suspensions were obtained after the second passage.

Radioactive Labelling

Cells were labelled in glass culture bottles (area 60 cm^2) with 100 µCi of (^{32}P)-orthophosphate (obtained as 1.0 mCi/ml in dilute HCl, Radiochemical Centre, Amersham) for 8 hours and chased overnight in EFT. This normally gave counts of 0.03-0.1 cpm/cell. Cerebral cells were labelled as large tissue pieces in suspension and were dissociated by aspiration using a Pasteur pipette after being chased. Neural retinal cells were plated at 4×10^7 cells per bottle for labelling. All other cell types were plated so as to be confluent 1-3 days after the addition of label.

Cell Sheet Collection Assay

A modification of the assay described by Walther et al (1973) was used. Monolayers or multilayers of the different cell types were plated in Linbro multiwell dishes (area of well 1.5 cm^2) about 15-20 hours before the assay. The numbers of cells seeded per well were: 6×10^5 for C13, HEF and HAK cells; 4.5×10^5 for CHO, L929 and PyAA cells 1.2×10^6 for embryonic chick fibroblasts; 8×10^6 for neural retinal cells; 6×10^5 for glial cells. Prior to the assay the sheets were inspected visually for the presence of gaps and were washed twice in Hanks-HEPES or Hanks-HEPES plus 10% serum.

Labelled cells were removed with 0.05% trypsin by the same procedure as for subculturing and were washed 3 times at 300 g for 3 mins in Hanks-HEPES plus 10% serum in order to remove all traces of trypsin from the medium and any label released from trypsin-damaged cells. The single cell suspensions were diluted to $1-2\times10^5$ cells/ml

and added to the sheets in 0.5 ml aliquots. After a given time interval, non-adherent or weakly attached cells were removed by three standardized washes using a water-driven suction pump. Two standards of washing force were used: a strong wash of 1 ml, delivered from a 10 ml disposable syringe, and a weak wash of 0.5 ml, in which the medium, CMF-HEPES at 37°C, was gently emptied from an automatic pipette.

The attachment of labelled cells to serum-coated plastic was also measured using this technique. The wells were precoated with 0.5 ml of 100% bovine or foetal bovine serum and rinsed with CMF-HEPES or saline prior to the assay. In some experiments, serum-coated wells were used as controls for the attachment of cells to monolayers.

Cell Fixation

Cell sheets were fixed for 30 mins in 2% formaldehyde, or in 1% glutaraldehyde, in PBS and washed 10 times with PBS and once with Hanks-HEPES. In some experiments, fixed monolayers were treated with sodium periodate (25 mM) for four hours or overnight followed by 2 washes in 0.5% glycerol and two washes in Hanks-HEPES. Cell suspensions were fixed in the same manner and were washed 3 times in Hanks-HEPES at 300 g for 3 mins.

Time Lapse Filming

Secondary culture glial cells were plated onto 32X32 mm glass coverslips (2.5 or 5.0×10^5 cells/coverslip) and were incubated overnight in 60mm Petri dishes. Prior to filming, a filming chamber was prepared by sealing a clean coverslip onto one side of a 1mm thick steel plate having a

circular aperture of 17 mm, using a 1:1 mixture of paraffin wax and vaseline. The chamber was filled with Hanks-HEPES and the coverslip bearing the cells was sealed onto the opposite side of the plate, using the same wax mixture. The filming was carried out in the presence of an air curtain incubator set at 37 °C, using a Leitz Ortholux microscope with an attached Bolex camera set at 15 secs per frame. Kodak Plus-X reversal film was used, and was developed by Brent Laboratories Ltd.

Preparation of Hemagglutinating Activity

Whole neural retinal, optic lobe or cerebral organs were placed in ice-cold Hanks-HEPES, carefully cleaned of all surrounding tissues and rinsed twice with cold Hanks-HEPES to remove the remaining blood cells. The homogenization and fractionation procedures were based on the methods of Merrell and Glaser (1973) and Dysart and Edwards (1977). The tissues were diluted with 7 volumes of PBS and placed in a Potter-Elvejhem homogenizer with a close-fitting Teflon pestle set at 2000 rpm. About 30 strokes were needed to disrupt about 90% of the cells as judged by phase contrast microscopy. In some cases, the PBS was supplemented with 50 µg/ml DNAase, 1.5 mM EDTA, or 1.0 mM $CaCl_2$ + 1.0 mM $MgCl_2.6H_2O$.

The homogenate was spun at 2500 g for 5 mins in an MSE 25 bench centrifuge (this step was optional) and at 13,500 g for 15 mins in an MSE 65 superspeed centrifuge set at 2 C, using the 10 ml rotor. The pellet was resuspended in 1.0 ml PBS and the supernatant was spun at 90,000 g for 90 mins under the same conditions. The

resulting supernatant was collected and the pellet redispersed in 0.1-0.3 mls PBS. The various fractions were kept on ice and either assayed immediately or stored in 1.0 ml aliquots at -20 C.

Preparation of Conditioned Medium

Neural retinal, optic lobe and cerebral cells were incubated for 24 hours in EFT, either as pieces of tissue in suspension or as cell monolayers (neural retina only). The conditioned media were obtained during a further incubation of the cells or tissues for 24 hours in either Eagles medium or EF, supplemented in some cases with Trasylol (200 units/ml), and were assayed directly, or after low speed centrifugation (300 g for 5 mins) to remove suspended cells.

Hemagglutination Assay

Rabbit, sheep and chicken red blood cells were fixed by the method of Butler (1963). The cells were washed three times in 20 volumes of PBS, diluted 1:1 with 3% formaldehyde and incubated for 18 hours at 37°C on a gyratory shaker. The fixed cells were washed five times in 2.5 volumes of PBS and stored as a 10% suspension in PBS. In some cases, the cells were trypsinized prior to fixation by the method used by Den et al (1976). A 4% suspension of red blood cells was incubated in 0.1% trypsin (Difco 1:250) for 1 hour at 20°C. The trypsinized cells were washed five times in PBS prior to fixation in formaldehyde. The hemagglutination assay was performed by adding a 50 µl aliquot of the fraction to be tested to V-shaped microtitre

wells, and making serial two-fold dilutions in the wells. Following this, 50 µl of a 2.5% suspension of erythrocytes was added to each well. When sugar inhibition was to be tested, 25 µl of sugar solution was added prior to the red blood cells to make a total of 100 µl per well. The wells were sealed with paraffin, shaken briefly and left for at least two hours before the determinations were made.

Statistical Test

The Wilcoxon-Mann-Whitney test was used. This test has the advantage of being non-parametric, and is quite accurrate for >4 samples from each group. The formula used is as follows:

$$\hat{z} = \frac{R_1 - n(n+m+1)/2}{\sqrt{n.m(n+m+1)/12}} ,$$

where R_1 = Rank sum for n samples

n,m = numbers of samples in each group

When P = 0.05, $\hat{z} \simeq t_\infty$ = 1.96. Values >1.96 are significant.

When P = 0.01, $\hat{z} \simeq t_\infty$ = 2.58

RESULTS AND DISCUSSION

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I. Adhesion of Fibroblasts

The adhesive interactions between a number of fibroblastic cell types were examined in order to determine:

1) Whether fibroblastic cells have the capacity to show specific adhesion with other fibroblasts, and whether there is any evidence that different fibroblasts have different adhesive mechanisms; and

2) The range of adhesiveness that can be found between different types of fibroblast, both between fibroblasts from different embryonic tissues and between normal and transformed fibroblastic cell lines.

RESULTS

1. Embryonic Chick Fibroblasts

Fibroblasts were isolated from four embryonic chick tissues, chosen to give a fairly representative selection of different regions of the embryo: anterior head skin, cerebrum meninges, heart ventricle and thighs. During the culturing of the cells, it was found that there were in some cases distinct morphological differences between fibroblasts from different tissues, both in growth phase and in confluent cultures. Heart fibroblasts in growth phase cultures could be readily identified by their smooth contours (Plate 1a), in contrast to the more jagged appearances of skin and meningeal (Plate 1b) fibroblasts. Confluent cultures of heart fibroblasts were characterized

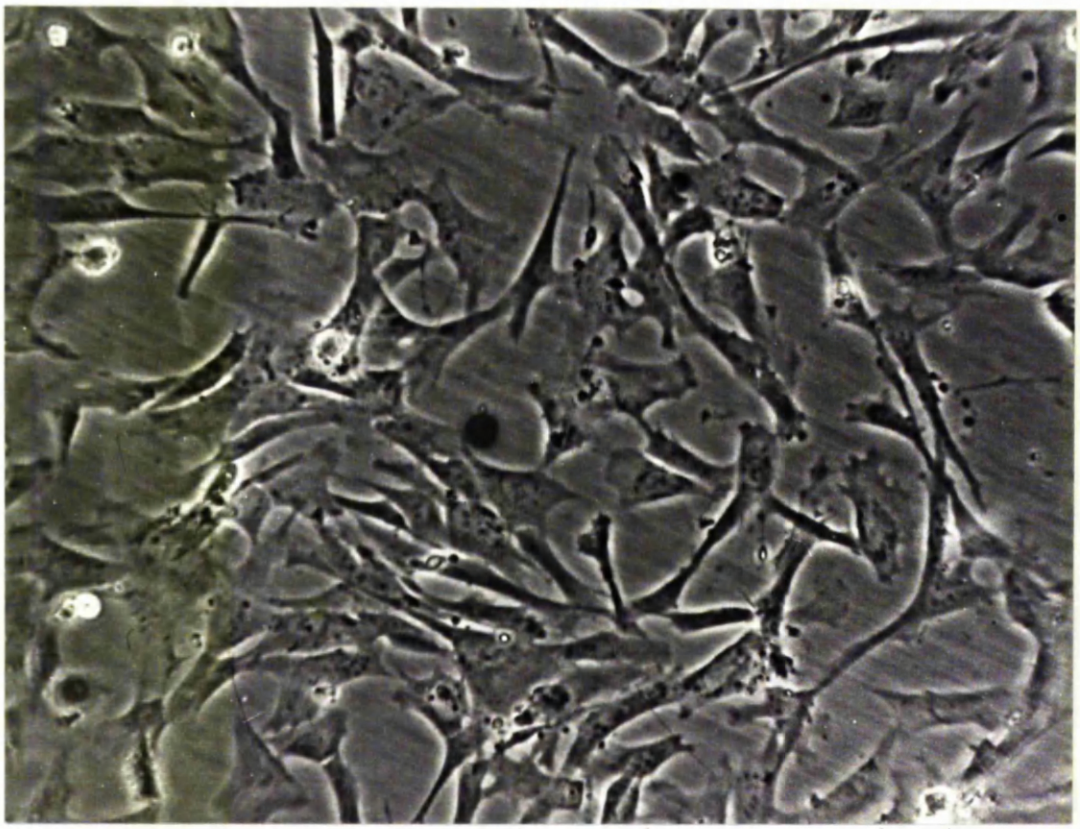

Plate 1 (a). Secondary culture embryonic chick heart fibroblasts. Magnification X 1400.

Plate 1 (b). Secondary culture embryonic chick meningeal fibroblasts. Magnification X 1400.

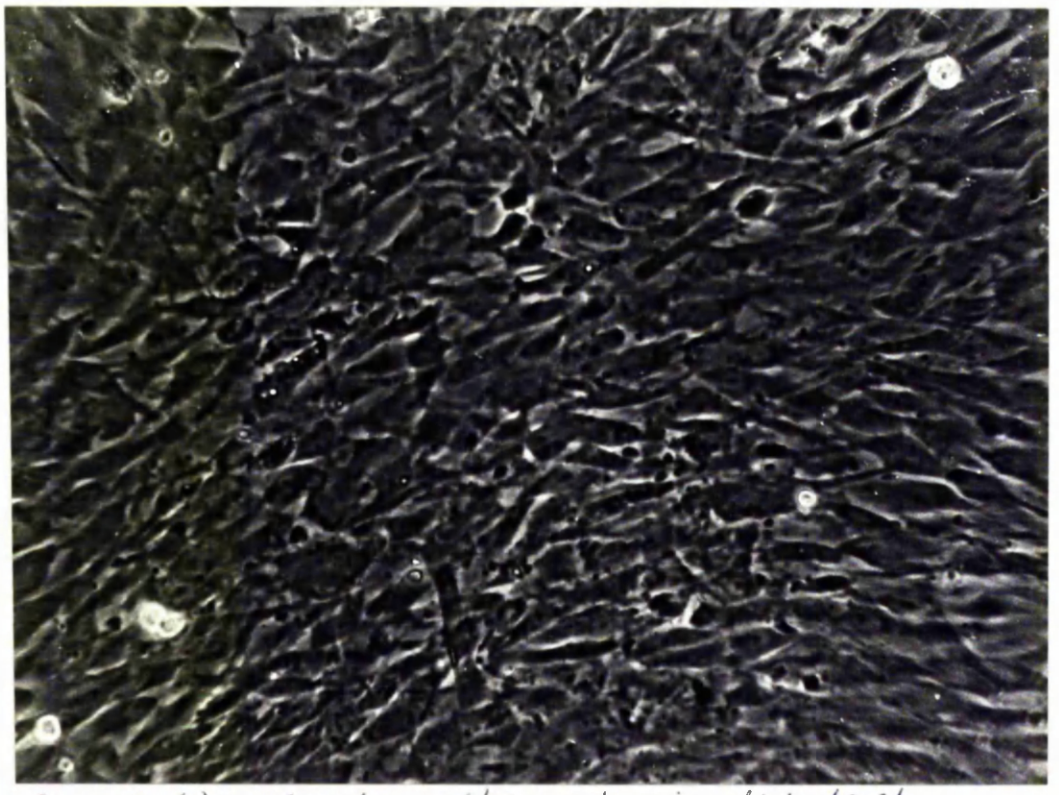

Plate 1 (c). Secondary culture embryonic chick heart fibroblasts. Confluent cultures. Magnification X 1100.

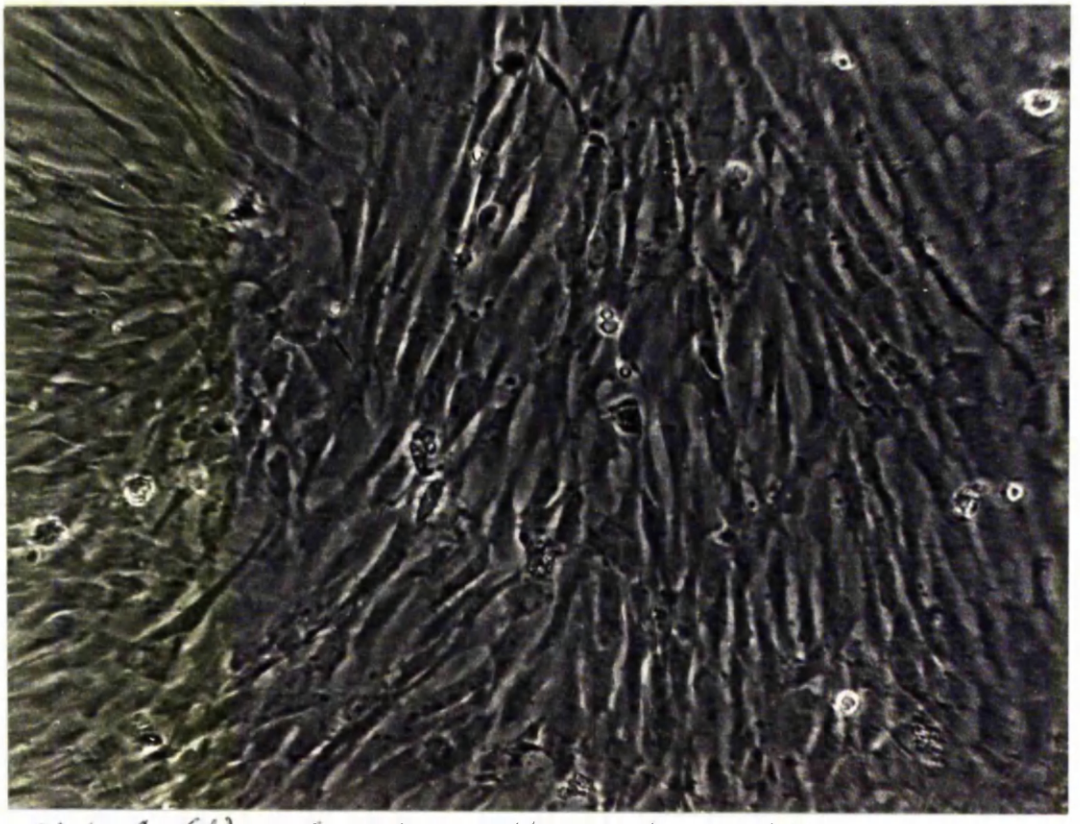

Plate 1 (d). Secondary culture embryonic chick skin fibroblasts. Confluent cultures. Magnification X 1100.

by poorly oriented cells with large overlapping processes (Plate 1c), whereas skin and limb fibroblasts were more elongated and were oriented in parallel arrays in confluent cultures.

The adhesive interactions between the four types of fibroblast were measured using the cell sheet collection assay (see Materials and Methods). This assay can be used to examine the adhesive interactions between reciprocal combinations of different cell types, and therefore provides a means of testing for the occurrence of specific cell adhesion, and also of determining the relative strengths of adhesion of different cell types under the same conditions. Some of the features of this assay will be described later.

The results of 3 assays are shown in Table 1. The fibroblasts did not show any specific adhesion, and the cell sheets of the different fibroblasts appeared to differ quantitatively in adhesiveness, such that skin $\gg$ limb $>$ heart. In all cases, the skin fibroblast cell sheets were more adhesive than those of heart cells. It is more difficult to compare the relative adhesivities of the seeded cells since these are likely to be influenced by extrinsic variables, such as the viability of the cells or the extents of their recovery from trypsinization.

Table 1 shows that the adhesivities of the cell sheets were generally very consistent within each experiment, but that there could be substantial differences between their adhesivities on different days. These

Table 1

Adhesion of Embryonic Chick Fibroblasts

Seeded Cells	Expt	Cell Sheets: Skin	Limb	Heart
Skin	1	78.2 ± 0.2		68.3 ± 0.6
	2	60.9 ± 2.5	50.1 ± 0.1	46.7 ± 1.6
	3	62.9 ± 2.1	62.6 ± 1.9	47.9 ± 4.4
	Ave	67.3 ± 8.4	56.3 ± 7.3	54.3 ± 11.1
Limb	2	57.4	50.0 ± 5.5	48.6 ± 2.0
	3	65.9 ± 1.1	69.7 ± 7.1	58.5 ± 3.5
	Ave	63.8 ± 4.3	59.8 ± 12.5	53.5 ± 6.1
Heart	1	74.1 ± 8.0		67.8 ± 0.3
	2	55.9 ± 3.3	44.7	37.6 ± 2.7
	3	61.2 ± 5.4	71.6 ± 1.1	48.3 ± 5.2
	Ave	63.7 ± 9.6	62.6 ± 15.5	51.2 ± 14.0
Meninx geal	2	60.7 ± 1.6		59.7 ± 4.3
	3	36.9 ± 2.0		33.1 ± 19.7
	Ave	48.8 ± 13.2		46.4 ± 19.2

Values are means ±S.D. based on individual duplicate or triplicate cell sheets in each experiment.

Averaged values ±S.D. are based on the total numbers of cell sheets used in the separate experiments.

Values are percentages of total radioactive counts in the cell suspensions which have attached to the cell sheets.

Assays were performed in HH + 10% Fetal Calf Serum.
Time = 40 mins.

differences could depend to a large degree on the extents to which the seeded cells had been damaged by trypsinization.

The results obtained suggest that fibroblasts from different embryonic chick tissues are likely to differ only to small extents in adhesiveness. Therefore, it was decided to test whether larger adhesive differences could be found between normal and transformed fibroblasts, and for this purpose a number of fibroblastic cell lines with marked morphological differences were examined. The availability of a series of fibroblastic cell types showing a wide range of adhesiveness would make it possible to test whether these cells adhered to each other in an additive manner (see Discussion 6), or whether there was evidence for the presence of different mechanisms of adhesion in different types of fibroblast.

2. Selection of Cell Lines

The normal and transformed fibroblastic cell lines which were tested could be grouped into 3 categories with respect to adhesiveness and morphology (Table 2). The most striking finding was a large difference in adhesiveness between two classes of transformed cell. These have been designated Type II and Type III fibroblasts, and the untransformed cells as Type I fibroblasts. The Type II and Type III fibroblasts had very different morphologies, the most noticeable difference being the lack or reduction of lamellar cytoplasm in the latter (Plates 2a-c). In order to test whether the restoration of the lamellar cytoplasm of Type III fibroblasts would increase their adhesiveness, sheets of CHO cells were treated with

Table 2

Phenotypes of Fibroblastic Lines

Type	Morphology	Adhesiveness	Examples
I	Normal	High to Very High	Cl3, HEF
II	Transformed, Criss-crossed	High to Very High	PyCl, S180
III	Transformed, Rounded	Low	CHO, PyAA, L-929

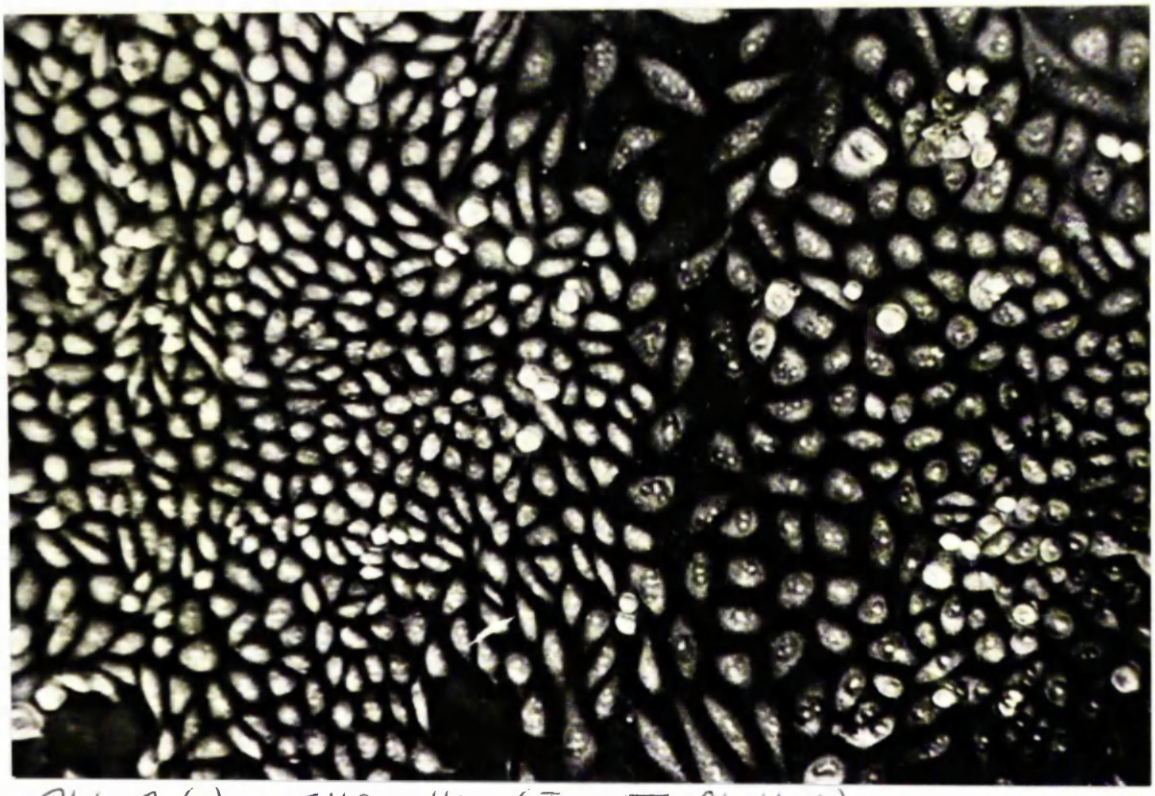

Plate 2 (a). CHO cells. (Type III fibroblast).
Magnification X 1100.

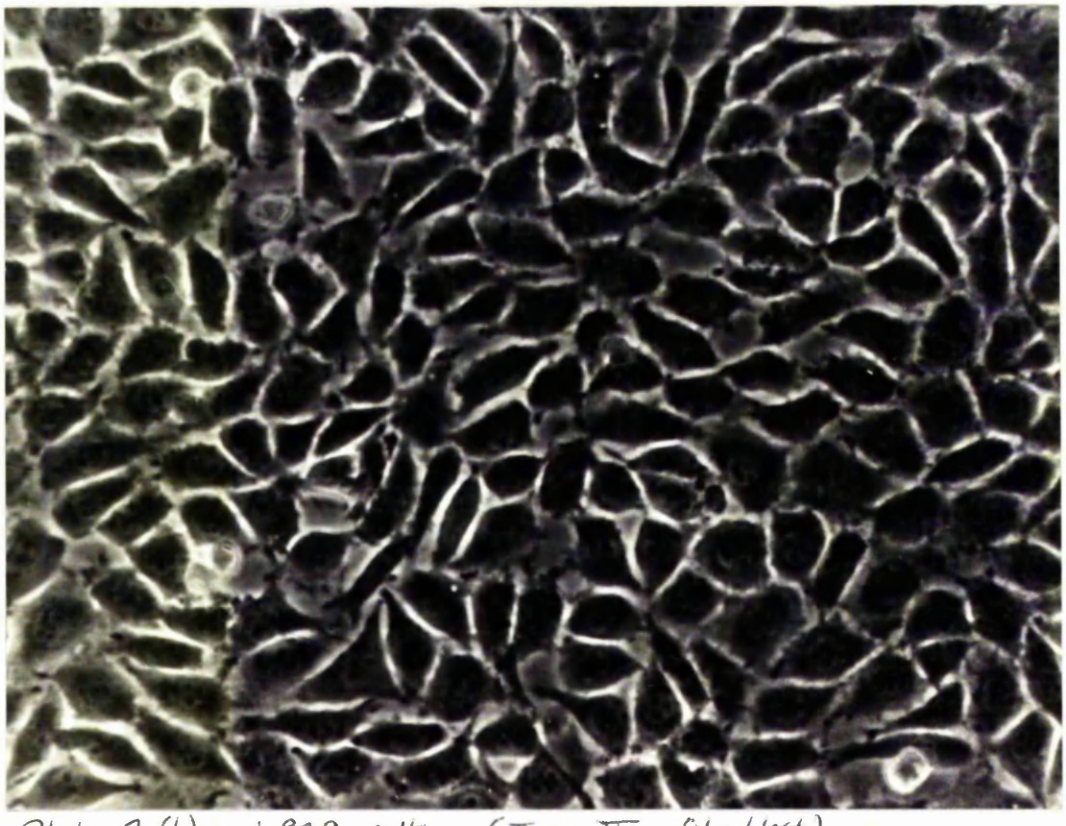

Plate 2 (b). L929 cells. (Type III fibroblast).
Magnification X1400.

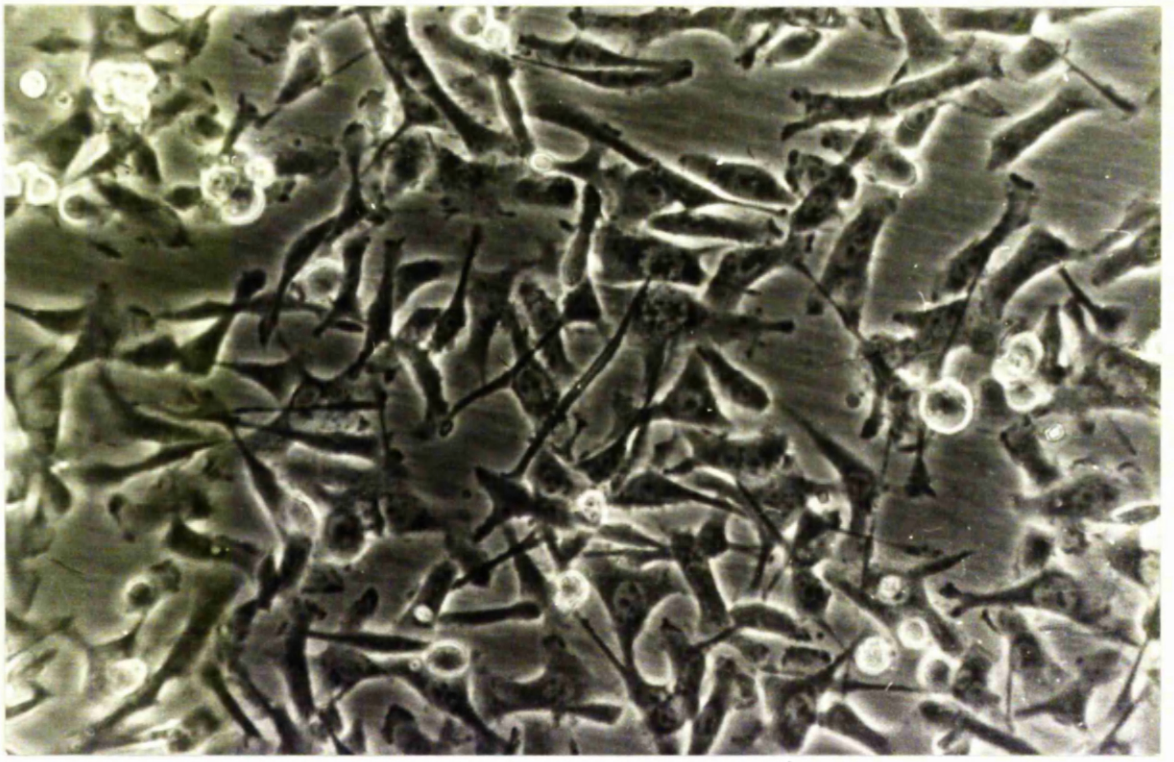

Plate 2 (c). PyC1 cells (Type II fibroblast).
Magnification X 1400.

1.0 mM dibutyryl cyclic AMP during the overnight incubation used for the preparation of the sheets. This drug has been shown to revert the morphology of CHO cells to that of Type I fibroblasts (Hsie and Puck, 1971).

3. Effect of Cyclic AMP

Treatment of CHO cell sheets overnight with dibutyryl cyclic AMP increased their adhesiveness by about 2.0-2.5 times (Table 3a). The rates of attachment of CHO cells to homologous cell sheets were approximately linear for at least the first 40 mins, both in the absence and in the presence of the drug. The degrees of stimulation of adhesion by this drug were similar at 20 and at 40 mins, and were comparable in the 3 experiments.

Although the adhesiveness of the cell sheets was only partially restored by treatment with dibutyryl cyclic AMP, it was also found that it was possible to obtain only a partial morphological transformation of the cells by this drug under the conditions in which it was administered. This was most likely due to the very high densities at which the CHO cells were plated in order to prepare the cell sheets. A complete degree of morphological transformation was obtained when relatively sparse cultures of the cells were used. These results therefore point to an apparently direct correspondance between the adhesiveness of the cell sheets and the amount of lamellar cytoplasm present.

The addition of dibutyryl cyclic AMP during the courses of the assays had no effect on the results obtained (Table 3b), indicating that the adhesive transformation required a relatively long period of treatment with the drug

Table 3a

Effect of Cyclic AMP on CHO Cell Adhesion

t 20	CHO Cell Sheets		
Expt	Control	b_2cAMP*	Ratio
2	5.8 ± 1.9	17.5 ± 4.1	0.33
3	12.5 ± 0.6	24.7 ± 6.3	0.51
Ave	9.2 ± 3.8	20.4 ± 5.8	0.45
t 40			
1	7.0 ± 4.1	35.7 ± 8.0	0.20
2	19.7 ± 1.6	42.8 ± 1.1	0.46
3	29.7 ± 1.0	55.1 ± 2.4	0.54
Ave	18.7 ± 10.0	43.2 ± 9.2	0.43

Values are means ±S.D. as for Table 1; expressed as per cent of attachment to serum-coated plastic.
*1.0 mM Dibutyryl Cyclic AMP added to cells overnight during the formation of the cell sheets.

Table 3b

Effect of Cyclic AMP on Seeded Cells

	CHO Cell Sheets		
Seeded Cells	Control	b_2cAMP	Plastic
Control	7.0 ± 4.1	35.7 ± 8.0	100.0 ± 8.6
b_2cAMP**	9.8 ± 5.5	37.7 ± 9.9	82.6 ±12.1

Values are means ±S.D. in one experiment.
** 1.0 mM Dibutyryl Cyclic AMP added to suspension of labelled cells for duration of assay (40 mins).

in order to be brought about, but was stable for at least the 40 min period of the assays.

4. Adhesive Interactions Between Cell Lines

In order to test whether different fibroblasts show quantitative (additive) adhesive interactions, four fibroblast lines were chosen to give as wide a range of adhesiveness as possible: C13, HEF, PyAA, and CHO cells. These cells were all derived from hamsters, and therefore the possibility of adhesive differences caused by phylogenetically-dependent properties was minimized. The rationale of this test is that if fibroblasts share the same mechanisms of adhesion, then their adhesive interactions should be quantitative, whereas if there are different types of adhesive mechanisms shared by different types of fibroblast, then these adhesive interactions would depend on the extents to which the adhesive mechanisms of the fibroblasts were matched. In the first case, it should be possible to arrange fibroblasts in a hierarchy of adhesiveness, in which adhesions between highly and weakly adhesive fibroblasts would be of intermediate value.

The possible relationship between the low adhesiveness of Type II fibroblasts and their epitheloid shape suggested that it might be possible to obtain an epithelial cell line which was further reduced in adhesiveness (see Discussion 4). A hamster-derived epithelial cell line, HAK cells, was found to form very poorly adhesive cell sheets, and was also used in the following experiments.

Preliminary experiments showed that the rates of attachment of both Type I and Type III fibroblasts to

homologous monolayers were approximately constant for the first 40 mins, and that the maximal differences between the adhesive rates of the different cell types were found at this time. Larger differences were found when a stronger washing procedure was used (see Materials and Methods). The leakage of radioactive material from the seeded cells during the courses of the assays was usually not more than 2-5% of the total label present.

The adhesive interactions between the 5 cell types are summarized in Table 4a. The cells appeared to show additive interactions, and the monolayers of the 5 types of cells could be arranged in a hierarchy of adhesiveness, such that Cl3 > HEF > PyAA ≫ CHO > HAK. The hierarchy was recognized by each of the cell types when used as seeded cells, and also by 4 other types of fibroblast (Table 4c). Table 4b shows that the relative adhesivities of the different monolayers were similar in each experiment. The absolute rates of attachment, however, differed somewhat between experiments, as was found previously when this assay was used (Table 1). Although the adhesive interactions appeared in each case to be additive, the adhesivities of the monolayers were more determining than those of the seeded cells. This was most striking in the case of HAK cells, which were able to attach well to fibroblast monolayers, but which formed very non-adhesive monolayers. It was found in subsequent experiments (eg. Table 5c) that the rates of attachment of cells to HAK monolayers could be increased by decreasing the strength of the washing procedure, suggesting that these monolayers will only support the formation of weak adhesions.

Table 4a

Adhesion of Cell Lines

Seeded Cells	Cell Sheets				
	HAK	CHO	PyAA	HEF	Cl3
HAK	3.7 ±1.7(11)	12.2 ±4.4(9)	21.0 ±9.0(12)	44.7 ±15.2(12)	73.5 ±23.4(6)
CHO	6.1 ±2.5(6)	15.7 ±2.1(9)	31.3 ±10.3(9)	62.7 ±17.8(9)	84.2 ±6.6(3)
PyAA	5.7 ±2.9(15)	26.5 ±17.5(9)	42.8 ±21.0(13)	61.7 ±14.4(12)	91.9 ±25.1(8)
HEF	8.8 ±5.5(15)	37.4 ±14.1(9)	39.4 ±22.0(15)	58.5 ±16.8(15)	94.7 ±32.4(9)
Cl3	15.2 ±10.3(15)	72.1 ±15.2(15)	63.1 ±22.9(9)	100.9 ±15.5(12)	110.8 ±24.0(9)

Values are means ±S.D. as for Table 1; expressed as per cent of attachment to serum-coated plastic.
Total numbers of cell sheets shown in brackets.
Individual experiments shown in Table 4b.

Attachment to plastic = 60-90% of total seeded cells.
Time = 40 mins.

Table 4b

Adhesion of Cell Lines: Individual Experiments

Seeded Cells	Expt	Cell Sheets				
		HAK	CHO	PyAA	HEF	Cl3
HAK	1	5.2 ±1.4	17.7 ±2.3	26.3 ±7.6	67.3 ±2.9	
	2	1.9 ±0.1	8.2 ±0.6	10.9 ±2.7	37.2 ±3.7	52.4 ±4.8
	3	4.8 ±1.3	10.9 ±1.7	16.0 ±5.4	44.5 ±7.1	
	5	2.8 ±0.4		30.2 ±1.8	29.8 ±4.1	94.6 ±2.9
	Ave	3.7 ±1.7	12.2 ±4.4	21.0 ±9.0	44.7 ±15.2	73.5 ±23.4
CHO	1		16.3 ±1.7	35.0 ±10.3	81.1 ±12.8	
	2	7.7 ±2.8	16.0 ±3.2	38.5 ±5.7	45.8 ±8.9	84.2 ±6.6
	3	4.6 ±0.4	14.9 ±1.7	20.3 ±1.1	61.4 ±8.9	
	Ave	6.1 ±2.5	15.7 ±2.1	31.3 ±10.3	62.7 ±17.8	84.2 ±6.6
PyAA	1	9.1 ±1.7	33.7 ±15.7	46.8 ±4.1	81.0 ±6.9	
	2	3.8 ±1.5	35.7	42.6 ±10.9	52.1 ±1.1	58.6 ±5.6
	3	4.8 ±0.8	9.8 ±1.3	13.5 ±2.9	47.2 ±1.2	
	4	2.7 ±0.4		41.2 ±4.9		65.4 ±6.3
	5	8.0 ±2.7		70.6 ±5.4	66.5 ±5.6	109.5 ±8.4

Table 4b Cont.

Adhesion of Cell Lines: Individual Experiments

Seeded Cells	Expt	Cell Sheets				
		HAK	CHO	PyAA	HEF	C13
HEF	1	15.9 ± 1.5	40.4 ± 12.0	60.6 ± 4.4	65.4 ± 2.3	
	2	13.9 ± 2.8	50.9 ± 7.6	24.5 ± 1.7	61.2 ± 1.6	77.7 ± 3.6
	3	3.0 ± 0.5	25.3 ± 10.5	17.7 ± 3.3	41.0 ± 6.5	
	4	4.3 ± 0.7		25.6 ± 2.3	41.9 ± 6.2	70.0 ± 0.8
	5	7.0 ± 0.3		68.8 ± 7.4	83.1 ± 5.6	136.6 ± 14.2
	Ave	8.8 ± 5.5	37.4 ± 14.1	39.4 ± 22.0	58.5 ± 16.8	94.7 ± 32.4
C13	1	28.6 ± 11.9	86.8 ± 8.1	65.3 ± 8.9	101.2 ± 7.1	
	2	18.4 ± 10.1	65.1 ± 8.9	63.0 ± 3.2	102.9 ± 2.0	102.8 ± 6.8
	3	6.1 ± 1.7	62.1 ± 13.3	33.2 ± 5.2	80.5 ± 4.0	
	4	8.3 ± 3.5		61.5 ± 3.4		88.4 ± 2.1
	5	14.4 ± 1.5		106.9 ± 5.7	119.0 ± 12.0	141.2 ± 3.3
	Ave	15.2 ± 10.3	72.1 ± 15.2	63.1 ± 22.9	100.9 ± 15.5	110.8 ± 24.0

Values are means ± S.D. as for Table 1.

Table 4c

Adhesion of Other Fibroblast Lines

Seeded Cells	Cell Sheets					
	HAK	CHO	PyAA	HEF	Cl3	Homotypic
L929	3.5 ±0.5	28.6 ±2.3	32.9 ±5.7	87.0 ±18.1		38.5 ±2.9
S180	3.8 ±1.2 7.4 ±1.4	91.1 ±3.8	51.8 ±4.8 74.8 ±3.2	102.4 ± 2.3 87.3 ± 1.2	108.1 ±14.6	87.9 ±2.3 105.8 ±4.7
HEP		61.2 ±7.3	63.1 ±2.7	95.7 ± 2.5		
PyCl				74.3 ± 4.4	100.0 ± 4.7	99.3 ±4.5

Values are means ±S.D. based on duplicate or triplicate cell sheets; expressed as per cent of attachment to serum-coated plastic.

5. Effect of Fixation

The effect of formaldehyde fixation on the adhesiveness of Cl3 cells is shown in Table 5a. It can be seen that Cl3 cells were able to attach to fixed monolayers, although at a reduced rate. Fixation of the cell suspensions resulted in a somewhat larger degree of inhibition of attachment. Fixation of both cell suspensions and monolayers completely inhibited attachment. Table 5b shows that periodate treatment of fixed monolayers of Cl3, HEF, and HAK cells increased, rather than inhibited, the rates of attachment of Cl3 cells to these monolayers.

A comparison of the effects of both formaldehyde and glutaraldehyde fixation on the adhesiveness of these 3 cell types is shown in Table 5c. The Cl3 cells retained their relatively high adhesiveness after fixation, whereas the adhesiveness of the HEF cells appeared to be quite sensitive to fixation. The monolayers were in each case slightly more adhesive after glutaraldehyde than after formaldehyde treatment.

Table 5a

Fixation of C13 Cells

Seeded Cells	Expt	Cell Sheets	
		Control	Fixed
Control	1	100.0 ± 4.3	82.5 ± 9.3
	2	100.0 ± 5.4	65.4 ±10.0
	Ave	100.0 ± 4.4	73.9 ±12.7
Fixed	1	45.8 ±14.4	7.1 ± 4.3
	2	35.8 ± 4.9	1.8 ± 0.2
	Ave	40.8 ±11.0	4.8 ± 4.4

Values of individual experiments ± S.D. as for Table 1.

Cell sheets or seeded cells fixed with 2% formaldehyde.

Table 5b

Periodate Treatment of Fixed Monolayers

Type of Sheet	Expt	Cell Sheets		
		Control	Fixed	Periodate *
C13	1	100.0 ±11.2	48.2 ± 6.5	57.7 ± 7.3
	2	100.0 ± 6.2	62.6 ±10.6	74.1 ± 6.1
	3	100.0 ± 3.2	60.2 ±12.5	80.6 ±17.7
	Ave	100.0 ± 7.3	57.0 ±11.0	70.8 ±14.3
HEF			35.0 ± 3.7	49.4 ± 7.6
HAK			21.7 ± 3.9	51.1 ± 9.4

Values are means ±S.D. as for Table 1.

* Fixed cell sheets treated with 25 mM Sodium Periodate.

Table 5c

Adhesion of C13 Cells to Fixed Cell Sheets

Fixation of Sheets	Expt	Cell Sheets		
		C13	HEF	HAK
Control	1	100.0 ±11.4	96.3 ±13.3	52.1 ± 8.8
	2	100.0 ± 5.4	95.2 ± 5.2	69.4 ± 5.5
	3		82.0 ± 4.4	22.2 ± 3.1
	4		97.5 ±19.0	37.5 ± 7.3
	Ave	100.0 ± 7.8	92.2 ±12.5	45.3 ±19.1
Formal-dehyde	1	44.6 ± 4.7	23.4 ± 3.5	13.4 ± 2.7
	2	65.4 ±10.0	33.1 ±12.4	33.1 ± 3.5
	3	46.8 ± 8.0	16.2 ± 6.2	21.3 ± 0.8
	4	53.5 ± 7.4	31.9 ± 2.5	20.6 ± 0.6
	Ave	50.9 ±12.2	25.6 ± 9.7	22.1 ± 7.6
Glutaral-dehyde	3	56.8 ± 1.0	38.4 ± 4.0	42.9 ± 1.9
	4	51.3 ± 2.3	22.1 ± 2.7	20.6 ± 7.2
	Ave	54.1 ± 3.4	30.2 ± 9.5	31.7 ± 13.1

Values of individual experiments ±S.D. as for Table 1.

DISCUSSION

1. Cell Sheet Collection Assay

The main features of the cell sheet collection assay have been described by Walther et al (1973). They found that the attachment of fibroblasts to sheets of homologous cells proceeded at an approximately linear rate for the first 30 mins, after an initial lag period of 5 mins. Similar results were obtained in this work, although no lag period was observed, possibly due to the different conditions under which the assays were performed (see below). Walther et al showed that the settling times of the seeded cells appeared to be negligible, that the degree of detachment of cells from the cell sheets during the courses of the assays was minimal, and that the release of radioactive material by the seeded cells into the medium was very low. Therefore the results obtained using this assay are likely to be a direct reflection of the adhesiveness of the seeded cells for the cell sheets.

It is important to know that the seeded cells are attaching directly to the cell sheets and not to the substrate through gaps in the cell sheet. There is a substantial amount of evidence in support of this. Howard et al (1975) showed by scanning electron microscopy that seeded 3T3 cells made direct contacts with the upper surfaces of the cells in the cell sheets. In this work, the cell sheets (for most cell types, multilayers) were formed by seeding the cells into wells at very high densities, making it unlikely that the labelled cells

would have access to the substrate during the course of the assay. No gaps could be detected in the cell sheets when these were monitored by phase contrast microscopy. In addition to this, the neural cells which were used in subsequent experiments adhered very poorly to the substrate, and were therefore evidently attaching to the cell sheets (eg. Plate 3c). However, it is possible that the dorsal surfaces of the cells in the cell sheets are predominantly non-adhesive, and that the seeded cells are attaching mainly to the exposed lamellar regions of these cells (DiPasquale and Bell, 1974).

The collection assay used in this work was modified slightly from the method introduced by Walther et al (1973), mainly in that the cells were allowed to attach under stationary conditions, as opposed to in a reciprocating shaker. A similar modification was used by Buultjens and Edwards (1977). In the stationary assay, the rate of attachment is likely to be determined solely by the numbers of cells which can form adhesions of a given strength after a given time interval, whereas when the assay is performed in a reciprocating shaker the results may depend to some extent on the abilities of the cells to form rapid adhesions, and under poorly defined flow conditions. Therefore, the results obtained using the former method should be more easily interpretable. It is also relevant to note that, with only a few exceptions, normal adhesive interactions between cells do not take place under conditions of flow. Two possible disadvantages of the stationary method which were mentioned by Walther et al

are that the variability of the results may be greater and the degrees of specific adhesion found may be less when this method is used.

2. Embryonic Chick Fibroblasts

Very few comparative studies have been made on the behavioural properties of fibroblasts cultured from different tissues. Two recent reports suggest that distinct differences between such fibroblasts are likely to exist. Conrad et al (1977) found quantitative differences between the saturation densities and sensitivities to detachment of embryonic chick skin, heart and corneal fibroblasts. They also described morphological differences between the skin and the heart fibroblasts, similar to those which were found in this study (see Results 1), and in addition to this, showed that corneal fibroblasts formed monolayers of polygonally-shaped cells, which could be readily distinguished from the multilayers formed by skin and heart fibroblasts. It is possible that the quantitative adhesive differences found between, for example skin and heart fibroblasts (Table 1), could account to some extent for the morphological differences observed between these cell types. Brunette et al (1977) obtained preliminary results that mouse gingival and periodontal ligament fibroblasts might show adhesive preferences for homologous epithelia, although only small numbers of cells were examined.

The diversity of the fibroblastic types which were used in this study suggests that only relatively small differences in adhesiveness are likely to be found between most types of embryonic chick fibroblast, in terms of their

interactions with other fibroblastic cell types. However, one way in which such quantitative differences could play a significant role in cell positioning will be described later (Discussion II).

3. Adhesion of Transformed Fibroblasts

Several workers have proposed that alterations in adhesive properties may be responsible for much of the abnormal behaviour of malignant cells. Cell transformation has been found to both increase and decrease cell-to-cell adhesion. Coman (1961) reported that the malignant transformation of rabbit epithelial cells decreased their mutual adhesiveness, measured by the forces needed to pull the cells apart by micromanipulation, but appeared to increase the adhesiveness of the cells to the culture substrate. Edwards et al (1971) showed that transformation of BHK fibroblasts with polyoma virus markedly decreased the abilities of the cells to aggregate, however, Halpern et al (1966) found that the transformation of a rat cell line with Rous sarcoma virus increased the aggregability of these cells. Dorsey and Roth (1973) found that 3T3 cell aggregates collected many SV40 3T3 cells but few 3T12 cells, whereas aggregates of the two transformed cell types did not show this selectivity, collecting only high or low numbers of cells, respectively. The authors suggested that in these cases the transformation of 3T3 cells had resulted in a decreased degree of cellular recognition, however, these results could be more easily explained by quantitative differences in the adhesiveness of the three cell types, where SV40 3T3 > 3T3 > 3T12.

Cell transformation appears to lead more consistently to a reduction in cell-to-substrate adhesion. This has been shown to be the case for BHK-Cl3 fibroblasts following transformation by polyoma virus (Shields and Pollock, 1974) and for embryonic chick fibroblasts transformed by Rous sarcoma virus (Yamada et al, 1976a). Tumour cells are thought to be generally more readily detachable from culture substrates and can often grow in suspension (Clarke et al, 1970; Nomura et al, 1973). Culp (1974) found that the adhesiveness of the microexudate deposited by 3T3 cells decreased after the transformation of the cells by Rous sarcoma virus.

It is possible that some of the changes in the adhesive properties of cells following transformation could involve specific alterations in the recognition properties of these cells. This has not been reported for fibroblastic cells, but Nicholson and Winkelhake (1975) have presented some evidence for altered adhesive recognition properties of metastatic variants of a melanoma cell line. One variant which showed a high tendency to form lung metastases, B16-F10, was found to have a high adhesive affinity for disaggregated lung cells, but adhered poorly to liver, heart, spleen and some other cell types. A low metastatic variant, B16-F1, adhered moderately to all of the above cell types.

It would be useful to have assays which could be used to distinguish between different types of transformed cell, in terms of their adhesive and other behavioural properties. The cell sheet collection assay is potentially well suited to this because of the possibility it offers of

making direct comparisons between adhesive, morphological and behavioural properties of different transformed cell types. In the work described here, I have shown that the cell sheet collection assay can be used to detect major differences in the adhesive properties of two types of transformed fibroblast. These differences may be directly related to the different morphologies of these cell types (see Table 2).

The adhesive differences between Type II and Type III fibroblasts could be due to: 1) quantitative differences in the same altered property, or 2) two distinct types of alteration, following transformation.

The first possibility is suggested by the relatively permissive conditions of the cell sheet collection assay, when compared to other adhesive assays. For example, PyCl cells are considerably reduced in their ability to aggregate (Edwards et al, 1971) but not in their rate of attachment to homologous cell sheets, when compared to untransformed BHK cells. One explanation for this could be the reduced ability of these cells to form rapid adhesions, as this alteration would be less detectable in the cell sheet collection assay. It is possible, if this is the case, that Type III fibroblasts are quantitatively more altered in this same property such that their decreased adhesiveness can be readily measured using this assay.

However, there are some reasons for thinking that the second of these two possibilities is the more likely. Cyclic AMP treatment reverts Type III fibroblasts directly into cells having the phenotype of Type I fibroblasts,

indicating that the Type II phenotype is not intermediate to the phenotypes of Type I and Type III cells. In some cases, Type II fibroblasts can be reverted to Type I cells by the addition of LETS protein (see Introduction 4). However, although Type III fibroblasts may also be reduced in LETS protein, the extremely low levels of LETS protein in some Type II fibroblasts make it unlikely that the different phenotypical properties of Type III fibroblasts are caused by quantitatively lower amounts of this protein. In the following two sections, the possibility of a direct relationship between the decreased adhesiveness and the reduction of lamellar cytoplasm of Type III fibroblasts will be discussed.

4. Relationship of Adhesiveness to Lamellar Cytoplasm

A number of studies have suggested that the adhesive sites of fibroblastic and epithelial cells may be restricted to the lamellar regions of these cells. Bragnina et al (1976) and Heaysman and Pegrum (1976) have shown that the ability of fibroblasts to form stable adhesive structures may be restricted to lamellar areas, both in cell-to-substrate and in cell-to-cell adhesion. DiPasquale and Bell (1974) found that latex particles and concanavalin A-treated red blood cells, as well as seeded cells and microspikes and processes of adjacent cells, did not attach to the upper surfaces of spread fibroblasts or epithelial cells, but could adhere to the free margins of these cells and subsequently be transported centripetally onto their upper surfaces. Abercrombie et al (1970) and Harris (1973c) postulated that membrane is inserted at the leading edge, transported centripetally backwards over the dorsal surfaces

of the cells and disassembled over the nucleus.

The low adhesiveness of some epithelial cell monolayers may be a direct consequence of the restriction of adhesive surface to lamellar regions. DeRidder et al (1974) seeded grafts of fibroblastic lines onto various epithelial organ fragments taken from the chick embryo. They found that the apical surfaces of limiting epithelia were very non-adhesive for the seeded cells, but that the basal surfaces of these tissues, as well as the surfaces of parenchymal epithelia, would support the attachments of the grafts. This finding is compatible with the report of DiPasquale and Bell (1974) of the non-adhesiveness of the dorsal (upper) surfaces of embryonic chick skin, gut and corneal epithelial cells, when cultured as explants. Vasiliev et al (1975) also found that only the cells at the free margins of epithelial cell monolayers showed a capacity for phagocytosis, and suggested that this might be due to in inabilities of the other cells to form adhesive contacts with the seeded particles. The authors mentioned that the non-adhesiveness of the epithelial monolayers could be due to the adhesive sites of the cells being used up in lateral contacts between the cells.

The possibility of a relationship between the reduced adhesiveness of Type III fibroblasts and their epitheloid morphology is suggested by the report of Vasiliev et al (1975) that the adhesivities of monolayers of a series of normal and transformed epithelial cell lines were inversely correlated with the extents to which these cells showed typical "epithelioid" properties. Cells which

were less tightly bound together in monolayers and which could move as single cells, formed monolayers of intermediate adhesiveness (16-42% attachment), whereas more typical epithelial cells which moved as a coherent cell sheet, were very non-adhesive (0-12% attachment), when fibroblasts were seeded onto the monolayers. The adhesiveness of the first of these cell types is very similar to that of Type III fibroblasts, which also have very similar morphological properties. Therefore, in terms of adhesiveness, morphology and cell behaviour on culture substrates, there may be intermediate forms between epithelial and fibroblastic cells.

Cell sheets of Type I and Type II fibroblasts would be likely to have sufficient amounts of exposed lamellar cytoplasm in order to be highly adhesive, if the dependence of adhesiveness on lamellar regions of the cells proves to be the case. Therefore, the differences in adhesiveness between Type II and Type III fibroblasts could be attributed to the lack of lamellar cytoplasm in the latter. However, it is likely that this reduction of lamellar cytoplasm may be a direct consequence of a defect in the adhesive properties of Type III fibroblasts. The finding (see Results 4) that the abilities of cells to attach to sheets of Type III fibroblasts were considerably improved by reducing the strength of the washing force, suggests that the Type II cells may have a defect in their ability to form stable adhesive contacts. In relation to this, Domnina et al (1972) have reported that the reduction of lamellar cytoplasm in L cells (Type III) appeared to be due to the disintegration of newly formed lamellar cytoplasm in these cells. Johnson

and Pastan (1971) showed that L-929 cells did have an absolute defiency of lamellar cytoplasm, but that these cells showed temporal fluctuations in the production and retraction of lamellae. At any given time, a certain fraction of these cells had extended lamellar regions. This finding is in accord with the possibility of an inability of these cells to form stable adhesion. Type II fibroblasts have been shown to have a reduced adhesiveness to the substrate (eg. Yamada et al, 1976a; Shields and Pollock, 1974) and also to aggregate poorly (Edwards et al, 1971), however, the adhesive defects responsible for these altered properties are likely to be qualitatively different from the adhesive defects present in Type III cells, for example, in being decreased but stable, as opposed to unstable.

5. Effect of Cyclic AMP

Further evidence for the relationship between the reduced adhesiveness and lack of lamellar cytoplasm in Type III cells was the finding that dibutyryl cyclic AMP partially restored both of these properties, to similar extents. However, it is possible that this relationship could in this case be an indirect one. Hsie et al (1971) showed that the reverse transformation of CHO cells by cyclic AMP resulted in the alterations of several cellular properties, including the increased production of collagen and the abilities of the cells to be agglutinated by concanavalin A. However, evidence has been presented that cyclic AMP can increase cell adhesiveness by stabilizing the cytoskeletal support for adhesive sites. This has been suggested by Shields and Pollock (1974) for the ability of

cyclic AMP to increase the adhesiveness of attached fibroblasts to the substrate (Johnson and Pastan, 1972; Shields and Pollock, 1974). Willingham and Pastan (1975) proposed that cyclic AMP stimulated microtubule assembly in L-929 cells, preventing the retraction of processes put out by these cells. The possibility that the altered properties of Type III fibroblasts result from a defect in the coupling of microtubule assembly to newly formed adhesive contacts is suggested by the morphological similarities between these cells and colchicine-treated Type I cells. The importance of microtubule assembly in stabilizing or permitting the formation of adhesive contacts between cells has also been shown by the inhibition of cell aggregation by microtubule-disrupting plant alkaloids (Waddell et al, 1974; Lackie, 1974).

The ability of cyclic AMP to increase the adhesiveness of CHO cells after long term (overnight) but not after short term (40 mins) treatment (Table 3b), indicates that the increase parallels the induction of the formation of lamellar cytoplasm, and is not due to artefactual side effects of the drug.

6. Quantitative Adhesion of Fibroblasts

It would be of interest to know whether different types of fibroblast, both normal and transformed, have the same mechanism(s) of adhesion, or whether some of the morphological and behavioural differences between different fibroblasts result from the existence of distinct types of adhesive mechanism, specific for different classes of these cells. In the second case, it would be expected that fibroblasts which have similar mechanisms of adhesion would

show strong adhesive affinities for each other, but would adhere poorly to other types of fibroblast. On the other hand, if different fibroblasts share in common very similar, if not identical, adhesive mechanisms, and differ only quantitatively in adhesiveness, then it should be possible to place these cells in a hierarchy of adhesiveness, such that the adhesive interactions between any two types of fibroblast would be an additive function of the adhesivities of the individual cell types.

The ability to assign different cell types relative, or absolute (Curtis, 1969) adhesive values would be useful in terms of understanding other properties of these cells which may be related to adhesiveness. Curtis (1969) was able to obtain values for the collision efficiencies of different embryonic chick cell types using a modified Couette viscometer, however, the use of this technique to determine the relative adhesivities of cells based on their mutual adhesive interactions would involve a considerable amount of time. Steinberg (1970) showed that several embryonic chick cell types could be arranged in a hierarchy of sorting out, but the relationship between this property and adhesiveness is not clear.

The results obtained in this work (Tables 1,4a,c) argue strongly for quantitative differences in the adhesiveness of different fibroblastic cell types, although it is not possible to generalise that this would also be true of other fibroblasts not tested in this study. There appeared to be no alteration in the quantitative adhesive interactions of these cells after two types of transformation. The different mechanisms of fibroblastic cell adhesion which have been proposed are discussed in Introduction 4.

7. Effects of Fixation and Periodate Treatment

A few previous reports have been made of the effects of fixation on cell adhesion, and the results suggest that these effects can vary both between different cell types and between different types of fixative. Walther and Roseman (1975) found that glutaraldehyde treatment of 3T3 cell sheets completely abolished their adhesiveness for homologous cells, whereas a similar treatment of the seeded cells reduced their rates of attachment to normal cell sheets by 60%. DeBono (1976), in a study of the attachment of pig lymphocytes to monolayers of pig aortic endothelial cells, showed that the adhesiveness of the monolayers was completely inhibited by fixation with 2% glutaraldehyde, but was not affected by fixation with 4% formaldehyde. Fixation of the lymphocytes with 4% formaldehyde inhibited their adhesiveness by 90%. Vasiliev et al (1974) reported that the fixation of very poorly adhesive monolayers of MPTR epithelial cells increased their adhesiveness, whereas a similar treatment of highly adhesive fibroblast monolayers somewhat decreased their adhesiveness, although they still remained 2-3 times more adhesive than the fixed MPTR cell monolayers. In both cases, 2% glutaraldehyde was used.

Roth (1968), using the collecting aggregate assay, found that when either the labelled cells or the aggregates were fixed with 8% glutaraldehyde the rates of collection of the cells were considerably reduced, although there appeared to be a small degree of retention of the preference for homotypic over heterotypic adhesions after fixation, when neural retinal and liver cells were used. Cassiman

and Bernfield (1976) seeded 24 hour aggregates of chick neural retinal, heart and liver cells onto homologous or heterologous cell sheets. They found that sheets of neural retinal cells were very sensitive to fixation with glutaraldehyde, but that the adhesiveness of heart and liver cell sheets was increased by treatment with low concentrations of glutaraldehyde and decreased when higher concentrations of glutaraldehyde were used. It was also found that the specificity of adhesion between neural retinal and heart cells was abolished by treatment of either of the cell types with glutaraldehyde. Heaysman and Turin (1976) showed that zinc-fixed chick heart fibroblasts were capable of eliciting a contact inhibition response in living fibroblasts, whereas this has not been observed with glutaraldehyde-fixed cells (Vesely and Weiss, 1973). It is possible that some degree of specific adhesion could be found if one of the cell types used was zinc-fixed.

The results presented in Tables 5a and 5c show that the relative degrees of adhesiveness of C13, HEF and HAK cells were to some extent preserved after fixation, but that the HEF cells appeared to be considerably more sensitive to fixation than the other two cell types. The effects of formaldehyde and glutaraldehyde were very similar, in all three cell types. It is possible that the non-adhesiveness of glutaraldehyde-treated fibroblastic cell sheets found by Walther and Roseman (1975) could have resulted from the use of a reciprocating shaker. The results obtained in this work, and also those reported by Vasiliev et al (1974) were based on adhesive assays performed under stationary

conditions.

The effects of fixation on the adhesiveness of C13, HEF and HAK cells could be accounted for in two ways: 1) These cells have two types of adhesive site, fixation-sensitive and fixation-insensitive. If this is the case, the HEF cells would appear to have relatively few of the latter, and therefore be more sensitive to fixation. 2) The adhesion of cells to fixed cell sheets is analogous to the attachment of the cells to solid substrates of moderate wettability (see Introduction 2). Vasiliev et al (1974) suggested that the effect of fixation of cell sheets was to form a leathery coat covering the surfaces of the cells, and that the relative abilities of seeded cells to attach to the fixed cell sheets depended on the extents to which the fixed cell surfaces were supported by cytoskeletal structures. Electrostatic differences between the fixed surfaces of different cell types could also explain some of the results obtained. In the latter two cases, the cytoskeletal or electrostatic properties of different cell types could be partially preserved after fixation.

Periodate treatment has been reported to inhibit cell aggregation (Moscona, 1965; Glaeser et al, 1968) and collection of cells by aggregates (McGuire, 1976), however, in these cases the periodate would have been likely to have indirect effects on cell adhesion, for example, by reacting with the glucose in the medium (see Edwards, 1977). The use of fixed cell sheets, on the other hand, avoids the possibility of artefactual effects of periodate treatment, and can be used as a direct test of the possible

role of carbonydrate groups in cell adhesion. The finding that seeded cells could attach to cell sheets which had been treated with both formaldehyde and periodate makes it very unlikely that the adhesion of live cells to fixed cell sheets involves any degree of molecular bonding. The three types of molecular bonds which could form between cells, protein-protein, protein-carbohydrate and carbohydrate-carbonydrate, should all be inhibited under these conditions. The fixed cells would also be unlikely to secrete any adhesive microexudates during the course of the assay. However, it is not clear whether the adhesion of cells to fixed and to normal cell sheets procede by the same mechanisms.

II. Adhesion Between Neural Retinal and Fibroblastic Cells

The finding of Buultjens and Edwards (1977) that embryonic chick choroid cells could show adhesive specificity with neural retinal cells suggested that fibroblasts in general might show poor adhesive associations with neural cells. Alternatively, this specificity could reflect a unique adaptation of choroid cells for cells of the adjacent neural retinal tissue. In order to test these possibilities, a study was made of the adhesive interactions between neural retinal cells and three types of fibroblast: embryonic chick skin fibroblasts and two cell lines, Cl3 and HEF cells.

RESULTS

The results of several cell sheet collection assays are shown in Tables 6a and 6b. The NR (neural retina) cells did not show any specific adhesion with either Cl3 cells or chick skin fibroblasts, but an approximately twofold degree of specificity was found between NR and HEF cells. The lack of specificity shown by the Cl3 cells provided a good control for the NR-HEF specificity, indicating that the latter was unlikely to be due to differences in the sizes of the NR and HEF cells. The adhesive interactions between the NR and HEF cells in the individual experiments are shown in Table 6c and a statistical analysis of the specific adhesion between these cells is given in Table 6d. The standard deviations given for each experiment are based on the duplicate or triplicate cell sheets which were used for each cell type, and are generally quite low. There was more variation in

the rates of attachment of the cells between experiments, however, in only one experiment was there an overlap in the adhesiveness of the NR and the HEF cell sheets (Table 6d). Using the Wilcoxon-Mann-Whitney test the degrees of adhesive specificity were found to be highly significant, both when NR and when HEF cells were seeded onto the cell sheets. In several additional experiments, in which only one of the cell types was seeded onto sheets of both cell types, a similar degree of specificity was obtained.

Table 6a

Adhesion Between NR and Fibroblast Lines

Seeded Cells	Cell Sheets		
	NR	HEF	C13
NR	98.1 ±22.5 (15)	36.5 ±13.4 (20)	100.0 ±9.6 (20)
HEF	44.4 ±18.5 (16)	77.5 ±14.6 (20)	100.0 ±4.4 (19)
C13	106.8 ±16.3 (10)	97.0 ±8.1 (14)	100.0 ±9.9 (15)

Values are means of 7 experiments ± S.D., based on the total numbers of cell sheets used (shown in brackets).

Table 6b

Adhesion Between NR and Skin Fibroblasts

Seeded Cells	Expt	Cell Sheets		
		NR	Skin	C13
NR	1	80.8 ± 6.2	77.0 ± 3.2	100.0 ±14.8
	2	75.6 ± 3.0	90.1 ±13.9	100.0 ± 3.6
	Ave	78.2 ± 5.1	84.9 ±12.8	100.0 ± 9.6
Skin	1	84.9 ± 6.5	108.1	100.0 ± 3.7
	2	76.4 ± 8.7	94.6 ± 3.7	100.0 ±10.0
	Ave	80.7 ± 8.3	99.1 ± 8.2	100.0 ± 6.8

Values of individual experiments ± S.D. as above.

Table 6c

Specific Adhesion Between NR and HEF Cells

Seeded Cells	Expt	Cell Sheets NR	Cell Sheets HEF	Ratio
NR	1	99.1 ± 5.1	45.2 ± 5.4	0.46
	2	121.1 ± 2.1	25.3 ± 2.1	0.21
	3	123.9 ± 5.6	28.8 ± 7.5	0.23
	4	97.4 ±12.8	28.5 ± 6.4	0.29
	5	73.1 ±10.7	39.6 ±19.4	0.54
	6	104.6	41.4 ±19.0	0.40
	7	71.1 ± 9.2	49.6 ± 9.2	0.70
	Ave	98.1 ±22.5	36.5 ±13.4	0.37
HEF	1	37.0 ± 5.7	93.0 ± 2.4	0.40
	2	32.4 ±11.4	67.1 ± 3.9	0.48
	3	28.3 ± 0.6	52.7 ± 1.1	0.53
	4	48.2 ±17.3	86.6 ± 2.1	0.56
	5	72.9 ±27.4	78.6 ±10.5	0.92
	6	45.2 ± 5.2	86.5 ± 6.3	0.52
	7	49.9 ±22.0	83.1 ±15.2	0.60
	Ave	44.4 ±18.5	77.5 ±14.6	0.57

Values of individual experiments ±S.D. as for Table 1.
Data expressed as % attachment to Cl3 cell sheets.

Table 6d

Stastical Analysis of NR/HEF Adhesive Specificity*

	Seeded Cells			
	NR		HEF	
RANK	Cell Sheets		Cell Sheets	
	HEF	NR	NR	HEF
1	25.3		28.3	
2	28.5		32.4	
3	28.8		37.0	
4	39.6		45.2	
5	41.4		48.2	
6	45.2		49.9	
7	49.6			52.7
8		71.1		67.1
9		73.1	72.9	
10		97.4		78.6
11		99.1		83.1
12		104.6		86.5
13		121.1		86.6
14		123.9		93.0

* The Wilcoxon-Mann-Whitney test. See Materials & Methods.

NR	HEF
$R_1 = 28$	$R_1 = 30$
$n = 14$	$n = 14$
$\hat{z} = 3.14$	$\hat{z} = 2.88$
$P < 0.01$	$P < 0.01$

DISCUSSION

It has previously been reported that choroid fibroblasts and NR (neural retina) cells can show specific adhesion (Buultjens and Edwards, 1977). The results obtained in this work (Tables 6a,b) show that HEF (Hamster Embryo Fibroblast) cells also have the capacity to show specific adhesion with NR cells, but that this capacity appears to be restricted to certain types of fibroblast. Two other studies have suggested that neural cells may have low adhesive affinities for meningeal fibroblasts. Sensenbrenner and Mandel (1974) found that embryonic chick cerebrum cells adhered very poorly to monolayers of meningeal, as well as of 6 day embryonic chick, fibroblasts. Nichols and Weston (1976) reported that meningeal cell monolayers were a poor adhesive substrate for chick dorsal root ganglion neuroblasts. However, no quantitative adhesive measurements were made or reciprocal combinations tested in these studies. In contrast to this, Luduena (1973) showed that dorsal root ganglion neuroblasts adhered well to the upper surfaces of cultured chick heart fibroblasts. It may be significant that the fibroblasts which appear to show a poor adhesive association with neural cells, for example, choroid and meningeal cells, are those which normally interact with neural tissues during development, as opposed to, for example, skin and heart fibroblasts. In this respect, it may be surprising that a fibroblastic cell line, HEF cells, can also show specific adhesion with neural cells, however, Gottlieb and Glaser (1975) have also

found that another fibroblast line, CHO cells, did not attach to monolayers of cerebrum cells. These results could be explained in two ways:

1) It is possible that the poor adhesive association between neural and fibroblastic cells is a general property of these cell types, but that some fibroblasts are too generally sticky for any degree of specific adhesion to be detectable in adhesive assays. This possibility would be consistent with the ability of HEF, and possibly CHO,cells, but not of Cl3 cells, to show spefific adhesion with neural cells. The results obtained in this work suggest that skin fibroblasts are a generally very adhesive cell type (Tables 1,7), although it is not known whether choroid or meningeal cells are generally less adhesive than skin fibroblasts, for example, in their quantitative adhesive interactions with other fibroblastic cell types.

2) Alternatively, the ability to show specific adhesion with neural cells may be restricted to certain types of fibroblast. This possibility is suggested by the apparent relationship between fibroblasts which have this ability and their positioning with respect to neural tissues (see above), indicating that this specific adhesion could have a functional role in the patterning or in the maintenance of neural organs. It would clearly be of interest to know the extents to which different fibroblastic, and also different neural,cell types can show this specific adhesion. The findings described above suggest that several neural cell types, including NR, cerebrum and dorsal root ganglion cells, have this ability, and that it is the fibroblastic cell types

which are restricted in this capacity. The ability of HEF cells to show specific adhesion with neural retina cells could be explained by the former having retained the properties responsible for the specificity in culture. If this is the case, it might be expected that other cell lines could be found which also show specific adhesion with neural cells. It would be a considerable advantage to be able to work with cell lines in the study of adhesive specificity, because of the large numbers of cells which could be obtained, and therefore the neural-fibroblast system could be useful in this respect.

The finding of specific adhesion between NR and HEF cells is the first demonstration of the ability of a cell line to show specificity, however, Pessac and Defendi (1972b) obtained evidence for the presence of aggregation factors and receptors in several mammalian cell lines, suggesting that these cells might also be able to show specificity. Pessac and Defendi found that one cell line, P388, did not produce a factor but that its aggregation was stimulated by factors secreted by other cell lines. Another cell line, NCT2555, produced a factor which stimulated the aggregation of heterologous cells, but was unable to aggregate in the presence of its own factor. These results were taken as indicating the presence of distinct factors and receptors. The proposed factors and receptors were inactivated by hyaluronidase and trypsin, respectively. Although these findings support the occurrence of bridging mechanisms of adhesion between these cell lines, it is less likely that adhesive specificity would be found in a hyaluronic acid-

mediated adhesive system. Although most studies of specific adhesion have been made using embryonic cell types (see Introduction 3), Walther et al (1973) described an adhesive specificity between adult mouse kidney cells and mouse teratoma cells.

The neural-fibroblast system also provides a clear demonstration of the abilities of cells to adhere by both specific and non-specific means. The HEF cells show specific adhesion with NR cells but only quantitative adhesion with other fibroblastic cell types. The NR cells appear to show specific adhesion with some fibroblasts but not with others. A similar dual adhesive capacity was found for mouse adlult kidney and mouse teratoma cells which showed specific adhesion with each other but not with several fibroblasts. These findings argue against the suggestion of Roth (1968) that cells which had regained their states of being able to show specific adhesion following trypsinization would only be able to adhere to other cell types having the same adhesive mechanism. However, it is possible that the ability to adhere non-specifically to cells capable of showing specific adhesion is restricted to fibroblasts.

The relationship between specific and non-specific adhesion is not clear, although it appears that the first of these involves a decrease in the adhesiveness of one cell type in the presence of another cell type. However, this decrease may be a general one, affecting the non-specific adhesive properties of the responsive cells, or it may be specific for the formation of adhesive contacts between the two cell types.

The approximately two-fold degree of specificity between HEF and NR cells is comparable to that found between choroid fibroblasts and NR cells (Buultjens and Edwards, 1977). These authors suggested that the poor adhesive association of these two cell types could play a role in maintaining the segregation of choroid and neural retina tissues, and also in the separation of these tissues during development. The striking segregation of these tissues, as well as of meningeal and neural tissues, which can be seen during dissection could be explained by specific adhesive differences between these cell types. The possible significance of the relatively small differences in adhesiveness found in these assays in cell positioning is indicated by the experiments of Carter (1967b) and Harris (1973a), who found that cell patterning was determined by quantitative adhesive differences in the substrate.

III A. Culturing of Glial Cells

The ability to isolate and culture glial cells would provide a valuable means of analysing some of the possible roles that adhesive and other behavioural interactions between different cell types might play in the patterning of the nervous system. The evidence that adhesive interactions between neural and glial cells may contribute to the histogenesis of neural organs will be discussed later (see General Discussion).

Previous studies using cultured explants of vertebrate neural tissues have shown that in most or in all cases outgrowths of flattened, epithelioid cells appear in the cultures after a few days and usually grow to form a monolayer underneath the neural cells. Similar cells have also been described in cultures of dispersed neural tissues. These epithelioid cells have been found in a large number of neural tissues, including in fetal (Shein, 1965) and adult (Ponten et al, 1969) human brain, rat brain (Lim et al, 1973), embryonic chick cerebrum (Varon and Raiborn, 1969; Sensenbrenner et al, 1972) and neural retina (Itoh et al, 1975), and in embryonic chick and mouse peripheral neural tissues (Spooner et al, 1971; Varon et al, 1974; Letourneau, 1975a). In the above studies, the epithelioid cells were either referred to directly as glial cells (Ponten et al, 1969; Sensenbrenner et al,1972; Spooner et al, 1971; Letourneau, 1975a) or were thought very likely to be glial cells (Shein, 1965; Lim et al, 1973; Varon and Raiborn, 1969; Varon et al, 1974; Itoh et al, 1975). However, there

is likely to be a certain degree of contamination with mesenchymal cells in these cultures, and it would be important to be able to differentiate between these two cell types. The evidence for the identification of these cells as glial will be considered in the Discussion.

RESULTS

The glial cells used in this study were isolated from embryonic chick neural retina, optic lobe and cerebrum. The glial cells were obtained by plating clumps of tissue of about 25 μ in diameter (see Materials and Methods). Very few glial cells were observed in cultures of single cell suspensions, and cultures made of larger pieces were too heavily contaminated with neural cells. Almost every clump of tissue of moderate size (25-50 μ in diameter) gave rise to an outgrowth of glial cells after 2-4 days. A typical outgrowth is shown in Plate 3a. It can be seen that the neuroblasts, having originated from a relatively small clump of tissue, have been carried outwards on the dorsal surfaces of the glial cells during the formation of the outgrowths. This suggests that the neural cells are likely to have a higher adhesive affinity for the glial cells than for other neural cells in the tissue clumps. The neural cells also appear to spread well on the glial outgrowths. A similar degree of spreading was found when mechanically dispersed neural cells were seeded onto monolayers of secondary culture glial cells. In contrast to this, neural cells were found to neither migrate out of tissue clumps onto the culture substrate in the absence of a glial cell outgrowth, nor to visibly spread when seeded onto the

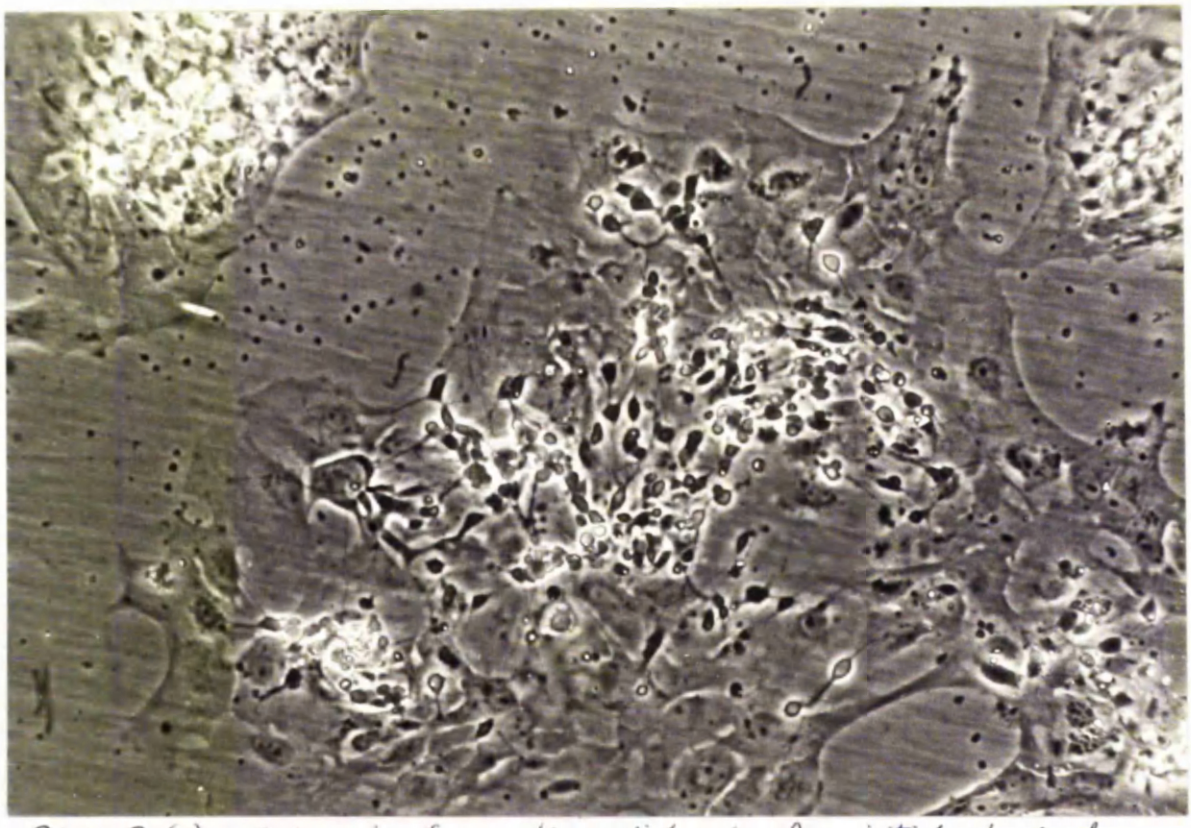

Plate 3 (a). Outgrowth of cerebrum glial cells from initial clump of tissue. Note spreading out of neuroblasts on glial cells. Magnification x 1100.

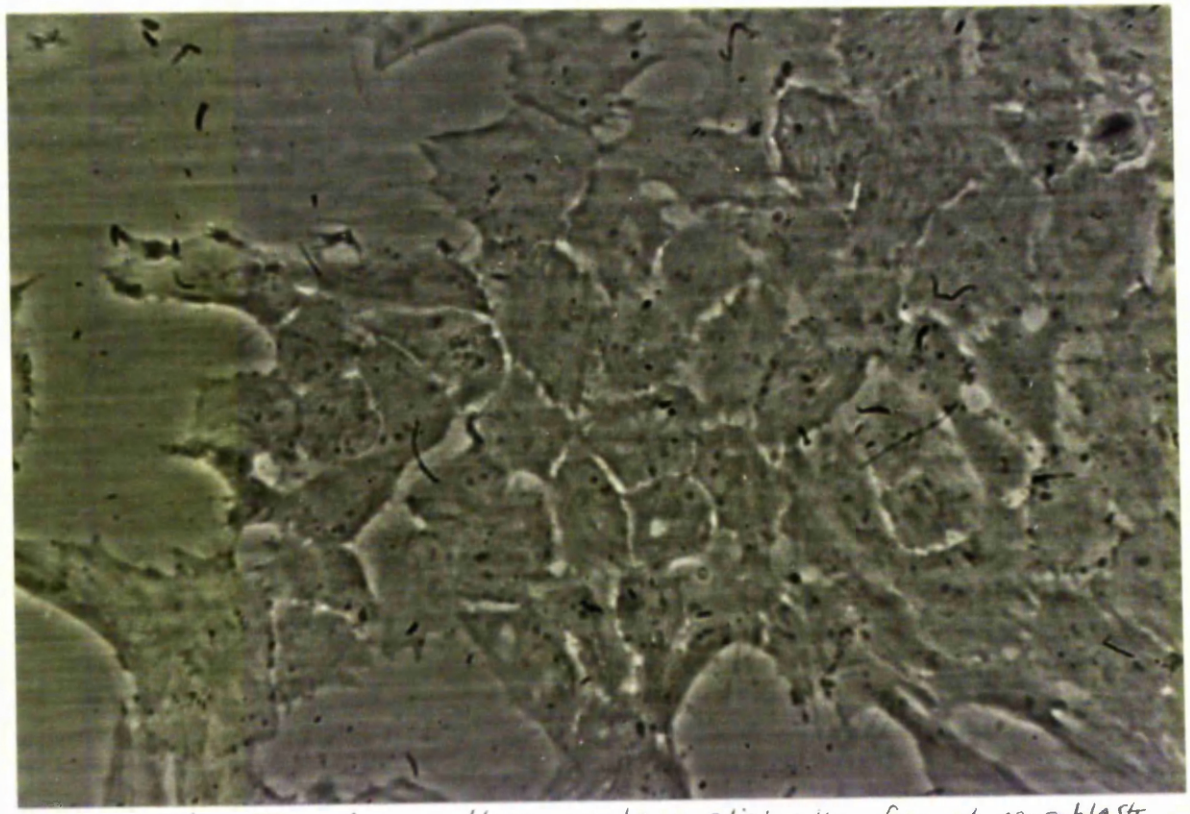

Plate 3 (b). Secondary culture cerebrum glial cells, free of neuroblasts. Magnification X 1400.

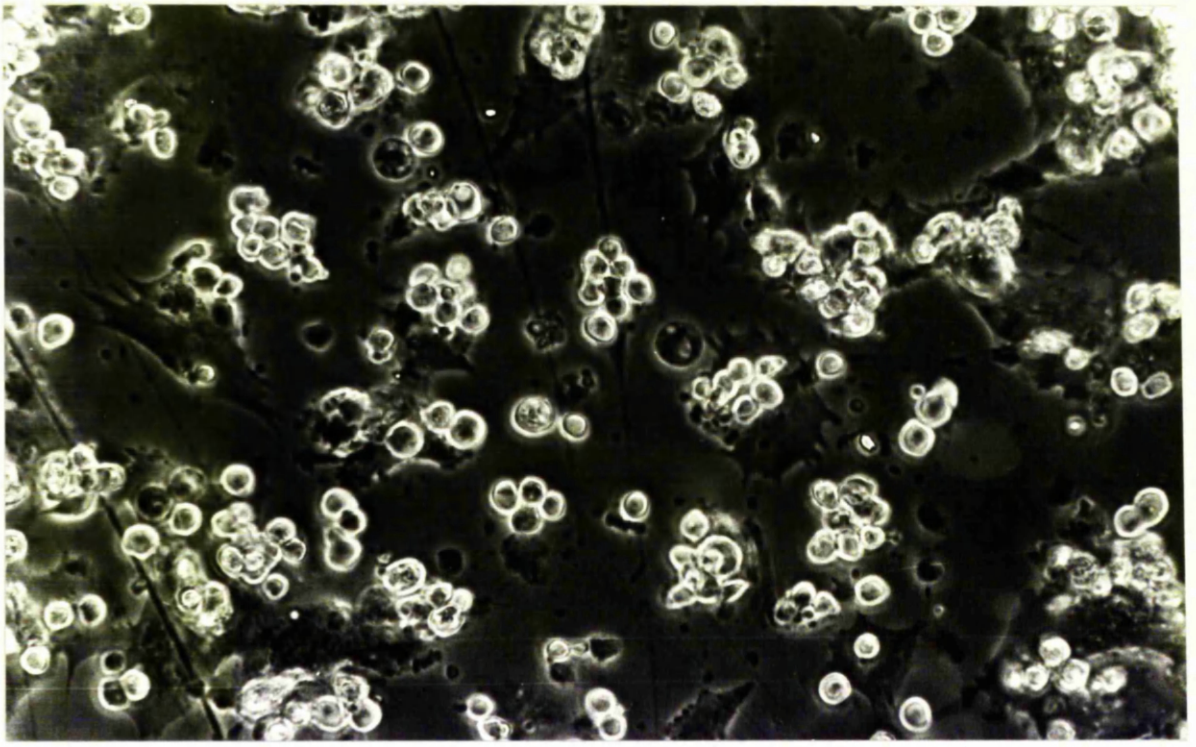

Plate 3 (c). Cerebrum neuroblasts (rounded cells) seeded onto sparse cultures of cerebrum glial cells.
Magnification X 1400.

culture substrate. Evidence that this was due to the relatively higher adhesiveness of the glial cell surfaces for the neural cells was obtained by seeding disaggregated neural cells onto sparse cultures of glial cells. Plate 3c shows that the seeded neural cells attached predominantly to the glial cells and avoided large areas of culture substrate.

The neural cells appeared to be firmly attached to the glial cell outgrowths and monolayers in primary cultures, but became detached during subculturing. After 2-3 subcultures, the cell cultures were substantially free of neural cell contaminants (Plate 3b). The growth rates, detachabilities and morphology of the glial cells did not appear to alter for at least 6 subcultures. After the second, or in some cases third, subculture predominantly single cell suspensions of the glial cells could be obtained.

The glial cells obtained from cultures of all three neural tissues appeared to have the same properties. The morphological features of these cultures were intermediate to those of cultures of epithelial and fibroblastic cells. Very few free cells were observed, either during the initial outgrowths of the cells or during the growth of the cells in secondary cultures. This observation indicates that the degree of contamination of the cultures with mesenchymal cells is likely to be low.

The presence of some mesenchymal cells in these cultures was indicated by two sets of observations. In both cases, it appeared that the mesenchymal-like cells were restricted to a relatively small proportion of the cultured cells. It was found by time lapse filming that a small

fraction of the cells, apparently only present at the edges of the glial cell sheets, migrated out of the sheets at intervals. The migration was often preceeded by a vigorous blebbing activity at the anterior end of the cell, and in some cases the cells appeared to move entirely by the translocation of blebbed membrane. In such cases, new blebs were formed at the anterior ends of the cells at approximately 15 second intervals. The blebs were not likely to be artefacts caused by the conditions of time lapse filming, since small numbers of migrating, blebbed cells were also observed in normal cultures. During their migration, the blebbed cells generally became elongated and appeared to be distinct from what were thought to be glial cells, which showed a fibroblast-like movement. The blebbed cells were quite likely contaminant macrophages. These cells showed stopped movement upon contacting a cell sheet, and during such contacts the blebbing immediately ceased and the macrophage-like cells gradually spread out along the adjacent cells in the cell sheet. Blebbing was occasionally seen in peripheral cells, but appeared to be withdrawn if the cells did not leave the sheet.

It was also found that in some cultures of glial cells in which the medium had not been changed and which were likely to have contained some degree of necrotic cells, a small proportion of the cultured cells appeared to transform into another type of macrophage-like cell. These were characterized by their small size and by having several very large, rapidly moving membraneous expansions. These cells moved rapidly on and off the glial cell sheets, and showed a

striking tendency to align along grooves in the culture surface or along cellular fibrillar processes. They were identified as microglial cells, a mesenchymally-derived macrophage of the nervous system (see Nakai, 1963). Olsson et al (1965) reported the appearance of differentiated mast cells in necrotic cultures of mammalian neural retinal cells, however, similar cells were not seen in this study. The presence of the mast cells was most likely due to the vascularity of the mammalian neural retinal tissues, whereas the chick neural retina is avascular.

No glial cells were found in cultures made of the dissecting medium or of surrounding tissues, indicating that the epitheloid cells were derived exclusively from the neural tissues. The large numbers of epithelioid cells obtained from each neural tissue make it unlikely that many of these cells were derived from the residual mesenchymal cells present in the neural tissues. No glial cells could be obtained from embryos younger than 4 days, but large numbers of these cells were found in cultures of 15 day embryos. Hansson and Sourander (1964) showed that these cells could be readily obtained from adult neural tissues. Therefore, it is unlikely that the epithelioid cells are dedifferentiated neural cells or residual undifferentiated ependymal cells. The presence of at least some glial cells in the cultures was indicated by the appearance, after a few days of culturing, of a thin, membraneous sheath which appeared to cover a substantial portion of the cultures. This was also found in similar cultures by Hansson (1971), although he attributed the sheath to a small number of morphologically differentiated glial cells.

DISCUSSION

Detailed studies of the epitheloid cells derived from the embryonic rat brain and from embryonic chick and mouse peripheral neural tissues have provided evidence that these are predominantly glial cells.

The embryonic rat brain epithelioid cells have been shown to contain two glial cell-specific proteins: S-100 (Lim et al, 1977a) and glial fibrillar acidic protein (Bock et al, 1975; Lim et al, 1977b, unpublished results). It has also been found that monobutyryl (Shapiro, 1973) and dibutyryl (Lim et al, 1973) cyclic AMP, and also a "glial maturation factor" prepared from rat brain extracts (Lim et al, 1973), will transform the morphology of these cells from a thinly spread, epithelioid shape to that of a typical fibrous astrocyte, having several long processes radiating from a thickened cell body and forming cultures of interconnected cellular processes. Using time lapse filming, Lim et al (1976) found that these astrocyte-like cells showed pulsating and tugging activity, a characteristic of differentiated glial cells in culture (Nakai, 1963).

The transformation of the epithelioid cells by the glial maturation factor has been studied by scanning and transmission electron microscopy (Lim et al, 1977b). The main changes which were observed during the transformation were from bundles of microfilaments to 10 nm filaments and from desmosomes to a variation of the zonula adherens. No change was found in the number of microtubules present, however, in the transformed cells these were reoriented to be parallel to the 10 nm filaments.

Lim et al (1977b) also found that the same morphological changes occurred after a few weeks in aggregates of the epithelioid cells in the absence of the maturation factor. Glycogen granules were also found in these cells, providing further evidence for their glial nature. Lim et al suggested that the maturation of the cells in aggregates in the absence of the factor could be explained by the requirement of this process for either a high degree of intercellular contact or for a certain extracellular concentration of some factor. They also proposed that the maturation factor was likely to be a membrane-bound protein, based on its molecular weight of 350,000 (Lim and Mitsunobu, 1974).

The morphological transformation of the epithelioid cells has also been found to occur in serum-free media (Lim et al, 1973). However, Moonen et al (1976) showed that in this case the transformation was not accompanied by ultrastructural differentiation, and attributed it to changes in the shapes of the cells caused passively by the greater adhesiveness of the substrate in serum-free media. On the other hand, cells transformed by cyclic AMP underwent similar ultrastructural changes as those which were transformed by the maturation factor, including the formation of 9 nm filaments. The transformations of the cells by dibutyryl cyclic AMP and by the maturation factor differed in their time courses, requiring 2-3 hours and 3 days respectively (Lim et al, 1973), indicating that the factor is unlikely to act by increasing the levels of cyclic AMP. Moonen et al (1975) found that the transforming activity

of the cyclic AMP was dependent on the composition of the medium, in a way that is difficult to explain.

Sensenbrenner et al (1972) showed that embryonic chick brain extracts had a similar ability to induce the glial-like differentiation of the epitheloid cells obtained from cultures of homologous tissues, indicating that these cells, the same as those used in this study, are very likely to be homologous with the rat brain epitheloid cells. The characteristic presence of epitheloid cells in cultures of most or of all neural tissues, but not of other tissues, with the possible exception of some endothelial cell cultures (eg. Maruyama, 1963), further suggests that these cells are homologous in different neural tissues, although the possibility of some differences cannot be ruled out.

Varon et al (1974) have provided evidence that the epitheloid cells found in cultures of peripheral neural tissues also have glial cell-specific properties. They have shown that these cells have a selective capacity to enhance the differentiation of neuroblasts from homologous tissues, and that this capacity is present in the epitheloid cells in their morphologically partially undifferentiated state. Varon et al also found that secondary cultures of dorsal root ganglia, which consisted exclusively of epitheloid cells, were equally effective in the selective capacity to support neuroblasts as the more heterogenous primary cultures, indicating that this glial-specific property is preserved in these cells during culturing. Therefore, the epitheloid cells used in this

study could be expected to have some glial-specific properties. The epithelioid cells are perhaps best described as incompletely differentiated glioblasts, some of the dedifferentiation being an artefact of the conditions of culturing.

However, it would be useful to have a means of monitoring the epithelioid cell cultures for the presence of mesenchymal cells, and of selectively enriching these cultures for glial cells. Brockes et al (1977) found that the addition of Thy-1 antibody plus complement did not affect Schwann cells whereas this treatment had been shown to kill fibroblasts (Stern, 1973), and suggested that this could be used to select for Schwann cells. A similar immunological approach could be useful for the isolation of different cell types from the central nervous system, for example for the Muller glial cells of the neural retina, which make up almost the entire glial cell population of this tissue (Meller and Glees, 1965).

Hansson (1971) reported that the epithelioid cells of the rabbit neural retina appeared to be mainly mesenchymal, based on their histochemical properties, but that a small number of histochemically differentiated glial (Muller) cells could be distinguished as well. However, these results could be accounted for by the low degree of differentiation of the epithelioid glial cells. For example, it is likely that the epithelioid cells would stain for phosphorylase when cultured in aggregates, since the cells contain glycogen granules under these conditions (Lim et al, 1977b), but not when grown as monolayers. It is difficult to

account for the presence of differentiated glia-like cells in the cultures described by Hansson, as these have not been reported by other workers. However, the presence of these cells may have resulted from the use of newborn rabbit neural retina, in which case some of the mature glial cells may not have dedifferentiated. In addition to the likely presence of some mesenchymal cells in the epitheloid cell cultures, Itoh et al (1975) have shown that small numbers of cells, presumed to be part of the epitheloid cell population, have the capacity to transform onto pigmented retinal epithelial cells.

III B. Adhesion of Glial Cells

RESULTS

Some preliminary experiments were carried out to examine the adhesive interactions between glial cells and neural and fibroblastic cells, using the cell sheet collection assay. The results of several assays are shown in Table 7. The attachment of the cells to cell sheets of skin fibroblasts was used as a control since all of the cell types showed optimal rates of adhesion to these sheets. For example, NR cells adhered equally well to sheets of skin fibroblasts and NR cells (Table 6b) but attached poorly to serum-coated plastic (Table 7).

Table 7 shows that NR and cerebrum cells adhered to glial cell sheets approximately two-fold better than did three types of fibroblast. The glial cell sheets may be the first case of a substrate to which neural cells will attach better than will fibroblasts. Although the two-fold degree of selectivity is relatively low, it is similar to other cases where specific adhesion has been detected using this assay (Table 6a; Buultjens and Edwards, 1977), and there are several reasons for thinking that this could be increased considerably by making some alterations in the conditions of the assay:

1) Purification of the glial cell cultures should improve the degree of selectivity shown. It is possible that the seeded fibroblastic cells were adhering to some

Table 7

Adhesion of Glial Cells

Seeded Cells	Cell Sheets		
	Skin Fibroblast	Glial Cell*	Plastic
Skin Fib	100.0 ± 2.1(14)	52.1 ±21.2(12)	93.4 (6)
Heart Fib	100.0 ± 7.8 (6)	64.2 ± 4.5 (3)	
Limb Fib	100.0 ± 1.6 (3)	34.2 ± 3.2 (2)	
NR	100.0 ±12.5 (4)	88.4 ±33.7 (4)	69.5 (2)
Cerebrum	100.0 ± 3.0 (3)	101.4 ± 3.6 (2) 93.5 ± 4.1 (2)*	16.2 (3)
Glial*	100.0 ± 5.1 (5)	32.1 ± 6.7 (5)	99.1 (5)

Values are means ±S.D. (n) based on total numbers of cell sheets used in separate assays.

* Cerebral glial cells were used in all assays; NR glial cells were also used as cell sheets in one assay, see asterisk.

extent to contaminant mesenchymal cells. The proportions of these cells present in the cultures may have increased during the several subcultures that were made prior to the assays.

2) The experiments shown in Table 7 were made using fibroblastic cell types which may be non-specifically highly adhesive. It is likely that a considerably larger degree of selectivity would be obtained if fibroblasts which are normally contiguous with neural tissues, for example choroid or possibly meningeal fibroblasts, were used (see Discussion II).

3) The degrees of selectivity obtained may have been limited by some of the features of the cell sheet collection assay. The neural cells appeared to attach to the glial cell sheets at approximately their optimal rates under the conditions of this assay. Therefore, more stringent attachment conditions would be required to demonstrate larger adhesive differences between the seeded neural and fibroblastic cells. The use of the collecting aggregate technique, for example, might increase the degree of selectivity (see Introduction 3), and would have the additional advantage of allowing the differentiation of the glial cells prior to the assay (Lim et al, 1977b).

The low adhesiveness of glial cells for homologous cell sheets was striking (Table 7). Most cell types adhere well to homologous cell sheets, with the exception of some epithelial and transformed fibroblastic cell types (see Results I), and in the latter two cases, the cell sheets formed are generally non-adhesive for other cell types. It is possible that the surfaces of glial cells may be

specialized for interactions with neural cells, rather than with other glial cells, as might be expected from the "dispersed" distribution of glial cells in neural tissues. The low rates of attachment of glial cells to glial cell sheets appeared to be dependent on the organization of the cell sheets (in the case of glial cells, monolayers). In separate experiments, it was found that when glial cells were seeded into wells at high densities ($1.0-1.5 \times 10^6$ cells/well), a monolayer of flattened cells was rapidly formed, with numerous rounded cells attached. After 2-3 hours of incubation, at least 90% of these cells were strongly attached to the monolayer cells, and could not be removed by moderate shaking. However, after overnight incubation about 60-70% of the cells were either loose in the medium or could be detached by gentle stirring of the medium. This may have resulted from the recovery of the monolayer cells from trypsinization or from some aspect of the organization of the spread cells into monolayers. It is unlikely that the detachment or decreased adhesion of the rounded cells after overnight incubation was due to changes in the adhesive properties of these cells since freshly seeded cells showed a comparable degree of non-adhesiveness for monolayers which had been incubated overnight.

DISCUSSION

Very few previous studies have been made of the adhesive properties of glial cells, and none of these, as far as I am aware of, have attempted to make quantitative measurements of these properties. Sensenbrenner and Mandel (1974) reported that mechanically disaggregated embryonic chick cerebrum cells were able to attach to monolayers of homologous glial cells by 2 hours, but not to sheets of meningeal or 6 day embryonic chick fibroblasts for 24 hours. This report demonstrates another example of a low adhesive affinity of neural for fibroblastic cells (see Discussion II), however, no reciprocal cell combinations were tested. The main difference between this report and the results obtained in this work is likely to be in the different fibroblastic cell types that were used. Considerably higher rates of attachment of the cerebrum cells were found in this work , and it is possible that this may have been due to some degree of cellular damage caused to the cerebrum cells by the disaggregation procedure used by Sensenbrenner and Mandel (1974). Care was taken in the work described here to minimize the forces used to disperse the cerebral tissues, since excess tissue disruption was found to markedly decrease the ability of the cells to attach to the culture substrate. Sensenbrenner and Mandel also mentioned that trypsinization of the cerebrum cells prevented their attachment to the glial monolayers.

Since no reciprocal cell combinations were tested in the report described above, the results could be explained

by the glial cell monolayers having a non-specifically high adhesiveness, or the fibroblastic cells a non-specifically low adhesiveness, under the conditions of the assay. The work described here both indicates that neural cells appear to show some degree of selective adhesive affinity for glial cell sheets, even under conditions of high rates of cell attachment, and that fibroblasts may show poor adhesive associations with glial cells.

Letourneau (1975a, b) suggested that dorsal root ganglion neural cells were likely to adhere strongly to the dorsal surfaces of homologous glial cells, as the patterns of axonal growth on the glial cells corresponded to those found on surfaces of high adhesivity. Luduena (1973) also showed that the attachment of the same neural cells to cultured glial cells enhanced the differentiation of the neural cells, but found that chick heart fibroblasts were equally effective in promoting the differentiation. However, in both of these studies, only small numbers of cells were tested, and no attempts were made to identify the spread cells as glial or mesenchymal.

GENERAL DISCUSSION

1) Specific Cell Adhesion in Neural Organogenesis

Although the importance of both temporal and spatial gradients in the patterning of the vertebrate nervous system is well established (see Jacobsen, 1970), very little is known about which cellular properties cause or respond to these gradients. The migration of neural cells prior to their morphological differentiation and the formation of specific synapses are two processes which involve adhesive interactions between cells, however,it has not been possible to show whether these interactions play causal or secondary roles in the regulation of these processes. Some of the proposed mechanisms of cell positioning which emphasize the role of cell adhesion have already been discussed (Introduction 1). It is also possible that different mechanisms may control different aspects of neural organogenesis, for example the migration and the final positioning of neural cells could be under separate controls.

Most of the evidence for specific cell adhesion has been obtained using cells which have no relation to each other in terms of tissue patterning (see Introduction 3). The embryonic chick central nervous system is well suited to the study of the possible role of adhesive interactions in organogenesis in that several different types of normally interacting cell types can be isolated, and in addition to this, large numbers of cells can be obtained.

The results obtained in this work support the possibility that selective adhesive interactions between

neural, glial and fibroblastic cells could contribute to the positioning of these cells. Fibroblasts appear to show a relatively low adhesive affinity for glial cells (Table 7), and a second example of specific adhesion between fibroblastic and neural cells has been described (Table 6a; see also Buultjens and Edwards, 1977). It is possible that the lack of invasiveness of neural by fibroblastic tissues and the distinct segregation of these two types of tissue during development (see Discussion II) could be determined by specific adhesive interactions. The apparently high affinity of glial for neural cells, but not for other glial cells, could be significant for the organization of neural tissues. These findings provide a further demonstration of the potential usefulness of relatively well-defined adhesive assays in analysing the nature of the behavioural interactions between contiguous tissue cells.

The results described in this work also show that the adhesive properties of cells may to a large extent be shared by cells of the same general type, for example, by different neural or by different fibroblastic cells. This is suggested by the demonstration of quantitative adhesive interactions between different fibroblastic cell types (Tables 1, 4a) and by the apparently similar affinities of different fibroblastic and different neural cell types for glial cells (Table 7). If cell adhesion does play a role in morphogenesis, then the occurrence of such generally shared adhesive properties would suggest that similar types of adhesive interactions are involved in the patterning of analogous organs, for example, different neural or different glandular organs. However, there also appear to be some

significant exceptions to this rule, for example, in the restriction of the ability of fibroblasts to show selective adhesion with neural cells to some types of fibroblast (Tables 6a,b). These restrictions could also be relevant to the possible role of cell adhesion in cell positioning (see Discussion II), and if so, would provide a useful means of examining the nature of this role.

Some attempts have been made to further isolate subpopulations of neural cells, for example from embryonic chick cerebrum (Varon and Raiborn, 1969), mammalian cerebrum (Giorgi, 1971) and cerebellum (Barkeley et al, 1973) and turtle neural retina (Lam et al, 1972), in all cases using differential centrifugation. McClay and Baker (1975) fractionated embryonic chick cerebellum on a Ficoll gradient, but the fractions were not characterized. Schachner and Hammerling (1976) were able to separate different mouse brain cells by a mixed adsorption hybrid antibody test, in which subpopulations of neural cells which became rosetted by sheep red blood cells in a discontinuous gradient could then be readily separated from the remainder of the cells. Other possible means of obtaining purified populations of neural cells could be the exploitation of their differential sensitivities to toxins (Hansson, 1966), or the irradiation of whole tissues with X-rays at different stages of development (Hicks and D'Amato, 1966).

2) Adhesive Interactions Between Neural and Glial Cells

There is evidence from several sources that adhesive interactions between neural and glial cells, which may involve some degree of specificity, may be important both in the histogenesis of the nervous system and in the differentiation of neural cells. It is also possible that the types of adhesive interaction involved in these two processes may be related (see later).

Autoradiographic and electron microscopic studies have shown that the patterning of both the mammalian (see Sidman, 1973) and the avian (Fujita and Horii, 1963) nervous system is achieved largely by the migrations of neuroblasts from the germinative neuroepithelium to the positions they will occupy in adult tissues. These migrations appear to be normally oriented along the radial processes of either neural or glial cells, and there is some evidence that these processes may direct the migrating cells by means of contact guidance.

This possibility has been most clearly demonstrated to be likely in the mammalian cerebrum and cerebellum. Rakic (1972) studied the migration of neuroblasts to the cortical layer of the rhesus monkey cerebrum. He found that the migrating cells followed the radially-oriented processes of glial cells, which span the entire molecular layer of the cerebrum, and that at all times the neuroblasts were attached to the glial cell processes but apparently not to the adjacent neural cell processes or blood vessels. This was taken to be some indication that the neuroblasts

might be selectively adhering to the glial cells, although the possibility could be not ruled out that the migrating neuroblasts were simply taking taking the path of least resistance through the tissue. In a more detailed study of this migration using serial reconstruction of electron micrographs, Rakic et al (1974) found that the neuroblasts were in all cases attached to the glial cell processes by several filipodial extensions which were intertwined about the processes. This is highly suggestive of the occurrence of contact guidance by the glial cells, being analogous to the mechanism of guided movement of neural cells on culture substrates (Bunge, 1975; Letourneau, 1975b).

Some evidence for the contact guidance of migrating neuroblasts by glial cells has also come from studies on the development of the mouse cerebellum. The latter process is characterized by a double migration across the molecular layer of the neuroblasts which are destined to become granule cells. The migrating cells appear to be at all times closely applied to the Bergman glial fibers which span the molecular layer. Rakic and Sidman (1973) proposed that the failure of the pre-granule neuroblasts to take up their correct positions in the granule layer in Weaver mutant mice could be due to a primary defect in the Bergmann cells. They showed that in heterozygotes, granule cells which were adjacent to normally oriented glial cells were correctly aligned, whereas those which were next to abnormal glial cells were incorrectly aligned or had in some cases detached from the glial cells. Rakic and Sidman argued that the primary lesion was likely to be in

the glial cells, both because a dose response was found between the degrees of abnormality of the glial cell morphology in heterozygotes and in homozygotes, and because the morphological alterations of the glial cells were quite distinct from those those found when these cells atrophy as a result of extrinsic factors. This study also provides some indication that the migration and the final positioning or alignment of neuroblasts may be to some extent controlled in different ways. Sidman (1973) has also suggested that glial cell processes might serve to orient the radially-directed axons of the T-shaped granule cells of the cerebellum, as these axons grow out along the Bergmann glial cell fibers. In some cases, glial fibers do not appear to be necessary for the guided migration of neuroblasts. For example, Das et al (1974) reported that in the mouse cerebellum the glial fibers are not well differentiated at the time of the migration of the pre-cortical neuroblasts, and that the latter appear to be able to migrate along neural fibers and blood vessels as well as along glial fibers. Hinds and Hinds (1974) found that during the differentiation of the ganglion cells in embryonic mouse retina, the migrating growth cones of these cells were wrapped around radially-oriented axon shafts. The migrations of different neuroblasts may occur by more than one mechanism, since three morphologically distinct types of growth cone have been observed during the migrations of different neuroblasts (see Hinds and Hinds, 1974).

The process of the reconstruction of neural tissues

in cellular reaggregates may also involve specific adhesive interactions, both between different types of neural cell and between neural and glial cells. Embryonic mouse (DeLong, 1970) and chick (Adler, 1970; Garber and Moscona, 1972a) neural cells reaggregate and reconstruct tissues of an approximately normal morphology, although these workers have shown that these processes are very dependent on the stages of differentiation of the tissues from which the cells are taken. In some cases, the capacities of disaggregated cells to reconstruct tissues appears to be narrowly restricted to certain stages of development, for example, between 17.5-18.5 days for the mouse cerebrum (DeLong and Sidman, 1970). The relevance of reconstruction to normal tissue patterning is indicated by the formation of disoriented patterns by Reeler mutant mice cerebellar cells in reaggregates, similar to the abnormal patterning that occurs in vivo (DeLong and Sidman, 1970).

The process of reconstruction of the chick neural retina has been studied in detail. The first stages of this process are the random aggregation of the cells, followed by the the development of cell polarity and rosette formation, and later by the fusion of rosettes and the radial layering of the different cell types (Sheffield and Moscona, 1969; Sheffield, 1970; Fujisawa et al, 1974). The formation of rosettes appears to depend on the polarization of adhesive sites on the surfaces of the neural cells, and may also involve some degree of specific adhesion between different cell types.

Electron microscopic observations have provided some evidence that the initial adhesions formed between the disaggregated cells are random with respect both to cell type (Sheffield, 1970) and to the arrangement of adhesive sites on the cell surfaces (Sheffield, 1970; Fujisawa, 1975). In both studies, it was found that after a few hours the sites of intercellular contact had become polarized in the cells, leading to the formation of rosettes with the same side of each cell facing inwards. This occurred both with 7-14 day embryonic cells aggregating in suspension (Sheffield, 1970) and with 5½ day embryonic cells aggregating on a culture substrate (Fujisawa, 1975).

Sheffield and Fujisawa described two types of rosette: lumicentric, found in aggregates of cells taken from 5½, 7 and 10 day embryos, and axocentric, formed by 10 and 14 day embryonic cells. The different cell types which formed these two types of rosette appeared not to mix in aggregates of 10 day embryonic cells, suggesting the possibility that some form of specific cell adhesion may have taken place.

The relevance of rosette formation to normal histogenetic processes is suggested by the finding that the lumicentric rosette-forming cells, thought to be derived from the outer limiting membrane, adhere to each other by zonula adherentes junctions once the polarized adhesive contacts have formed. These cells form similar junctions in vivo (Sheffield, 1970; Fujisawa, 1975). Hicks and D'Amato (1966) have also found that the process of recovery of the mouse neural retina from irradiation with X-rays involves the initial organization of the remaining viable

cells into rosettes. This shows that the formation of rosettes can occur *in situ* and indicates that the cellular properties determining rosette formation, which may involve some degree of specific adhesion (see above), must be interconvertible with the properties that underlie normal histogenesis. The polarization of adhesive sites in neural cells may be of general importance in the development . This is, for example, indicated by the specific localization of acetylcholine receptors at the pre-synaptic sites of cultured embryonic chick neural retinal cells (Nirenbirg and Vogel, 1976).

Glial cells appear to show high adhesive affinities for early rosettes, and may play an important role in the radial patterning of the neural cells after the rosettes have formed. Fujisawa et al (1974) showed that Muller glial cells were ultrastructurally well differentiated during the reconstruction of the neural retina, and proposed that these cells were required for the radial layering of the neural cells. Muller cells have been shown to be associated with newly formed rosettes, both in aggregates (Sheffield, 1970; Fujisawa et al, 1974) and on a culture substrate (Hansson and Sourander, 1964). The fact that this association can take place when the cells are seeded onto a substrate indicates the glial cells are likely to attach to the rosettes prior to the radial organisation of the neural cells. If the glial cells also have polarized adhesive sites, these cells could play a direct role in radially organising the neural cells.

Recent studies on cultures of the peripheral nervous system have shown that glial cells appear to have the ability to selectively enhance the differentiation of isolated neural cells. Two criteria which have been commonly used to measure neural cell differentiation in culture are the capacity of these cells for long term survival and the rate of fiber production. Varon and Raiborn (1972) showed that the presence of NGF (Nerve Growth Factor) was not in itself sufficient to maintain the long term survival of embryonic chick sympathetic ganglion neuroblasts, but that the presence of some non-neural cells was also required. Burnham et al (1972) found that, in addition to this, the requirement for NGF could be reduced or completely abolished if the numbers of non-neural cells were increased, the amount of reduction being determined by the numbers of cells added. This indicated that the non-neural cells could perform two functions in the maintenance of neural cell differentiation, one NGF-like role and another role which could not be replaced by NGF. Burnham et al suggested that the normal function of NGF might be to supplement the supportive capacity of glial cells when these were present in insufficient numbers, for example, in early development. It was also shown that embryonic chick fibroblasts could not be substituted for the non-neural cells in their NGF-like role.

Varon et al (1974) found that the ability of non-neural cells to substitute for NGF was highly tissue specific. They showed that the NGF-like capacity of mouse DRG (dorsal root ganglion) cells for supporting

homologous neuroblasts was greater by several orders of magnitude than that of heterologous non-neural cells, tested from over 20 different tissues. A slight amount of species- and age- specificity was also found. Varon et al concluded that the effective cells in the non-neural cell cultures were presumably glial cells. On the other hand, heterologous non-neural cells, as well as fibroblasts, were found to be equally effective in providing NGF-independent support.

Varon et al (1974) also found that mouse DRG neuroblasts required either NGF or the presence of non-neural cells in order to attach to the collagen substrate, although in this case heterologous and fibroblastic cells could be substituted for the homologous cells. The NGF or non-neural cell requirement for the attachment of the mouse DRG cells appeared to be a property of the majority of these cells, but some other types of neural cell did not have this requirement.

There have been several reports of factors which enhance the differentiation of cultured neural cells, as well as of glial cells (see Discussion III A), although their relationships to the activities of NGF or non-neural cells are not known. Monard et al (1973) found that the morphological differentiation of a neuroblastoma line was induced by the conditioned media of several cell lines, the most effective cell line being a glioma. The extent of differentiation of the neuroblastoma cells, measured by the rate of fiber production, increased linearly with the amount of conditioned medium added. However, high amounts

of activity were also found in the conditioned media of several fibroblastic lines, indicating that the active factor was likely to have had a non-specific effect.

Luduena (1973) showed that the conditioned medium obtained from mixed cultures of embryonic chick DRG cells enhanced the differentiation of DRG neural cells taken from 8 day, but not from 3.5 day, embryos. It was not tested whether this activity was derived from the neural or from the glial cells present in the cultures, although it was found that the age-specific activity was shown by NGF. Sensenbrenner et al (1972) showed that the morphological differentiation of cultured embryonic chick cerebrum cells was enhanced by a cerebrum extract.

It is not known whether these factors are derived from the cell surface, however, this possibility is suggested by the finding of Gombos et al (1972) that the differentiati n of neural cells in culture could be enhanced by membrane-derived glycopeptides extracted from the same cells. Also, Varon et al (1974) mentioned that NGF-like molecules appeared to be present on the surfaces of mouse DRG non-neural, or most likely glial, cells. This suggests that the effects of NGF on cultured cells may be to mimic a cell-cell contact interaction between neural and glial cells which normally is present under physiological conditions. Lim and Mitsunobu (1974) also proposed that a glial maturation factor was likely to be a cell membrane protein.

It is possible that one mode of action of these factors in enhancing neural cell differentiation could be

to increase the adhesiveness of the neural cells or of the substrate to which the neural cells are attached. Letourneau (1975a) showed that the rate of fiber production of DRG neural cells varied directly with the adhesiveness of the substrate, measured by detaching the cells with blasts of air. However, it has not been studied ultrastructurally whether this type of enhancement is similar to that induced by NGF, glial cells or other factors. The stimulation of the differentiation of glial cells by highly adhesive substrates was found to not lead to ultrastructural changes in these cells (see Discussion III A). Monard et al (1973) have suggested that the enhancements of neural cell differentiation by highly adhesive substrates or by cyclic AMP are unphysiological in that they decrease the growth rates of the neural cells.

It is not clear how NGF acts to promote the attachment of mouse DRG neural cells to the substrate. Varon et al (1974) suggested that NGF might exert its effect by adsorbing to the substrate, since this compound has been shown to adsorb to a variety of other substrates (Pearce et al, 1972). Alternatively, NGF might act directly on the adhesiveness of the neural cells. In either case, it is possible that a gradient of adhesive substances, analogous to NGF, present on the surfaces of glial cells,could provide one means of contact guidance for neural cells, either if bound to the cell surface or if released locally. If secreted into the medium, these substances could guide neural cells by chemotaxis. Varon et al (1974) suggested that the tissue specificity of the NGF-like glial cell activity could be

explained by the presence of a class of NGF-like proteins distributed between the cells of different neural tissues. This could be compatible with the role of adhesive gradients in the interactions between neural and glial cells during development.

Several previous studies have indicated that specific adhesive affinities of neural for glial cells are likely. Abercrombie et al (1949) described an apparently high affinity of rabbit Schwann cells for peripheral neural cells. Several workers (eg. Varon et al, 1974) have commented on the tendency of many DRG neural cells to have satellite (glial) cells strongly attached to them after the tissues have been dispersed. These satellite cells have also been shown to have a high affinity for dispersed neural cells when these celltypes are coaggregated (Meller, 1974). Meller and Waelsch (1975) showed that if the glial membranes encapsulating DRG neural cells in culture were destroyed by treatment with puromycin, the membranes would be resynthesized and would reassociate with the neural cells after the removal of the drug. The newly formed glial cell membranes were found to adhere closely to and to show numerous interdigitations with the neural cells, similar to the interactions between the membranes of these cell types which is found in vivo. It is likely that this capacity of the glial cells to encapsulate neural cells is specific for neural cells, and it is also possible that some tissue specificity of this capacity might be shown. Varon and Raiborn (1969) fractionated embryonic chick cerebrum cells by differential centrifugation. They

observed that one class of small neural cell appeared to be strongly attached to the epitheloid non-neural cells, which the authors suggested to be glial-like cells. Koenig (1967) was able to isolate the different cell types of the rabbit neural retina by microdissection after fixation of the tissue. He found that Muller cells which had been dissected free of the neural retina had clusters of photoreceptor cells tightly bound to one end, suggesting a particularily high adhesive affinity of these cells for the Muller cells. Hansson (1971) also reported that differentiated Muller cells in cultures of adult neural retina tended to become associated with photoreceptor cells which could also be distinguished in these cultures.

IV. Cell-to-Substrate Adhesion

The previous three sections have pointed to some differences between the adhesive properties of neural, glial and fibroblastic cells. In this section it will be examined whether there are differences in the mechanisms by which these three cell types attach to culture-type substrates, in terms of their requirements for divalent cations and their sensitivities to cytochalasin B. Previous studies by other workers on cell-to-substrate adhesion have used almost exclusively mesenchymally-derived cells, and it would be of interest to know whether the relatively low rates of attachment of NR cells, and even more so of cerebrum cells (see Table 7), are due to quantitatively or qualitatively different adhesive properties of these cell types.

RESULTS

1. Effects of Divalent Cations

The effects of Ca^{2+}, Mg^{2+} and Mn^{2+} ions on the cell-to-substrate attachment of NR, glial and fibroblastic cells are shown in Table 8a. Details of the individual experiments are given in Table 8c. The effect of Mn^{2+} ions on the attachment of cerebrum cells in one experiment is shown in Table 8b.

Table 8a shows that for each cell type 10^{-5} M Mn^{2+} ions was more effective in promoting attachment than 10^{-3} M Mg^{2+} or Ca^{2+} ions. The Mn^{2+} ions were optimally effective at 10^{-4} M, but did not stimulate attachment at 10^{-3} M.

Table 8a

Effects of Divalent Cations on Adhesion

Molarity	Mn^{2+}	Ca^{2+}	Mg^{2+}
NR Cells (4)			
10^{-6}	0.6 ± 0.3		
10^{-5}	48.8 ± 22.1	0.7 ± 0.3	0.8 ± 0.6
10^{-4}	64.5 ± 15.8	7.5 ± 10.2	1.1 ± 0.8
10^{-3}		23.7 ± 22.4	34.0 ± 15.7
2×10^{-3}		38.4 ± 24.2	$59.8^{*} \pm 6.7$
Skin Fibroblasts (5)			
10^{-6}	8.6 ± 9.4		
10^{-5}	50.3 ± 23.4	8.3 ± 6.0	5.0 ± 2.9
10^{-4}	77.2 ± 19.1	17.2 ± 10.2	21.0 ± 16.4
10^{-3}		23.4 ± 14.7	64.0 ± 33.2
2×10^{-3}		20.3 ± 14.7	$41.5^{*} \pm 26.4$
Glial Cells (1)			
10^{-6}	2.9 ± 0.8		
10^{-5}	24.8 ± 1.5	1.7 ± 0.4	1.3 ± 0.2
10^{-4}	46.7 ± 4.7	4.1 ± 0.9	6.5 ± 1.0
10^{-3}		6.5 ± 1.0	$28.1^{*} \pm 1.0$

Values are % attachment to serum-coated plastic after 20 mins. Means of several experiments (Table 8c).

* 10^{-3} M Mg^{2+} + 10^{-3} M Ca^{2+}

Table 8b

Adhesion of Cerebrum Cells

	40 mins	80 mins
HH	5.6 ± 0.7	7.4 ± 1.5
+ Mn^{2+} *	7.0 ± 0.6	7.7 ± 2.4

Per cent attachment to serum-coated plastic

* 10^{-4} M Mn^{2+} added to HH.

Table 8c

Divalent Cations: Individual Experiments

NR Cells

Ion	Expt	10^{-6}	10^{-5}	10^{-4}	10^{-3}	2×10^{-3}
Mn^{2+}	1			45.6 ± 7.5	12.4 ± 2.0	
	2	0.4 ± 0.1	46.3 ± 3.1	69.5 ± 6.5		
	3	0.9 ± 0.4	75.2 ± 3.3	84.6 ± 5.3		
	4	0.5 ± 0.2	24.8 ± 4.9	58.2 ± 3.6		
	Ave	0.6 ± 0.3	48.8 ± 22.1	64.5 ± 15.8		
Ca^{2+}	1			4.0 ± 0.4	9.8 ± 2.0	
	2		0.5 ± 0.1	1.0 ± 0.2	22.1 ± 4.0	43.0 ± 4.2
	3		0.9 ± 0.2	24.1 ± 3.5	57.4 ± 15.3	63.3 ± 7.4
	4		0.7 ± 0.2	1.1 ± 0.1	5.3 ± 2.2	8.9 ± 2.0
	Ave		0.7 ± 0.3	7.5 ± 10.2	23.7 ± 22.4	38.4 ± 24.2
Mg^{2+}	2		0.7 ± 0.3	0.5 ± 0.2	29.7 ± 2.6	54.4 ± 2.9
	3		1.3 ± 0.9	2.0 ± 0.4	53.7 ± 1.7	65.4 ± 3.2
	4		0.5 ± 0.2	0.6 ± 0.2	18.5 ± 2.3	
	Ave		0.8 ± 0.6	1.1 ± 0.8	34.0 ± 15.7	59.8 ± 6.7

Values are means ±S.D. as for Table 1; expressed as per cent of total radioactive counts in cell suspensions.

Table 8c Cont.

Divalent Cations: Individual Experiments

Skin Fibroblasts

Ion	Expt	10^{-6}	10^{-5}	10^{-4}	10^{-3}	2×10^{-3}
Mn^{2+}	1		72.7 ±4.3	81.8 ±3.7	11.7 ± 2.1	
	2	5.4 ±0.8	34.0 ±2.5	78.7 ±17.4		
	3	6.9 ±1.6	50.1 ±1.8	77.0 ± 5.4		
	4	1.4 ±0.7	17.8 ±2.6	49.0 ±10.5		
	5	26.8 ±4.0	76.8 ±4.1	100.5 ± 5.1		
	Ave	8.6 ±9.4	50.3 ±23.4	77.2 ±19.1		
Ca^{2+}	1		10.5 ±3.1	14.5 ± 4.3	16.7 ± 3.6	
	2			41.4 ± 2.8	37.8 ±10.3	
	3		5.1 ±1.1	15.7 ± 1.7	22.4 ± 2.2	26.3 ±15.1
	4		1.5 ±0.9	1.7 ± 0.8	1.8 ± 0.5	4.5 ± 3.5
	5		16.2 ±0.8	29.4 ± 6.6	34.6 ± 7.1	32.3 ± 0.9
Mg^{2+}	1					72.0 ± 6.6
	2		3.3 ±1.1	12.4 ± 3.4	56.1 ± 6.3	17.3 ± 1.6
	3		4.4 ±2.1	15.4 ± 1.5	74.5 ±19.7	42.6 ± 8.6
	4		2.9 ±1.5	7.4 ± 1.4	19.8 ± 5.0	9.4 ± 3.3
	5		9.3 ±0.3	47.0 ± 4.0	103.9 ± 3.0	66.2 ± 3.1
	Ave		5.0 ±2.9	21.0 ±16.4	64.0 ±33.2	41.5 ±26.4

The Mg^{2+} ions were in all cases more effective than Ca^{2+} ions, although the rates of attachment in the presence of these two ions varied considerably between experiments (Table 8c). There appeared to be less variation when Mn^{2+} ions were used. The variation was most likely due largely to damage to the cells caused by their preincubation in Ca^{2+}- and Mg^{2+}- free medium prior to the assays. This can also explain the low rates of attachment of the cells in 10^{-3} M Ca^{2+} + 10^{-3} M Mg^{2+} ions, the concentrations of these ions present in balanced salt solutions.

In contrast to these results, Mn^{2+} ions at 10^{-4} M failed to stimulate the attachment of cerebrum cells, either at 40 or at 80 mins.(Table 8b). These cells were consistently found to have very low rates of attachment to the substrate during the course of this work (see also Table 7). The same appeared to be true of optic lobe cells, but was not as marked in the case of NR cells. It was also found that cerebrum and optic lobe tissues were very brittle and could be dispersed into predominantly single cell suspensions by a mild mechanical dispersive treatment. Neural retinal tissues were somewhat more resistant to the same mechanical treatment, and substantial trypsinization was required in order to obtain good single cell suspensions from these tissues.

2. Effect of Cytochalasin B

Table 9 shows that cytochalasin B inhibited the attachment of neural, glial, fibroblastic and also liver epithelial cells, both at 1.0 μg/ml and at 5.0 μg/ml.

The neural cells were considerably more sensitive to inhibition by this drug than the other cell types, although the glial cells were also strongly affected by this drug when added at the higher of the two doses. The effects of cytochalasin B appeared to be similar after 20 and after 40 mins. The DMSO in which the drug was dissolved was shown to not be responsible for the inhibition of attachment.

Table 9

Effect of Cytochalasin B

Cell	Time	Control	0.5% DMSO	CB 1.0 µg/ml	CB 5.0 µg/ml
NR	20	100.0 ±14.7(2)		27.6 ± 6.4(3)	29.3 ± 5.9(3)
	40	100.0 ±24.5(6)	99.4 ± 7.5(3)	43.1 ±10.9(6)	21.4 ±16.4(6)
Skin	20	100.0 ± 8.6(3)		81.4 ± 6.8(3)	55.7 ±18.2(3)
	40	100.0 ± 4.4(3)		83.2 ± 5.4(3)	75.4 ±11.3(3)
Glial	20	100.0 ±11.0(3)	102.5 ± 0.9(2)	72.4 ± 7.3(2)	22.0 ± 8.1(3)
	40	100.0 ± 2.4(3)	100.8 ± 9.4(3)	73.1 ± 4.8(3)	28.9 ±21.4(3)
Liver	20	100.0 ± 7.6(6)	110.3 ±12.4(5)	76.3 ±12.9(6)	55.2 ±12.6(6)

Values are means ±S.D. based on total numbers of wells. Attachment of cells to serum-coated plastic expressed as per cent of total counts in cell suspensions. Time in mins.

CB = Cytochalasin B (dissolved in a final volume of 0.5% DMSO).

DISCUSSION

The finding that neural, glial and skin fibroblastic cells appear to have very similar divalent cation requirements for cell-to-substrate attachment suggests that these ions are likely to be exerting their effects in the same way in each cell type.

The results (see Table 8a) show that Mg^{2+} ions were somewhat more effective than Ca^{2+} ions in promoting cell attachment and that Mn^{2+} ions were highly effective at very low concentrations. Similar results have been reported for other cell types. Rabinowitch and DeStefano (1973a,b) first showed that Mn^{2+} ions at very low concentrations stimulated both the attachment to and the spreading on culture substrates of macrophages and Sarcoma I cells. Similar findings were made by Pegrum and Maroudas (1975) and by Witkowski and Brighton (1972) using fibroblasts, but apparently no previous reports have described the effects of Mn^{2+} ions on the rates of attachment of other cell types.

Takeichi and Okada (1972) found that Mg^{2+} ions were more effective than Ca^{2+} ions in stimulating the attachment of chick scleral fibroblasts to serum-coated plastic. This was also found by Rabinowitch and DeStefano (1973b) for Sarcoma I cells and by Garvin (1968) for rat polymorphonuclear neutrophils attaching to serum-coated glass beads. Therefore, it is possible that this may be the general case for the attachment of cells to culture substrates.

The effects of divalent cations on cell-to-cell adhesion appear to be more variable. Cultures of chick

scleral cells were found to have a higher index of dispersion in the presence of Mg^{2+} ions than in that of Ca^{2+} ions (see Okada et al, 1974). The index of dispersion is a measure of the extent to which cells grown on a substrate are randomly dispersed or are grouped together in clusters, and this result was taken to mean that Mg^{2+} ions preferentially increased cell-to-substrate adhesion whereas Ca^{2+} ions had a greater effect on cell-to-cell adhesion, in the case of scleral cells.

Some cell types have been shown to aggregate in the apparent absence of divalent cations (Beug et al, 1973; Edwards et al, 1975). Armstrong (1966) found that the abilities of several divalent cations to stimulate the aggregation of chick limb bud cells, when used at concentrations which resulted in similar electrophoretic mobilities of these cells, were, in order of decreasing effectiveness, Mg^{2+}, Ca^{2+}, $Sr^{2+} = Ba^{2+}$. Although Armstrong suggested that these results could not be explained on electrostatic grounds, this conclusion is not valid if the effects of the different concentrations of the ions used on the degrees of double layer repulsion of the cells are taken into consideration.

There have been some reports of a specific requirement for Ca^{2+} ions in cell adhesion. McGuire (1976) found that this appeared to be the case for the collection of chick liver cells by liver aggregates. Culp (1974) described a Ca^{2+} ion specificity for the attachment of 3T3 fibroblasts to substrate-attached material, which was shown to consist mainly of hyaluronic acid. However, Wateson et al,

(1973) showed that the hyaluronic acid-dependent aggregation of feline lymphoma cells occurred in the absence of divalent cations. Grinnell (1976b) reported that the attachment of BHK cells to a culture surface coated with a cell spreading factor isolated from serum required Ca^{2+} ions but not Mg^{2+} ions. Armstrong and Jones (1968) found that Ca^{2+} ions specifically prevented the disaggregation of embryonic tissues of Rana pipiens in the presence of EDTA. However, these different effects of divalent cations do not necessarily indicate that these ions affect cell adhesion by more than one mechanism (see below).

The effects of divalent cations on cell-to-substrate adhesion, as well as on cell-to-cell adhesion, could be explained in several ways: 1) The ions decrease the electrostatic forces of repulsion between the cells and substrate, or between cells. Most of the effects of divalent cations on cell adhesion could be readily explained by this mechanism. The relatively specific activities of Mn^{2+} or, in some cases, Ca^{2+} ions could be mainly due to the higher degrees of binding of these ions to the Stern layers of the cells. In other cases, differences in the effects of different divalent cations could be accounted for by the extents to which the ions reduce the negative electric charges of the double layer (see above). However, it is also possible that divalent cations could have effects on other properties related to cell adhesion, in addition to their electrostatic effects.

2) The ions have general effects on cell spreading movements, for example, by altering membrane rigidity or by regulating the sub-membraneous contractile system.

Grinnell (1974) presented some evidence for the involvement of divalent cations in spreading movements, rather than in the formation of adhesive contacts, during the attachment of BHK fibroblasts to serum-coated plastic (see Introduction 2). Rabinowitch and DeStefano (1973b) found that Ca^{2+} ions inhibited the stimulation of attachment of Sarcoma I cells by Mn^{2+} ions, and suggested that the latter ions could be replacing the normal function of Ca^{2+} ions in controlling the polymerization of microtubules and microfilaments. One argument against this proposal is the finding that Mn^{2+} ions also show a stimulatory effect on the aggregation of Dictyostelium cell ghosts (Sussman and Boswitch, 1975b). In general, there appears to be a good correlation between the requirements of cells for attachment and for spreading, for example, as can be seen in the effects of Mn^{2+} ions (see above), Ca^{2+} and Mg^{2+} ions (Okada et al, 1974), cytochalasin B (Grinnell, 1974b), and of cationic anesthetics (Rabinowitch and DeStefano, 1975). In addition to this, cells which can be visually observed to spread poorly, for example, neural, myoblastic and, to some extent, epithelial cells, also show low rates of attachment to serum-coated plastic, whereas fibroblastic and glial cells, which spread rapidly on this substrate, also attach rapidly (observed in this study).

3) The ions bind specifically to one or more molecular components of the cell surface, possibly to a molecule directly involved in the formation of adhesive bonds. There is very little evidence for this, although certain enzymes, for example, adenyl cyclases and glycosyl

transferases, are activated by Mn^{2+} ions. The similar effects of divalent cations on the three cell types used in this study indicate that if the ions are activating specific surface components, then the latter must be present on the surfaces of all three cell types.

The lack of a stimulatory effect on the attachment of cerebrum cells by Mn^{2+} ions could be explained either by these cells being quantitatively very non-adhesive, and outside the range in which the ions could be effective, or by the cells lacking some basic requirement of cell adhesion which is not directly related to the requirement for divalent cations. The very strong stimulatory effects of Mn^{2+} ions on the attachments of other cell types suggests that the second possibility is the most likely, if the effects of these ions are electrostatic ones or are to increase the rate of spreading. The cerebrum cells appear to increase in their abilities to attach to the substrate during a 1-3 day period in culture (see also Sensenbrenner and Mandel, 1974). This process may involve the synthesis of certain membrane proteins, analogous to the induction of adhesiveness of rat hepatoma cells by dexamethasone, a process taking several hours and requiring protein synthesis (Ballard and Tomkins, 1970). In sharp contrast to their inability to attach to substrates, newly dispersed cerebrum cells are able to rapidly aggregate (observed in this study), indicating a marked difference between cell-to-substrate and cell-to-cell adhesion in this case.

Cytochalasin B has previously been shown to inhibit the attachment of BHK fibroblasts to serum-coated plastic (Gail and Boone, 1972; Grinnell, 1974b; Shields and Pollock, 1974) and to slightly reduce the ability of Ehrlich ascites cells to attach to serum-coated glass or plastic (Weiss, 1972). Miranda et al (1974) showed that cytochalasin D inhibited the cell-to-substrate attachment of several fibroblastic cell lines. Both Shields and Pollock (1974) and Miranda et al (1974) found that, in contrast to this, cytochalasins B and D increased the adhesiveness of already attached cells to the substrate. Sanger (1974) observed that 5 μg/ml cytochalasin B, when added to cultures of embryonic chick striated muscle myoblasts and fibroblasts, selectively caused the detachment of the former after 24 hours. The fibroblasts became arborized and remained firmly attached. Cytochalasin B has also been shown to inhibit the aggregation of blood platelets (Haslam, 1972) and limpet hemocytes (Jones and Partridge, 1974).

In this study, it was found that NR cells, and possibly also glial cells, which are also neuroectodermally derived cells, appeared to be more sensitive to cytochalasin B than skin fibroblasts and epithelial cells. However, all four cell types were affected by the drug, indicating that a cytochalasin B-sensitive property is involved in the attachment of all of these cell types, and that the differences in sensitivity are likely to be quantitative ones.

The mechanism of action of cytochalasin B is not known. Both cytochalasins B and D have been shown to preferentially bind to cellular membranes (Mayhew et al, 1974;

Tannenbaum et al, 1973), findings which are consistent with the hydrophobic-hydrophilic natures of these molecules and with the ability of cytochalasin B to rapidly inhibit glucose uptake (Estensen and Plagemann, 1972; Zigmond and Hirsch, 1972). Hauschka (1973) and Mayhew et al (1974) obtained evidence against the binding of cytochalasin B to specific surface components. Therefore, cytochalasins B and D may act primarily by binding to the plasma membrane. It is unlikely that the inhibition of cell-to-substrate attachment by these drugs is a result of the inhibition of glucose transport, since Miranda et al (1974) found that the latter drug was more potent than the former in preventing cell attachment, but had relatively little effect on glucose uptake by the cells.

However, there is much evidence which suggests that the principal effects of cytochalasins B and D are on the cellular contractile system. Wessels et al (1971) proposed that the effects of cytochalasin B on various cell types were to interfere with the contraction of microfilaments. Miranda et al (1974) showed that the addition of cytochalasin D to attached fibroblasts, rather than disrupting cortical microfilaments, appeared to cause the microfilaments to become organized into compact masses which were frozen into their insertion points in the plasma membrane, as if in a state of contracture. Cytochalasin B has also been shown to have effects on isolated actomyosin and actin (see Miranda et al, 1974, for discussion) and cytochalasin D to bind to myosin (Puszkin et al,1973). Therefore, most of the effects of cytochalasins B and D

could be explained by their binding to the insertion points of actin or myosin in the plasma membrane, or by binding directly to these two components if these are present as normal integral membrane proteins. The effects of cytochalasins on cell attachment would then most likely be on cellular spreading movements, either by interfering with actomyosin-linked spreading, or by disrupting the control of membrane rigidity by the cytoskeletal system, suggested by the induction of membrane blebbing by cytochalasin D (Godman et al, 1975). It is less likely that other effects of cytochalasins, for example, the reduction of mucopolysaccharide synthesis (Sanger and Holtzer, 1972), would inhibit cell attachment in a short term assay.

V. Preparation of Hemagglutinating Activity

There is some indirect evidence for the possible involvement of carbohydrate-binding proteins, which may show hemagglutinating activity (HA), in cell adhesion (see Introduction 4). Teichberg et al (1975) and more recently Simpson et al (1977) described HA's in rat brain, and also in the electric organ of Electroplax and in a neuroblastoma cell line. Yamada et al (1975) showed that a cell surface protein present on embryonic chick fibroblasts also had HA, although this appeared to have different properties in that it was inhibited by EDTA and glutamine, but not by simple sugars. Therefore, it was examined whether HA was present in embryonic chick neural and glial cells, and whether any differences could be found in the types of HA which might be present in different neural cell types.

RESULTS

Homogenates of embryonic chick cerebrum, optic lobe and neural retinal cells, and also of cerebrum and optic lobe glial cells, agglutinated fixed rabbit erythrocytes (Table 10 and Plate 4a). Approximately the same yields of HA were obtained from each of the three neural tissues and the optimal activity appeared to be present in the second fraction (Table 4a), which was thought to represent the crude membrane fraction. The homogenization procedure which was used was shown by Merrell and Glaser (1973) to result in the formation of small membrane vesicles. The second fraction would therefore be expected to contain the crude membrane fraction,

Table 10

Hemagglutinating Activity

Source	Activity
Neural Tissues*	
Homogenate	++
Fraction 1 (Pellet 13,500 g)	++
Fraction 2 (Pellet 90,000 g)	+++
Fraction 3 (Supernatant)	None
Glial Cells	Same as above
Fetal Calf Serum	None
Conditioned Media**	None

* Similar yields obtained from cerebrum, optic lobe and neural retina. Fresh homogenates diluted 1:7 with PBS had approximately 500-1000 units HA. Fraction 2 appeared to be about 4 times enriched in activity, allowing for an estimation of the tissue:medium ratio.

** Conditioned media were prepared from all three neural tissues and from glial cell cultures as described in Materials and Methods.

Plate 4 (a)

Cerebrum HA

Dilution*	Row	Wells 1-6	Wells 7-12
1/50	A	Sheep erythrocytes +trypsin**	-trypsin
	B	Chicken erythrocytes +trypsin	-trypsin
	C	Rabbit erythrocytes(-trypsin)	
1/100	D	Lactose 50 mM	EDTA 6 mM
	E	D-(+)-Galactose 50 mM	p-Nitrophenyl B-D-Galactose 10
	F	Melibiose 50 mM	N-Acetylmannosamine 25 mM
	G	L-Fucose 50 mM	D-Fucose 50 mM
	H	N-Acetylgalactosamine 25 mM	Glutamine 50 mM

* Dilution of HA in Well #1, followed by serial two-fold dilutio

** Erythrocytes fixed and trypsinized as in Materials and Methods

Plate 4 (b)

	Wells 1-6	Wells 7-12
Row	Cerebrum HA Dilution 1/40	Optic Lobe HA Dilution 1/40
A	Control	Control
B	α-Methylmannoside 50 mM	Same
C	N-Acetylmannosamine 25 mM	Same
D	Control	Control
E	N-Acetylgalactosamine 25 mM	Same
F	Control	Control
G	D-Fucose 50 mM	Same
H	L-Fucose 50 mM	Same

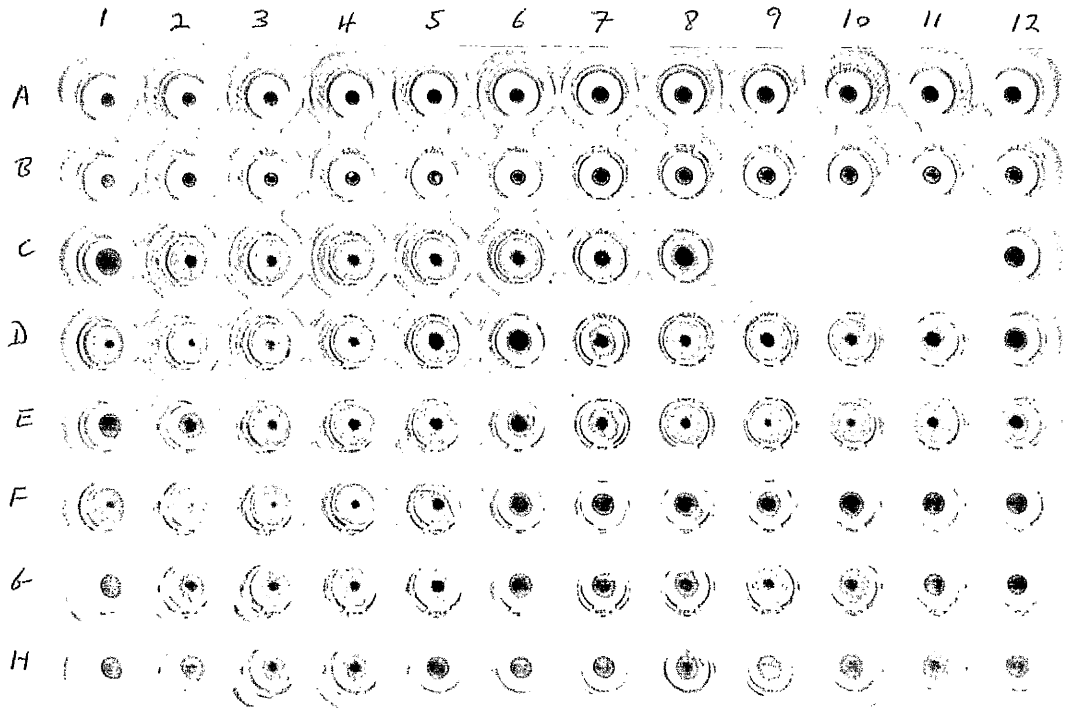

Plate 4 (a). Hemagglutination (see opposite page).

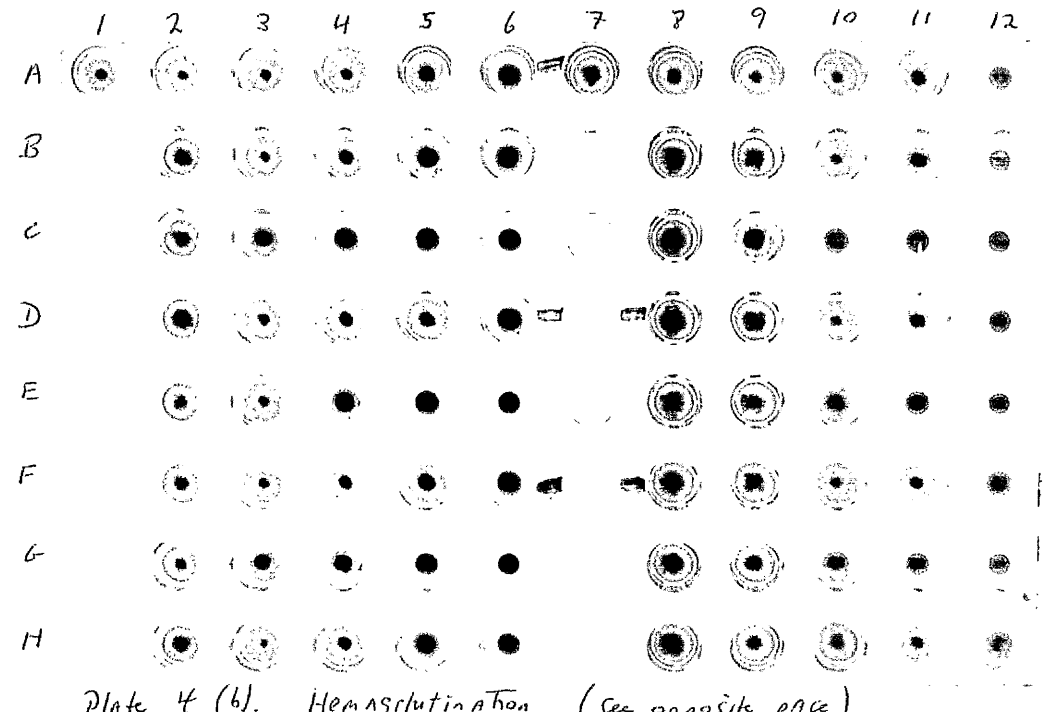

Plate 4 (b). Hemagglutination (see opposite page).

similar to the sedimentation of small membrane vesicles prepared form fibroblast cell lines (see Dysart and Edwards, 1977). However, since no characterization of the fractions was made in this work, it is possible that some degree of crude membrane was also present in the first fraction. The addition of 1% deoxycholate to the second fraction immediately cleared the suspension and also dissolved particles visible by phase contrast microscopy, indicating that this fraction was likely to be predominantly membrane-derived. No activity was present in the supernatant, in fetal calf serum, used to culture the glial cells, or in conditioned media obtained from 24 hour cultures of neural cells or pieces of neural tissue (see Materials and Methods). In some cases, the conditioned medium was prepared in the presence of 200 units/ml Trasylol to minimize proteolytic degregation of possible HA (see Rutishauser et al, 1976), and also in some cases in the presence of 10% fetal calf serum, to rule out the possibility of toxic effects caused by the serum-free medium.

Plate 4a, Row C shows the dose response of the HA prepared from cerebrum tissue (fraction 2). Well #12 shows the control button of unagglutinated cells. Wells 1-8 show the typical optimum dose response which was found at all times. Serial dilutions of the HA were made starting at well #1, and the total activity is about 6400 units, taking well #8 as the endpoint (where activity = reciprocal of the dilution at the endpoint). Rows A and B show that both sheep and chicken erythrocytes were not agglutinated by the cerebrum HA.

Some tentative evidence was obtained for the inhibition of the HA by specific sugars. Plates 4a and 4b show that N-Acetylmannosamine and N-Acetylgalactosamine at 25 mM, and to a lesser extent D-Fucose and L-Fucose at 50 mM, inhibited hemagglutination, the former in the presence of large amounts of HA. There was no detectable inhibition by D-Galactose or by three derivatives of D-Galactose, used at similar concentrations, indicating that the inhibition by the above sugars might be specific and not a non-specific effect of sugars at high concentrations. However, confirmation of this would require testing these sugars at lower concentrations. Glutamine at 50 mM also inhibited hemagglutination (Plate 4a) but EDTA at 6 mM was ineffective, suggesting that the HA has different properties from the hemagglutinin described by Yamada et al (1975). The cerebrum and optic lobe HA's had very similar sensitivities to the 5 sugars tested (Plate 4b), making it likely that these are similar compounds.

The HA was unlikely to have been an artefact of the homogenization or fractionation procedures. Similar yields of activity were obtained when these procedures were carried out in PBS, in HH or in PBS + 1.5 mM EDTA. The addition of 50 μg/ml DNAase did not affect the yield. The HA was very reproducible both on the same day (see Plate 4b) and after storage at -20°C. However, the HA was very sensitive to warm temperatures and fell off rapidly at room temperature (about 90% loss of activity after 1-2 hours). Approximately 80% of the activity was lost by storage overnight at 4°C.

DISCUSSION

The embryonic chick neural HA's described here appear to have similar properties to previously reported vertebrate cell hemagglutinins. These have been shown in most cases to be primarily membrane-bound (see Introduction 4), and to show erythrocyte specificity for hemagglutination (Teichberg et al, 1975; Dysart and Edwards, 1977; Stockert et al, 1974). Although the evidence for specific sugar inhibition of the HA's described in this work is only preliminary, it is of interest that Dysart and Edwards (1977) similarly found specific inhibition of a BHK cell HA by N-Acetylgalactosamine and D-Fucose, but not by D-Galactose, when the same erythrocyte type was used.

Some evidence for the possible involvement of specific carbohydrate-binding proteins in embryonic chick neural cell adhesion has been described by Roth et al (1971) and by Balsamo and Lilien (1975) (see Introduction 4), however, the apparent similarities of the HA's isolated from cerebrum and optic lobe (see Results) indicate that these are not likely to be related to the receptors for the factors described by Balsamo and Lilien. In the above studies it is also possible that the carbohydrate-binding proteins affected adhesion indirectly, for example by their biosynthetic activity. However, the investigation of the possible roles of carbohydrate-binding proteins in neural-neural and neural-glial cell interactions should be facilitated by the isolation of hemagglutinating activities from these cells. The hemagglutination assay allows considerable more chemical precision than aggregation assays, and also provides a convenient means of examining the spatial and

temporal distributions of at least one class of carbohydrate-binding protein in different neural tissues. Simpson et al (1977) have recently shown that the appearance of a rat brain hemagglutinating activity appears to be developmentally regulated.

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ERRATA

p. 65 line 12. Add "Radioactivity was measured in a Beckman Liquid Scintillation Counter (LS-200B). The wells containing the attached cells were normally cut out and placed in 10 ml toluene + 0.4% PPO in disposable scintillation vials. In some cases, the attached cells were dissolved in 1 N NaOH and diluted with Aquasol (1:9)."

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