A FINE STRUCTURAL STUDY OF
ADHESIVE CELL JUNCTIONS IN
HETEROTYPIC CELL AGGREGATES

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ABSTRACT
Mixed suspensions of cells obtained by dissociation of 7 day chicken embryo heart and pigmented retina were allowed to reaggregate in tissue culture. The reaggregates which resulted contained both kinds of cells. Establishment of homogeneous tissues by cell sorting out in these reaggregates was advanced by 20 hr in culture and was complete within 2 days. When sorting out was advanced, heterotypic aggregates were fixed, sectioned, and examined in the electron microscope. Particular attention was paid to the morphology of regions of contact between cells. No qualitative differences were observed in the contact junctions between like cells (heart-heart or pigmented retina-pigment retina junctions) and unlike cells (heart-pigmented retina junctions). Broad areas of undifferentiated cell contact with cell membranes separated by a 100–200 A gap were formed regardless of cell type. Specialized junctions of the fascia and macula adherens type were also present, not only between like cells but also between unlike cells.

INTRODUCTION
Many tissues and organs can be dissociated into suspensions of single cells upon exposure to agents which remove divalent cations from the external milieu, such as EDTA (Armstrong, 1966), or to proteolytic enzymes, such as trypsin (Rous and Jones, 1916; Moscona, 1952), or to combinations of these agents (Steinberg, 1967). Usually, upon return to conditions favorable for cell adhesion, the dissociated cells can reaggregate with one another and can reestablish coherent tissues. If suspensions of cells dissociated from two different tissues are combined, in most cases, the reaggregates which form will contain cells from both tissue types. Initially the different kinds of cells are randomly arranged in the reaggregate (Holtfreter, 1944; Townes and Holtfreter, 1955). As demonstrated initially by Holtfreter (1944) and Moscona (Moscona and Moscona, 1952), the random cell arrangement is soon replaced by a more orderly configuration in which the cells organize themselves into homogeneous tissue masses. The establishment of these homogeneous tissues occurs by a process known as “sorting-out”: cells move about in the reaggregate in such a fashion that like cells become sequestered together (Townes and Holtfreter, 1955; Moscona, 1957; Trinkaus and Gross, 1961; Trinkaus and Lentz, 1964; Garcia-Bellido, 1966).

A variety of hypotheses have been proposed to account for sorting out. Most of these assume that the process is mediated by adhesive differences between cells of the different tissues. These include what will be termed the specific adhesion hypothesis (Weiss, 1941, 1947; Moscona, 1962) and the differential adhesion hypothesis (Steinberg, 1963a, 1964). These models assume that surface properties, especially the adhesive properties of cells,
play the primary role in guiding and directing cell sorting out.

Although there have been several studies with the electron microscope of initial reaggregation of dissociated cells (Overton, 1962, 1969; Lesseps, 1963; Millonig and Guidice, 1967), electron microscope studies of cell sorting out are few in number (Hilfer et al., 1968). Information of fine structural aspects of cell sorting out is critical to an understanding of the mechanism of the process. In the following study, an examination will be made of the morphology of cell adhesions in heterotypic aggregates which have completed cell sorting out. Special attention will be paid to comparing the adhesions in heterotypic aggregates which have completed cell sorting out.

MATERIALS AND METHODS

The tissues used in this study were heart ventricle and pigmented retina from the eye, both taken from 7-day-old chick embryos (White Leghorns from a commercial in-cross-bred stock). Cells of both tissues could be identified unambiguously under the electron microscope: the former by the bundles of thick and thin myofilaments and the latter by cytoplasmic melanin granules.

Cell Dissociation

Heart ventricles from 8-12 embryos were dissected out and minced into tiny pieces. These were incubated in 16-ml screw cap test tubes in 2-3 ml of a solution which contained 0.1% crude trypsin (Difco 1:250, Difco Laboratories, Inc., Detroit, Mich.) dissolved in Ca++-, Mg++-free Hanks' solution containing 2 mm ethylenediaminetetraacetate (EDTA), pH 7.5, at 37°C. During incubation, the tissue pieces were agitated on a rotating drum test tube rack. After 25 min in trypsin solution, the tissue pieces were pelleted gently by a brief low speed centrifugation and the trypsin solution was removed. The pieces of tissue were washed four to five times with Hanks' solution and were finally suspended in 1 ml of tissue culture medium (Eagle's MEM + 10% Horse Serum (HS)). A few drops of 0.1 mg/ml stock solution of DNase II (Worthington Biochemical Corp., Freehold, N. J., crystallized two times) were added to digest any extracellular DNA-containing slime (Steinberg, 1963b). The actual dissociation of the tissue was accomplished by stirring the contents of the test tube on a mechanical test tube stirrer (Vari Whirl, Van Waters & Rogers Inc., San Francisco, Calif.) Care was taken at this step to prevent turbulence and cavitation in the solution.

The pigmented retina was dissected free from the other components of the eye and dissociated by a procedure modified from that initially developed by Whittaker (1963) and Trinkaus (1963). Intact eyeballs were preincubated in Ca+++, Mg++-free Hanks' solution, pH 7.3, for 15 min at 37°C. Removal of the back of the eye was accomplished following a 15 min incubation in a 3% trypsin (Difco 1:250) + 1% pancostatin (Difco 1:75) solution made up in Ca++-, Mg++-free Hanks' solution, pH 7.3, 37°C. Following incubation in this solution, the eyes were transferred with a widemouth pipet into tissue culture medium (MEM + 10% HS). The back of the eye, which had by now detached from the pigmented retina, was picked off with forceps, and the pigmented retina was peeled from the neural retina with needle knives. The isolated pigmented retinas were dissociated by a 45 min incubation at 37°C in a 0.1% solution of trypsin dissolved in Ca++-, Mg++-free Hanks' solution containing 2 mm Na2-EDTA, pH 7.3. Following trypsin treatment, the tissue was washed free of trypsin, suspended in culture medium containing horse serum and DNase, and dispersed with the test tube stirrer as described above for heart tissue.

Clumps of cells were removed from both cell suspensions by several low speed centrifugations. By this procedure healthy, well dispersed populations of cells were obtained. Each heart yielded about 1.4 X 10⁶ cells and each pigmented retina yielded about 0.4 X 10⁶ cells.

Cell Reaggregation

Cell concentrations were determined by counting on a hemocytometer. Reaggregation was accomplished in 25 ml Erlenmeyer flasks placed on a gyroratory water-bath culture shaker (New Brunswick Scientific Co., New Brunswick, N. J.) as described by Moscona (1961). Temperature was 37°-38°C. Initially, the shaker was rotated at 70 rpm but was speeded up to 100 rpm after 1 day to retard further fusion of cell aggregates. Each flask contained 2 X 10⁶ heart cells and 1 X 10⁶ pigmented retinal cells suspended in 3 ml of culture medium. Culture medium was Eagle's MEM containing 100 units/ml of penicillin and 100 μg/ml of streptomycin and 10% horse serum. Earle's buffered saline was used to prepare the culture medium. Each flask was gassed with a 5% CO₂—air mixture prior to being placed on the shaker. The flasks were then tightly stoppered with silicon rubber stoppers (Belco Glass, Inc., Vineland, N. J.). The pH of the culture medium remained constant at 7.2 for over 1 wk with this arrangement.

Electron Microscopy

Aggregates were removed from the shaker, washed two to three times in Hanks' saline, and fixed in cold
Heterotypic reaggregates of dissociated chick embryo heart ventricle and pigmented retina cells after 48 hr in tissue culture. Homogeneous tissue masses have been established by cell sorting out: heart cells are collected at the peripheries of the aggregates and pigmented retinal cells (distinguishable by their black color in 1a and by the presence of cytoplasmic melanin granules in 1b) form central masses of tissue. 1a, Living heart-pigmented retina reaggregates. X 213. 1b, Phase-microscope view of a 1.5 µ section through a heart-pigmented retina reaggregate. No pigmented retina cells are visible in the peripheral layer of heart tissue, and vice versa. Hematoxylin-safranin 0. X 730.

Karnovsky's fixative (as modified by Trelstad, Hay, and Revel, 1967) for 25 min. Aggregates were then washed for 10-20 min in cold 0.1 M sodium cacodylate buffer, pH 7.3, and postfixed for 1 hr in cold 1.2% OsO₄. Dehydration was carried out in acetone, and aggregates were embedded in Epon 812 or Bojax No. 1A. Silver-to-grey sections were cut on a Porter-Blum microtome (Ivan Sorvall Inc., Norwalk, Conn.) with glass knives. Sections were mounted on carbon-Formvar films on No. 150 copper grids, stained with uranyl acetate and lead citrate or with lead citrate alone, and examined in a Hitachi HU 11E electron microscope. Measurements were made with a magnifying measuring eyepiece on prints of known enlargement. Thick sections for light microscopy were also prepared and were stained with hematoxylin and safranin O (Schantz and Schecter, 1965).

OBSERVATIONS

The degree of dissociation of the initial suspensions of heart and pigmented retina was good. With both heart and pigmented retina, better than 80% of the cells were single cells, and cell clumps larger than four to five cells were absent. Cell viability, as judged by cell appearance under the phase-contrast microscope, was also high. Aggregation of dissociated cells, resulting in the formation of large aggregates containing both heart and pigmented retina cells, occurred within 6 hr after cells were

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placed in culture. Sorting out of heart and pigmented retinal cells was advanced by 19 hr of culture and was complete by 43 hr. Fig. 1 a shows several living 43-hr aggregates. Fig. 1 b shows a phase-contrast micrograph of a 1/3 micron section through a 43-hr aggregate. Pigmented retina cells are confined to the centers of the reaggregates; the peripheral or surrounding tissue consists solely of heart cells. By 12 hr in culture, the heart cells of some of the heterotypic aggregates contracted rhythmically.

**General Cytology of Cells**

In most cases, pigmented retinal cells were easy to distinguish from heart cells in thin sections since the cytoplasm of the former contained pigment granules in various stages of development and the heart cells contained myofilaments. Mature pigment granules were very dense and were membrane bounded (Fig. 2). Granules in earlier stages of melanin deposition had fibrous interiors. Heart cells contained bundles of thick and thin myofilaments (Fig. 2). The cytoplasm of pigmented retinal cells was often noticeably denser than that of heart cells, due to the presence of larger numbers of ribosomes. The nucleus of pigmented retinal cells sometimes was lobulated with several deep indentations of the cytoplasm.

Both cell types possessed prominent Golgi bodies (Fig. 3), numerous mitochondria, cytoplasmic microtubules (Fig. 11), and membrane-lined cytoplasmic vesicles. Some of the smaller cytoplasmic vesicles (Fig. 8) are identical in morphology to vesicles which were commonly observed at cell surfaces (Fig. 5). The cell surface vesicles present in both heart and pigmented retina cells in the aggregate are of the rough surfaced type (Fawcett, 1965). This kind of cell surface vesicle has been implicated in the transport of protein into cells (Roth and Porter, 1964) and has been observed in a variety of different cell types (Roth and Porter, 1962; Bruni and Porter, 1965; Pannese, 1969; Zachs and Saito, 1969; Bonnet, 1969). Pigmented retina cells contained small-to-moderate amounts of rough endoplasmic reticulum. In some cells the lumen of the rough endoplasmic reticulum was dilated and filled with moderately dense material.

**Fine Structure of Cell Contacts**

Three sorts of interfaces between cells were observable in the reaggregates: wide spaces between adjacent cells, regions of close apposition of plasma membranes where the plasma membranes are separated by a 100-200 A wide space, and specialized junctional regions of the fascia adherens and macula adherens types. Wide intercellular gaps were present between heart cells (Fig. 6), between pigmented retinal cells (Fig. 3), and between heart and pigmented retinal cells (Figs. 2 and 3). There was no obvious difference in abundance or extent of wide gaps in any of these three situations. Certainly the largest percentage of cell surface area faced on such spaces. Often cells sent long thin pseudopods into the wider intercellular spaces. The filipods which were sometimes quite numerous (Fig. 3) had a core of filaments which were oriented parallel to the long axis (Fig. 4). The filipods are similar to those described by Taylor (1966) in cultured chick embryo kidney cells. Cell debris in the form of membrane vesicles and flocculent material was also present in some of the wide gaps.

Less than half of the total cell surface area was involved in cell contact. The most common sort of cell-cell contact was simple apposition of plasma membranes (Figs. 5-7). Apposed plasma membranes were separated by a 100-200 A wide gap of low electron density. This sort of junction has

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**Figure 2** Electron micrograph of a portion of the interface between heart and pigmented retina tissue in a 19 hr old mixed aggregate. Only limited regions of close contact between cells are apparent (arrows). Pigmented retina cells (R) are distinguishable by the presence of melanin pigment granules (p) in the cytoplasm. Heart myoblasts (H) can be distinguished by the presence of myofilaments (m). × 26,000.

**Figure 3** Interface between pigmented retina and heart in a 19 hr old reaggregate. Filopod pseudopodia (f) project into wide intercellular lacunae (L). A cell junction (fascia adherens) (j) connects a heart cell process (H) to a pigmented retina cell (R). Small junctions between heart (j') and pigmented retina cells (j") are also present. Note that the interface between the two pigmented retina cells (R and R") has only limited regions of close cell-cell contact, much like the interface between the heart and pigmented retina cell in Fig. 2. G, Golgi body. 19 hr old reaggregate. × 21,500.
been observed in a variety of tissues, and it has been proposed that cells adhere to one another in these contact regions (Curtis, 1967). At least in those cases where cells adhere to artificial substrates, regions of simple apposition are probably regions of adhesion since, in the regions of closest approach to the substration, a 100-200 A gap still is present (Coon and Manasek, 1969; Cornell, 1969). However, it is not certain whether regions of plasma membrane apposition are also regions of cell-to-cell adhesion in the present system.

By 19 hr, numerous specialized regions of cell contact were also present. These were found joining not only heart cells with heart cells and pigmented retina cells with pigmented retina cells but, in addition, joining heart and pigmented retina cells (Figs. 3, 8-11). The specialized junctional complexes were both of the fascia adherens type and the macula adherens (desmosome) type. In both types of structure, apposed cell membranes were straight and parallel. The amount of electron-opaque material in the intercellular space was increased over that in regions of simple apposition, and in a few cases it appeared to be organized as fibers connecting apposed plasma membranes (Fig. 10). The cytoplasm subjacent to the junctional complex was condensed. In heart cells the fascia adherens was distinguished by the insertion of actin filaments into the condensed cytoplasm lying beneath the plasma membrane (Fawcett and McNutt, 1969). The junctional complexes involved in cell adhesion had specialized plaques present in both cells, but the degree of differentiation of the subjacent cytoplasm was not always equal in both cells (Fig. 10). In addition to the cylindrical pseudopodia which project free into intercellular lacunae, cell processes which are in contact with neighboring cells have been noted.

In some cases these appear to be spread over the surface of a second cell which fronts on a wide space in the aggregate (Figs. 5, 11), while in other cases they contact cells on both sides (Fig. 6, 12). The latter type are sometimes very long and very thin (Fig. 12).

**DISCUSSION**

Cell sorting out following dissociation and reaggregation has interested embryologists for several years because, under certain conditions, normal tissue relationships can be regenerated by the process (Moscona and Moscona, 1952; Townes and Holtfreter, 1955; Weiss and Taylor, 1960; Giudice, 1962; Garcia-Bellido, 1966). Although the morphogenetic events which give rise to these tissue relations in the course of normal embryonic development rarely involve cell sorting out from random mixtures of cells, it is hoped that knowledge of the cell properties which guide the sorting out process in vitro will aid in an understanding of the cell properties which guide normal morphogenetic movements during embryogenesis.

Of the various hypotheses which have been put forward to account for cell sorting out, the two which received the greatest attention are the specific adhesion hypothesis and the differential adhesion hypothesis. The specific adhesion hypothesis developed by Weiss (1941, 1947), Moscona (1962), Roth (1968) and others favors the idea that cell adhesions are specific in the sense that most kinds of cells adhere far better to cells of their own cell type than they do to other kinds of cells. These specific adhesions are thought to be mediated by characteristic adhesive materials residing at cell surfaces (Weiss, 1958; Moscona, 1962, 1968). Moscona has proposed that sorting

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**FIGURE 4** Filopods projecting into a broad lacuna between pigmented retina cells. Core filaments are shown. 19 hr old reaggregate. X 70,000.

**FIGURE 5** Interface between a pigmented retina cell (R) and a heart cell (H). A myofilament-containing process of the heart cell appears to be spreading over the surface of the pigmented retina cell (arrow). Rough-surfaced pinocytotic vesicles (v) are present. 19 hr old reaggregate. X 25,400.

**FIGURE 6** Interface between pigmented retina cell (R) and heart tissue (H). Note the wide space (L) between the heart cells, H1 and H2. A process from heart cell H1 lies between H2 and the pigmented retina cell. Note the ribosome helix present in the pigmented retina cell (arrow). n, nucleus; v, rough-surfaced vesicle. 19 hr old reaggregate. X 25,400.

**FIGURE 7** Broad areas of close contact interspaced by wide intercellular spaces at the juncture between heart and pigmented retina tissue. 19 hr old reaggregate. H, heart cell; R, pigmented retina cell; n, nucleus. X 18,900.
out occurs following regeneration of the surface adhesive macromolecular layer lost during dissociation. Once regeneration of these surface layers of material has occurred, like cells are able to recognize each other and make cell specific adhesions.

Good evidence exists for the action of specific cell surface adhesion-controlling substances in the formation of species-specific cell aggregates in interspecific mixtures of dissociated sponge cells (Humphreys, 1963; Moscona, 1963; MacLennan and Dodd, 1967). Recent evidence suggests that the initial reaggregation of dissociated avian cells may exhibit tissue specificity as well (Roth, 1968; Roth and Weston, 1967) and that tissue-specific surface macromolecules may be involved in the aggregation process (Lilien, 1968; 1969; Kuroda, 1966). However, it should be emphasized that these later observations deal with the initial reaggregation of dissociated cells and may not accurately describe those adhesive interactions of cells within established reaggregates which are thought to be responsible for sorting out.

An alternative model, termed the differential adhesion model, has been put forward by M. S. Steinberg (Steinberg, 1963а, 1964). This model assumes that cells adhere with characteristic strengths of adhesion which differ for different kinds of cells and that sorting out and determination of final tissue placement result from the maximization of the sum of the adhesive strengths of all the cells in the aggregate. Although, to a certain extent, the two hypotheses deal with different aspects of sorting out and, in these respects, are not in conflict, they do differ in their treatment of adhesions between different kinds of cells in those mixed reaggregates where sorting out results in a sphere-within-sphere configuration of tissues. The differential adhesion hypothesis assumes that the strength of adhesion between the unlike cells is stronger than between the cells which form the outer layer of tissue. In the present system, this hypothesis predicts that pigmented retina cells adhere more strongly to heart cells than heart cells do to each other. The specific adhesion hypothesis assumes that like cells adhere better to like cells than they do to other kinds of cells.

In the present system, we were unable to observe any morphological differences between the kinds of cell junctions in homotypic and heterotypic cell contacts, either in reaggregates which were in advanced stages of sorting out (19 hr) or which had completed the process (43 hr). Thus, if cells do actually have specific cell recognition sites on their surfaces, they do not affect the gross morphology of the kinds of cell contacts which are made. In addition, no evidence was found for the existence of voluminous extracellular substances which might be instrumental in binding cells together. Smaller amounts of material exterior to the plasma membrane probably are present but are not visualized clearly under the conditions of fixation and staining which were used (Rambourg and Leblond, 1967).

It should not be too surprising that specialized attachment devices are made between differing cell types in vitro, because this situation is known to occur in a variety of situations in vivo where different kinds of cells are in direct contact with each other without an intervening basement lamina. Desmosomes, septate desmosomes, tight junctions, or other specialized cell-connecting devices occur between a variety of different cell types: arteriole endothelial and smooth muscle cells (Rhodin and Gerdin, 1967), the different sorts of cells lining the stomach (Farquhar and Palade, 1965; Ito and Winchester, 1963), intestine (Farquhar and Palade, 1963), and insect midgut

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**Figure 8** Heart cell process (H) lying between two pigmented retina cells (R). In the plane of this section, the lower pigmented cell is separated from the main body of pigmented retinal cells by this heart cell process. By 19 hr in culture, pigmented retina cells isolated amongst heart cells were uncommon. Numerous heart-pigmented retina junctional complexes joining the heart cell process to pigmented retina cells have been formed (arrows). It is of some interest that specialized cell junctions connecting heart and pigmented retina cells are present even in regions where sorting out is as yet not completed. In the upper pigmented retina cell, helical clusters of ribosomes are evident. Rough-surfaced vesicles, both open to the outside and completely within the cytoplasm, are present. 19 hr old reaggregate. X 23,000.

**Figure 9** Specialized contact junction (j) between heart cell process (H) and pigmented retina cell (R). n, nucleus. 19 hr old reaggregate. X 38,200.
(Anderson and Harvey, 1966), trophoblast, and uterine endothelial cells (Enders and Schlafke, 1969), nerve and glial cells (Heuser and Doggenweiler, 1966; Coggeshall and Fawcett, 1964; Panneese, 1969), bipolar olfactory receptor cells and sustentacular cells in the olfactory epithelium (De Lorenzo, 1963; Reese, 1965), ear vestibular hair cells and supportive cells (Hurato, 1961; Kimura, 1966), rods or cones and Müller cells of the retina (Bloom and Fawcett, 1968), mammalian oocytes and follicle cells (Anderson and Beams, 1960; Adams and Hertig, 1964), photoreceptors and associated pigment cells in some invertebrates (Hermans and Cloney, 1966), and other cell types (Knoth, 1968; Barber and Wright, 1969; Hay, 1961; Rifkind, Chin, and Epler, 1969).

In addition, embryonic cells of different germ layers are electrically coupled, not only to adjacent cells in the same tissue but also to distant cells in other tissues, in both the squid embryo (Potter et al., 1966; Furshpan and Potter, 1968) and the chick embryo (Sheridan, 1968). Since electrical coupling requires that the cells be adherent to each other (Loewenstein, 1966), this is strong evidence

**Figure 10** Specialized junction between a heart cell (H) and a pigmented retina cell (R). The intercellular matrix of one junction (arrow) appears to be organized as fibers connecting the apposed plasma membranes. n, nucleus. 43 hr old reaggregate. × 55,900.

**Figure 11** Interface between heart (H) and pigmented retina (R). A cell contact junction (j) binds a myofilament-containing heart cell process to the pigmented retina cell. A second heart cell process appears to be spreading over the surface of the pigmented retina cell (arrow). t, microtubules. 19 hr old reaggregate. × 28,600.
that cells of different tissues can adhere quite well to one another in the intact embryo. Indeed, close- and tight-junctions have been observed between ectoderm and mesoderm cells and between mesoderm and endoderm cells in the gastrulating chick embryo (Trelstad et al., 1967). It would be interesting to know if electrical coupling occurs between cells of different tissues in the heterotypic cell aggregate after sorting out has been completed, as this might shed further light on the sorts of adhesions made between unlike cells in vitro.

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