CELL SORTING IN THE PRESENCE OF CYTOCHALASIN B

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ABSTRACT

The ability of cytochalasin B to inhibit ruffled membrane activity and cellular locomotion of vertebrate cells in monolayer culture prompted its use to study the necessity for this kind of active cellular locomotion in cell sorting in heterotypic cell aggregates. Cell sorting was inhibited in chick embryo heart-pigmented retina aggregates but a remarkable degree of sorting did occur in neural retina-pigmented retina aggregates. In these experiments, the levels of cytochalasin B employed (5 or 10 μg/ml) are sufficient to inhibit completely locomotion of these cell types in monolayer culture. It is proposed that the degree of cell movement achieved during sorting in neural retina-pigmented retina aggregates in the presence of cytochalasin B is the result of changes in cell contact resulting from adhesive interaction of cells. The effect of cytochalasin B on the initial aggregation of dissociated cells was also tested. With the cell types used in this study (chick embryo neural retina and limb bud), aggregation was not affected for a period of several hours.

INTRODUCTION

One of the principal means of locomotion of vertebrate cells in tissue culture involves a "ruffled" or "undulating" membrane located at the advancing end of the actively locomoting cell. The undulating membrane is a highly flattened portion of the cell with localized thickenings (or ruffles) which propagate from the leading edge backwards toward the main body of the cell. The ruffles appear to be intimately involved in the process of locomotion since cells contact the substratum primarily at the tips of ventral ruffles (Ambrose, 1961). The activity of the ruffled membrane (and consequently the motile activity of the cell) can be inhibited reversibly by the drug cytochalasin B (Carter, 1967 b, 1972). Inhibition of locomotion is rapid and complete at concentrations of 1–10 μg/ml.

The ability of cytochalasin B to inhibit active cellular locomotion in monolayer culture has suggested its use for investigating the movement of cells in three-dimensional cell aggregates during cell sorting. Cell sorting is the process that leads to the establishment of homogeneous tissues from initially disordered heterotypic cell aggregates. The aggregates in question are formed after aggregation of suspensions of dissociated cells that contain cells of two different cell types. As the cells aggregate from suspension, the aggregates contain both cell types mixed initially in a disordered fashion (Holtfreter, 1944; Moscona, 1957; Trinkaus and Gross, 1961; Okada, 1965; Burdick, 1968; Armstrong, 1971). When these aggregates are maintained in culture, the cells move about within the aggregate to construct homogeneous tissues (Townes and Holtfreter, 1955; Moscona, 1962; Trinkaus and Lentz, 1964; Trinkaus, 1969; Steinberg, 1963 b, 1970; Armstrong, 1970). Apparently, during cell sorting, cellular movement is guided by adhesive interactions between the cells (Weiss, 1961; Moscona, 1965; Roth, 1968; Steinberg, 1963 b, 1964, 1970). The present study was developed to examine the mechanism of the rearrangement of cells that occurs during cell sort-

ing. To what degree does this movement of cells require active cellular locomotion and to what degree is the movement of cells a passive result of changes in adhesive cell-to-cell contact between cells of different cell types in the aggregate? Cytochalasin B has been employed to help answer this question. In the present study, we observed that in certain tissue combinations cell sorting can proceed at least partially to completion, even in the presence of levels of the drug sufficient to abolish completely any sign of ruffled membrane activity of cells in monolayer culture. This suggests that a fair measure of cell sorting can occur in the absence of active cellular locomotion. Apparently, this degree of sorting is the result of changes in cell contact resulting from adhesive interaction of cells.

MATERIALS AND METHODS

The tissues used in this study were heart ventricle, neural retina, and retinal pigment epithelium from 7-day old chick embryos (White Leghorns from a commercial incrossbred stock) and limb bud mesenchyme from 4-day old chick embryos.

Cell Dissociation

Heart ventricle and pigmented retina were isolated and dissociated, employing a technique modified from that developed initially by Whittaker (1963) and Trinkaus (1963) as described by Armstrong (1971), and limb bud mesoderm, using the techniques of Moscona and Moscona (1952) as modified by Niederman and Armstrong (1972). Tissues were treated with a 0.1% solution of crude trypsin (Difco 1:250, Difco Labs, Inc., Detroit, Mich.) dissolved in Ca^{++}-, Mg^{++}-free Hanks' solution containing 2 mM ethylenediaminetetraacetate (EDTA), pH 7.5, 37°C. Trypsin-treated tissues were washed free of trypsin solution and were suspended in tissue culture medium (Eagle's Minimal Essential Medium [MEM] + 10% horse serum, Grand Island Biological Co., Grand Island, N. Y.) before dispersion. Dispersion of the trypsin-treated tissue fragments was accomplished using the shear produced by stirring with a mechanical test tube stirrer (Vari-Whirl, Van Waters & Rogers, Inc., San Francisco, Calif.).

Aggregation of Homotypic Cell Suspensions

Viability of dissociated cells (as judged by nigrosin exclusion, time-lapse microcinematographic studies of dissociated cells in monolayer culture, and the ability of dissociated cells to aggregate and of heterotypic aggregates to sort out) was high. For example,

in excess of 95% of the dissociated cells exclude nigrosin. Cell suspensions were centrifuged at low speed to remove all larger clumps of cells and the majority of small clumps (5–10% of the cells were present in clusters of two or three cells,larger clusters were absent). Concentrations of cells were determined using a hemocytometer. Stock suspensions of cells were then diluted to the desired concentration (usually 1 or 2 $\times$ 10^6 cells/ml) and were dispensed into 25-ml shaker flasks (Bellco Glass, Inc., Vineland, N. J., usually at a volume of 2 ml of cell suspension per flask). Culture medium was Eagle's MEM + 10% horse serum. DNase (Worthington Biochemical Corp., Freehold, N. J., twice crystalized) was added (1 μg/ml) to avoid any problems with DNA released during trypsinization (Steinberg, 1963 a). Aggregation was accomplished on a water-bath gyratory culture flask shaker (New Brunswick Scientific Co., New Brunswick, N. J.) set at 60 rpm, 37°C.

Cell Sorting

Cell sorting proceeds in heterotypic aggregates produced from mixed cell suspensions containing cells of two or more different cell types. Mixed cell suspensions were prepared by mixing in varying ratios suspensions of dissociated cells obtained from different tissues. In heart-pigmented retina combinations, the cells were mixed at a ratio of two heart to one pigmented retina. In neural retina-pigmented retina combinations, the ratio was three neural retina to one pigmented retina. Heterotypic aggregates were produced from these cell suspensions in two different ways: (a) Cell suspensions were allowed to aggregate in shaker flasks. Large aggregates formed rapidly if the volume of culture medium was increased to 4 ml and the shaker was set at a low speed (60 rpm). Within 2 hr, a majority of the cells were included in large heterotypic aggregates. These were cultured further in 25-ml shaker flasks containing 2 ml of culture medium with an increase in the speed of shaking to 80–90 rpm. These measures increased the shear produced by stirring and prevented large aggregates from fusing with each other. (b) Cell suspensions were centrifuged into pellets from small volumes of medium (2–3 drops) as described by Armstrong (1971), and the pellets were then broken into fragments which could be cultured as above. In some cases, aggregates were cultured singly in hanging drops as described by Armstrong (1971).

Monolayer Cultures

Explants of heart ventricle, neural retina, and pigmented retina were cultured on glass coverslips in Sykes-Moore chambers (Bellco Glass, Inc.) in

MEM + 10% horse serum. The medium over the cells was changed by injecting new medium through the gaskets of the culture chambers. Culture chambers were maintained at 37°C for photography and time-lapse microcinematography with a temperature regulated microscope stage (Wild Heerbrugg Instruments, Inc., Farmingdale, N. Y.) fitted with a specially designed insert to accommodate the Sykes-Moore chamber.

Histology

Aggregates were fixed in cold Karnovsky's fixative as modified by Trelstad et al. (1967). Aggregates were washed in cacodylate buffer, postfixed in 1.5% OsO_4, dehydrated in ethanol, and embedded in ERL-4206 (Spurr, 1969). 1 μ sections for light microscopy were stained in alkaline toluidine blue.

Cytochalasin B

Cytochalasin B (CB, obtained from Imperial Chemical Industries, Ltd., Alderly Park, Great Britain) was dissolved to a concentration of 1 mg/ml in dimethylsulfoxide. In experiments involving the effects of CB on cell sorting, heterotypic aggregates, prepared either by rotation-mediated aggregation or by centrifugation, were cultured in the presence of the drug (at concentrations of 5 and 10 µg/ml). For controls, heterotypic aggregates were placed in culture medium or in culture medium containing 1% dimethyl sulfoxide (DMSO). The potency of our preparations of CB were assayed periodically using heart fibroblast monolayers. At 5 or 10 µg/ml, the drug causes rapid abolition of ruffled membrane activity in these cells. In the experiments reported in this paper, two entirely different preparations of cytochalasin B were used. The results were identical with both preparations.

OBSERVATIONS

It has been reported that cytochalasin B causes an abolition of ruffled membrane activity in a variety of cell types in monolayer culture (Carter, 1967 b, 1972); Allison et al., 1971; Axline and Reaven, 1971; Gail and Boone, 1971; Goldman, 1971; Spooner et al., 1971; Wikswo and Szabo,

1971; Yamada et al., 1971; Zigmond and Hirsch, 1971). This has been confirmed for the cell types used in cell sorting experiments (Fig. 1). In these cell types, the effect is rapid, being evident within 5–10 min, and is reversible upon removal of the drug, even after 18 hr of treatment. Cell viability is not impaired by the drug at the levels used in the present experiments. Time-lapse microcinematographic studies of treated cells show that cellular locomotion and translocation is halted by drug treatment.

Effect of Cytochalasin B on Cell Sorting

The effect of cytochalasin B on cell sorting was ascertained in heterotypic aggregates containing either heart and pigmented retina or neural retina and pigmented retina. Aggregates produced both by rotation-mediated aggregation and by centrifugation were employed. In the latter case, centrifugation from small volumes of culture medium ensures that the initial aggregates approximate random mixtures of cells. Although it is not certain that the initial aggregates produced by rotation-mediated aggregation of binary cell suspensions are truly *random* mixtures of the two cell types, they certainly are highly *disordered* mixtures showing little sign of the organization of tissues that is produced by cell sorting (Fig. 3 a). In the experimental cultures, cytochalasin B was present at 5 or 10 µg/ml. At these concentrations, ruffled membrane activity of cells in monolayer culture was completely abolished. In most control cultures, DMSO at 1% was present. In some control cultures, this was omitted as well.

Control aggregates completed cell sorting within 18–24 hr (Figs. 2 a, 3 c). DMSO had no apparent effect on cell sorting in the concentrations used. In experimental cultures containing cytochalasin B at 5 or 10 µg/ml, the degree of sorting depended on the cell types employed. In heart-pigmented retina combinations, there

FIGURE 1 Phase-contrast micrographs of living cells showing the effect of cytochalasin B (10 µg/ml) on cells in monolayer culture. Shown are cells of the tissues used in the present study of cell sorting. (a), (c), and (e) are pictures taken just before administration of cytochalasin. (b), (d), and (f) are the same cells as shown in (a), (c), and (e), respectively, photographed 30 min after addition of the antibiotic. Temperature was maintained at 37°C with a microscope incubator stage. (a), (b) Chick embryo pigmented retina; (c), (d) chick embryo neural retina; (e), (f) chick embryo heart ventricle. × 400.
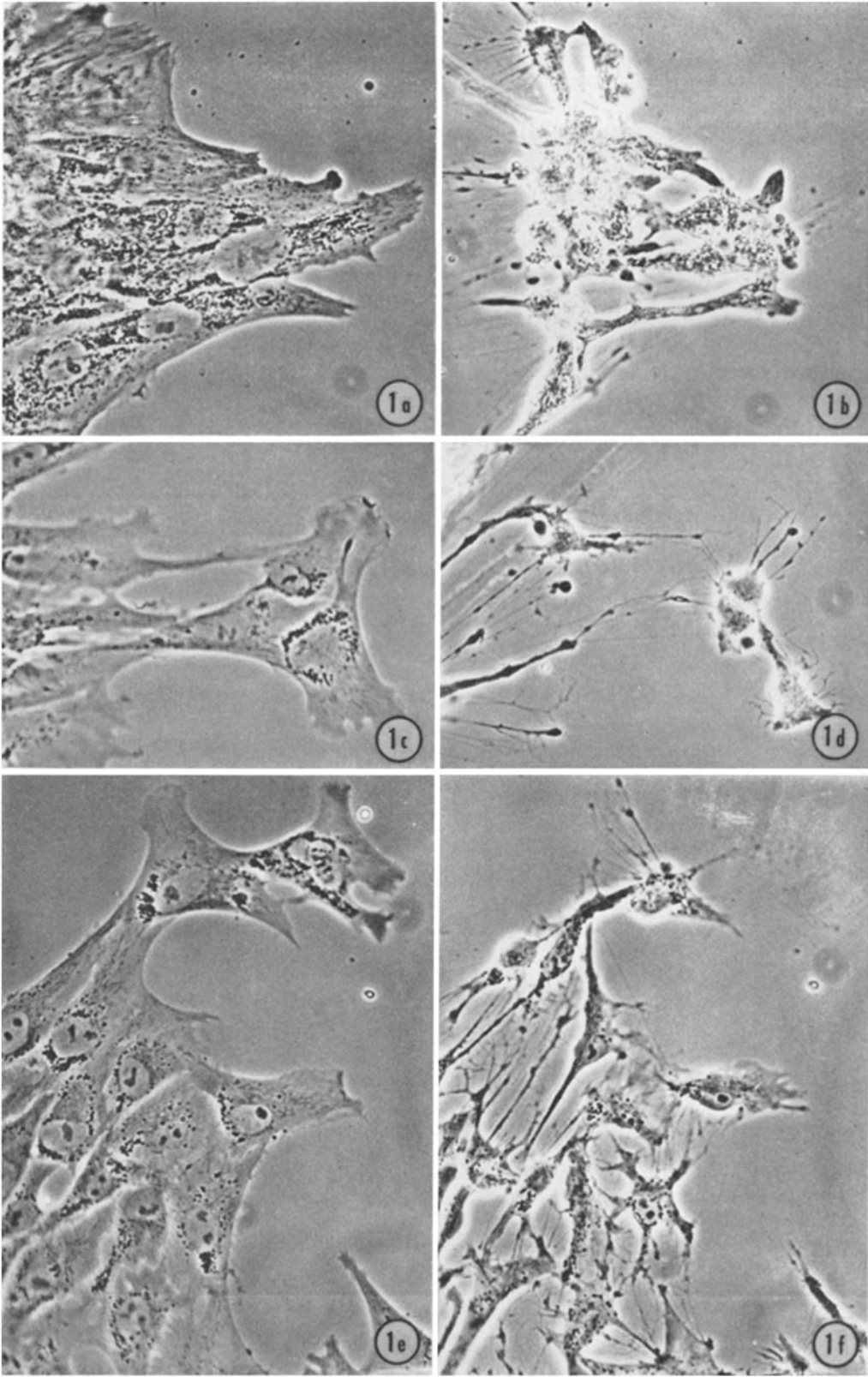
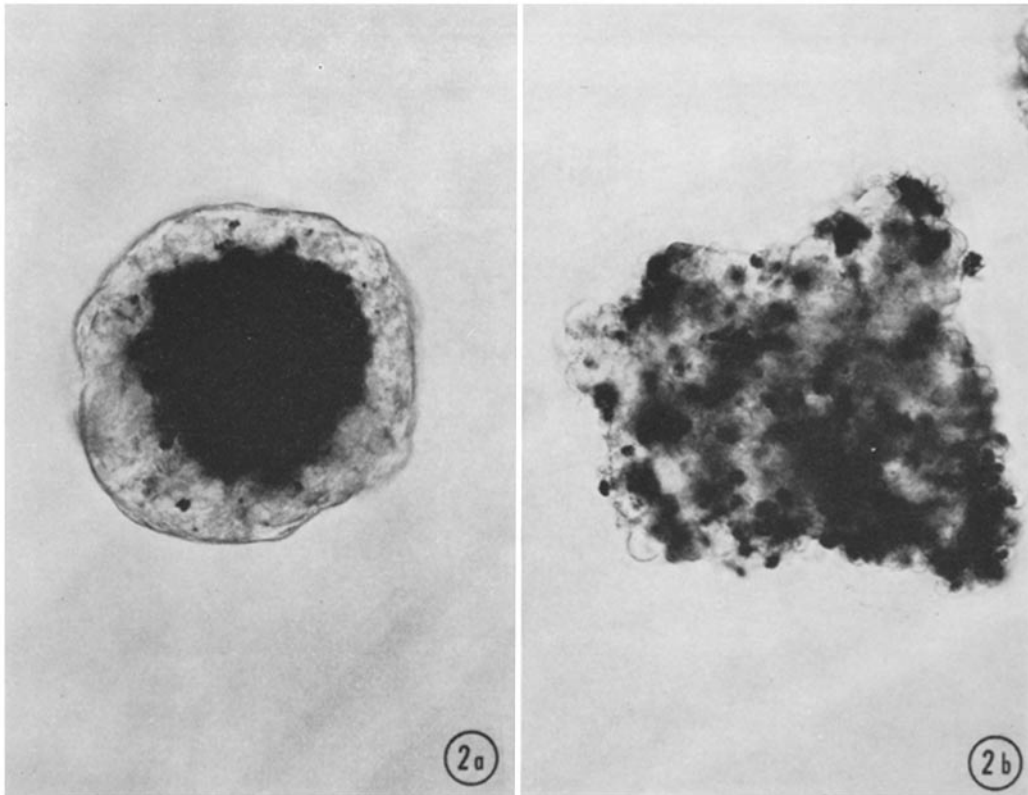FIGURE 2 The effect of cytochalasin B on cell sorting in heterotypic aggregates of 7-day chick embryo heart ventricle and 7-day chick embryo pigmented retina after 2 days in culture. Living aggregates, produced by rotation-mediated aggregation, were photographed on a hemocytometer. (a) Aggregate from control culture (cytochalasin is absent). Cell sorting is complete with heart cells present in the periphery and pigmented retinal cells present in the center of the aggregate. This stage of cell sorting is reached within 1 day of culture under the conditions employed in this experiment. (b) Aggregate from experimental culture (cytochalasin B concentration is 10 μg/ml). Although the aggregate is compact, observable sorting of heart and pigmented retinal cells has not occurred. × 230.

was no apparent sorting (Fig. 2 b). However, in neural retina-pigmented retina combinations, cell sorting did occur to a remarkable degree (Fig. 3 d). (Fig. 3 d can be compared with Fig. 3 a to appreciate the degree of cell sorting that has occurred.) Although sorting in the presence of cytochalasin B does not result in the single central pigmented retinal tissue mass character-

FIGURE 3 Time course of cell sorting in heterotypic aggregates of 7-day chick embryo neural retina and pigmented retina. As in Fig. 2, living aggregates were photographed on a hemocytometer stage. (a) An early aggregate (2 hr in culture) produced by rotation-mediated aggregation of a suspension of neural retinal and pigmented retinal cells, showing the lack of ordering of the two cell types. Pigmented retinal cells are black; neural cells are clear. (b) Aggregate from control culture in intermediate stage of sorting. (c) Aggregates from control culture after 1 day in culture, at the completion of cell sorting. (d) Aggregates from experimental culture (cytochalasin B is 10 μg/ml) after 1 day in culture, showing the degree of cell sorting achieved in the presence of cytochalasin B. Pigmented retinal cells are present as multiple discrete masses embedded in the neural retinal tissue. The degree of sorting achieved is about equivalent to that shown in Fig. 3b. × 230.
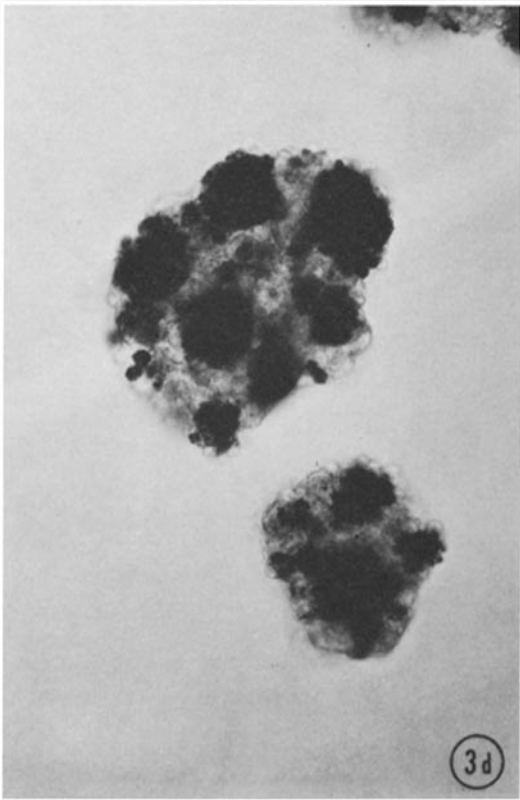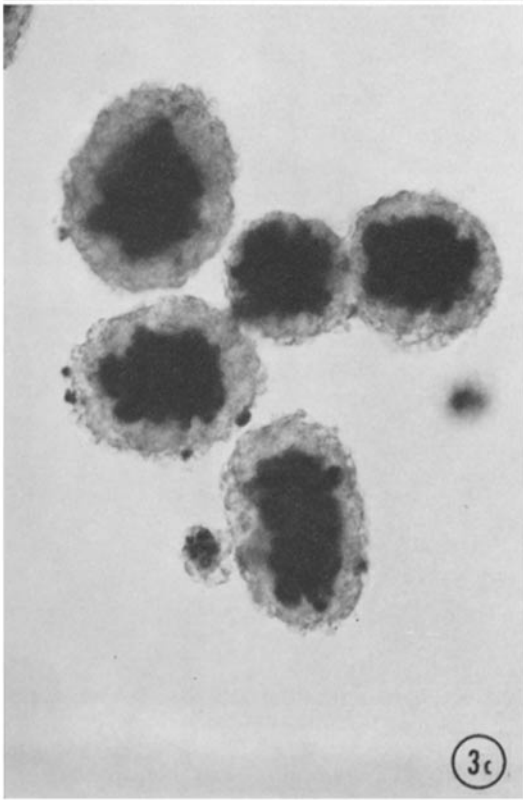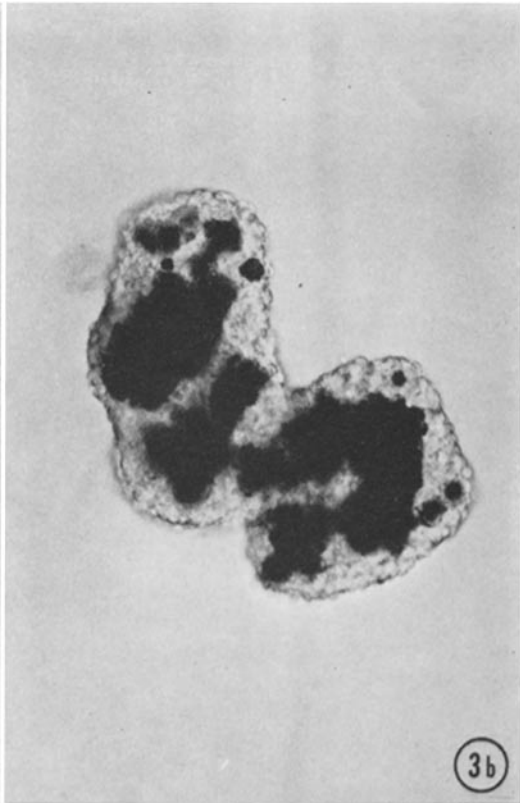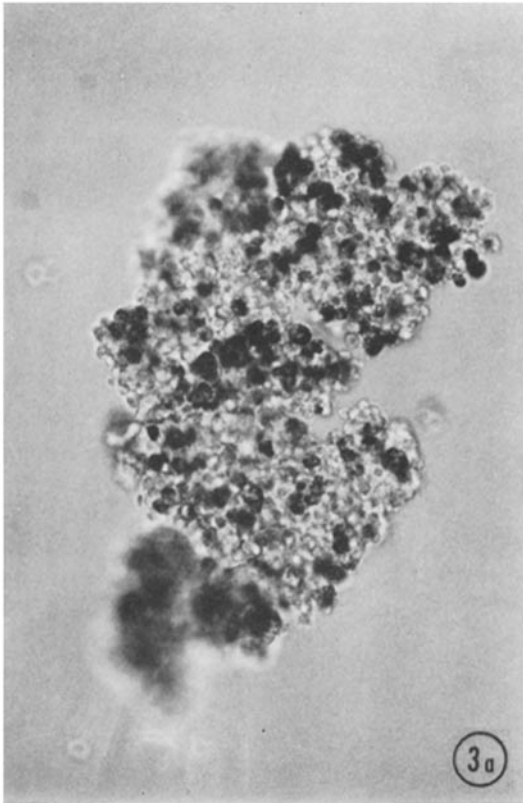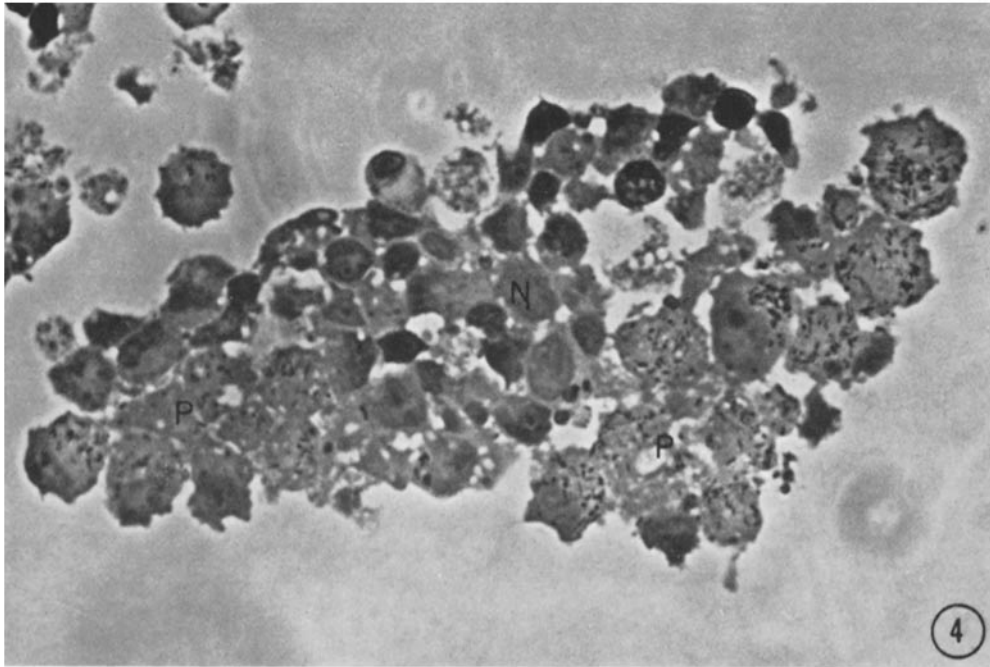
FIGURE 4   1 μ plastic section through an aggregate of neural retina and pigmented retina cultured for 1 day in the presence of cytochalasin B (i.e., an aggregate comparable with that shown in Fig. 3 d). Pigmented retinal cells can be distinguished by the presence of melanin granules in the cytoplasm and are present as two discrete tissue masses (P) with a mass of neural retinal cells (N) between them. Cells are obviously healthy and intact. × 1180.

istic of control aggregates (Fig. 3 c), pigmented retinal and neural retinal cells are clearly segregated from each other as relatively homogeneous tissue masses. In contrast to control aggregates which show single internal pigmented retinal masses, multiple pigmented retinal clumps are present at the completion of sorting. This arrangement of tissues resembles that found in control aggregates in intermediate stages of sorting (Fig. 3 b), except that not all pigmented retinal clusters in cytochalasin-treated aggregates are entirely surrounded by neural retinal cells (Fig. 4). The degree of sorting achieved was the same at cytochalasin levels of 5 and 10 μg/ml. The experiment studying the effect of cytochalasin B on neural retina-pigmented retina aggregates was repeated 10 times using two different preparations of cytochalasin B. Under the conditions of cell aggregation used in these experiments, 50 to several hundred aggregates were formed each time. In all these cases, the results were completely consistent.

In the presence of cytochalasin B, sorting proceeds no further than the arrangement of tissues illustrated in Figs. 2 b, 3 d, and 4, even in aggregates maintained 3–4 days in culture. Once sorting in neural retina-pigmented retina combinations has reached the arrangement shown in Fig. 3 d, removal of cytochalasin does not result in further observable sorting. Apparently the separate pigmented retina tissue masses, being separated from one another by neural retinal cells, are unable to establish contact to form a single central tissue.

Effects of Cytochalasin B on Aggregation in Homotypic Cell Suspensions

The effects of cytochalasin B (10 μg/ml) on aggregation of dissociated cells were variable, depending on the time of culture and the cell type employed. The effect on aggregation of trypsin-dissociated neural retinal cells was negligible. After 4 hr, experimental aggregates (Fig. 5 a) were approximately the same size as control aggregates (Fig. 5 b). Both experimental

and control aggregates are compact and have rounded contours. Similar results were observed in 18-hr cultures; experimental (Fig. 5 c) and control (Fig. 5 d) aggregates were comparable as to size and shape. In this case, DMSO was omitted from the culture medium of the controls. Similarly, aggregation of trypsin-dissociated limb bud mesodermal cells was unaffected after 4 hr by the antibiotic (Figs. 5 e, 5 f). However, at longer times, cytochalasin B did show an inhibitory effect on the aggregation of limb bud mesoderm cells. The aggregates present in experimental cultures after 18 hr of aggregation (Fig. 5 g) were markedly smaller and more ragged than those in control cultures (Fig. 5 h).

DISCUSSION

The most interesting observation of the present study is that partial sorting of cells into homogeneous tissue regions can occur in neural retina-pigmented retina combinations, even in the presence of levels of the drug cytochalasin B that are sufficient to inhibit active cellular locomotion in monolayer culture. Several explanations can be advanced to account for this observation. First, it can be suggested that, although cytochalasin B is able to inhibit active cellular locomotion in monolayer culture, it does not do so in cell aggregates, perhaps because a different method of active cellular motility is employed in aggregates. Although this appears to be unlikely, we are not aware of any evidence which can refute this contention. Some forms of active cellular motility are unaffected by cytochalasin B (i.e., the locomotion of large freshwater amoebae [Colby and Lanners, 1971] and the bending motility of blue-green algae and gliding motility of diatoms [J. L. Spangle, unpublished observations]).

A second explanation for our results is that the movement of cells during sorting is, at least in part, purely passive (i.e., movement does not require the production of specialized locomotory organelles and does not involve cytochalasin-sensitive cellular contractile systems). Curtis (1967) has emphasized the extensive degree of contact that is present between "like" cells even in completely randomized binary mixtures of cells. If the volume ratio of the two cell types is close to being equal, almost every cell in the aggregate is connected to numbers of other cells of its own type via long, highly branched chains of cells.

This raises the distinct possibility that the movement of cells that occurs during sorting may be, at least in part, a passive result of a broadening of the areas of contact between cells of the same type which are already in contact in the initial disordered aggregate. According to this hypothesis, active cellular locomotion is not required for sorting. This passive means of movement of cells in heterotypic aggregates, suggested by the results of the present study, is similar to that proposed by Carter (1967 a) as a general mechanism for cell locomotion and given, by him, the name "haptotaxis." Although there is considerable controversy over the general applicability of this theory (cf. Wolpert et al., 1969; Carter, 1970), our results suggest that passive cellular locomotion may be responsible for the degree of rearrangement of cells observed in neural retina-pigmented retina aggregates maintained in the presence of cytochalasin B.

Although an extensive degree of cell sorting can occur in combinations of neural retinal and pigmented retinal cells in the presence of cytochalasin B, the degree of sorting that we have observed is not so great as is observed in control aggregates. In control cultures, cells of the type comprising the interior cell mass sort out into a single inner mass of cells. Under conditions of cytochalasin inhibition, cells of this type form multiple discrete rounded masses. Thus, it appears that although sorting is to a large extent independent of active cellular locomotion, it is not completely so.

One problem of some interest results from the observation that recognizable sorting occurs only in certain combinations of tissues. In the present experiments, sorting was observed in combinations containing neural retinal and pigmented retinal cells but not in heart-pigmented retina combinations. Wiseman and Steinberg (1971) and Steinberg and Wiseman (1972) have reported that sorting does not occur to an appreciable degree in liver-heart combinations if cytochalasin B is present. Similarly, Sanger and Holtzer (1972) have reported that the self-organization of neural retinal aggregates into "rosettes of neural epithelium and masses of lens cells" and the sorting out of chondroblast-myoblast aggregates is suppressed by cytochalasin B.

Again, several explanations can be proposed. The simple explanation that ruffled membrane activity and active cellular locomotion is not

affected by cytochalasin B at the doses used (10 μg/ml) in neural retinal and pigmented retinal cells (but is affected in heart cells) can be ruled out by the studies of cell behavior in monolayer culture. In monolayer culture, cytochalasin B reversibly inhibits ruffled membrane activity, in all of the cell types studied, at a concentration much lower than that used in the cell sorting studies (i.e., 1.0 μg/ml). In fact, neural retinal cells appear to be more sensitive to cytochalasin B than heart cells. All cells showed retraction of ruffled membranes and filopods in neural retina monolayers at 1.0 μg/ml, but numbers of normal-appearing cells were observed in heart fibroblasts monolayers even after several hours at this concentration. With heart, no effect was observed at 0.1 μg/ml but retraction of cell processes was seen in a significant percentage of neural retinal cells at this concentration.

Alternatively, the magnitude of the difference in cohesive strength of the cell types employed may be important. According to Steinberg (1970), pigmented retinal cells are considerably more cohesive than neural retinal cells, but heart, pigmented retinal, and liver cells differ only slightly from one another in strengths of cellular adhesion. A possible explanation for the absence of sorting in the cell types studied by Sanger and Holtzer and Wiseman and Steinberg, and, in our hands, heart-pigmented retina, is that the differences in adhesiveness of the different cell types are too slight to result in observable sorting[1] under con-

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[1] Cell sorting proceeds by the exchange of contacts between dissimilar cells for contacts between like cells. Expressed in formal terms, the magnitude of sorting depends on the function $\frac{1}{2}(W_{AA} + W_{BB}) - W_{AB}$, where W_{AA}, W_{BB}, and W_{AB} are, respectively, the strengths of adhesion between two A cells, two B cells, and an A and a B cell. Thus, in general, one would not expect the magnitude of sorting to depend directly on $W_{AA} - W_{BB}$ (i.e., the difference in cohesiveness of the two cell types). However, if the strengths of adhesion of different cell types are determined by cell surface parameters as proposed by the site frequency model of Steinberg

ditions where active cellular locomotion is prevented. According to this explanation, the difference in adhesive strengths of neural retina and pigmented retina is large enough to bring about a sufficient degree of cellular movement to result in observable cell sorting.

The exact mechanism of action of cytochalasin B in inhibition of cellular motility (Carter, 1967 b, 1972), prevention of cytokinesis (Carter, 1967 b, 1972), and morphogenetic movement in epithelia (Wrenn and Wessells, 1970; Spooner and Wessells, 1970; Wrenn, 1971) is still a subject of controversy. It has been proposed that cytochalasin acts primarily on systems of contractile microfilaments (Schroeder, 1969; Wessells et al., 1971; Spooner et al., 1971; Yamada et al., 1971) or, alternatively, primarily on the cell surface and plasma membrane (i.e., by inhibiting cell surface glycoprotein metabolism [Sanger and Holtzer, 1971, 1972] or membran fusion reactions [Bluemink, 1971 a, b; Estensen, 1971; Estensen et al., 1971; Hamner et al., 1971]). The present study of the effects of cytochalasin B on aggregation of dissociated cells was conducted as an attempt to examine the possible effect of cytochalasin B on one example of cell-surface-mediated behavior (viz. cellular adhesiveness). Sanger and Holtzer (1972) have reported that cytochalasin B does inhibit aggregation of cells from trypsin-dissociated 3-day chick embryos. With the cell types used in our study, aggregation over a period of several hours was not markedly affected by the drug. This suggests that whatever effects cytochalasin B may have on the surfaces of cells of the cell types employed in our studies, these effects do not result in altered adhesiveness, at least for a period of several hours after treatment.

(1958, 1964), then W_{AB} would equal $(W_{AA} \cdot W_{BB})^{1/2}$. When this, and an expression for the difference between W_{AA} and W_{BB} (i.e., $W_{AA} - W_{BB} = K$) is substituted into the expression for the magnitude of sorting (S), this becomes $S = \frac{1}{2}K + W_{BB} - (KW_{BB} + W_{BB}^2)^{1/2}$. It is readily seen that S will be larger, the larger the value of K (i.e., the larger the difference between W_{AA} and W_{BB}).

FIGURE 5   Photographs of living intact aggregates showing the effect of cytochalasin B (10 μg/ml) on rotation-mediated aggregation of trypsin-dissociated chick embryo cells. Controls (cytochalasin B is absent) are in the right-hand column (b, d, f, and h); experimentals (cytochalasin B is 10 μg/ml) are in the left-hand column (a, c, e, and g). (a)-(d), 7-day neural retina; (e)-(h), 4-day limb bud mesoderm; (a), (b), (e), (f), 4 hr in culture; (c), (d), (g), (h), 18 hr in culture. × 25.
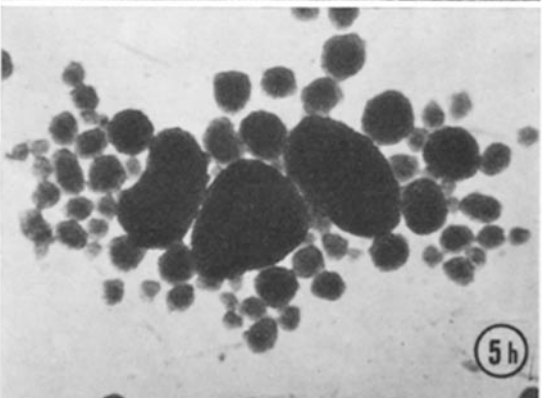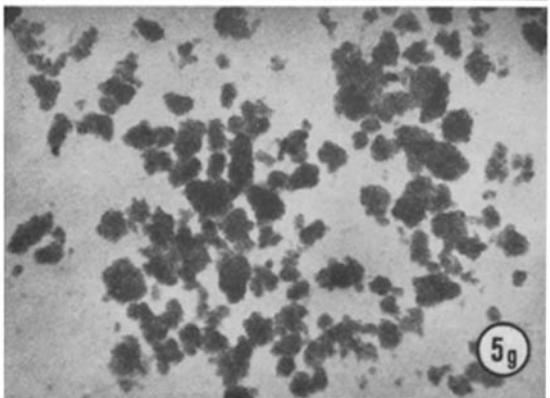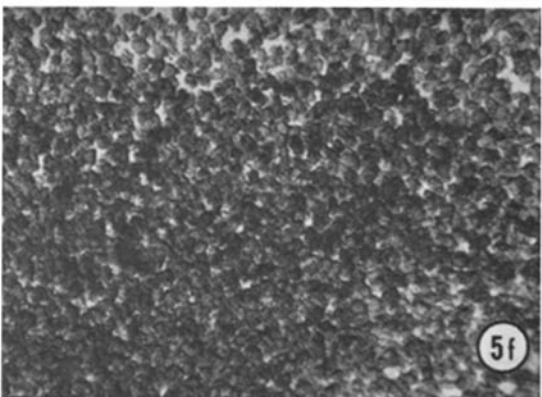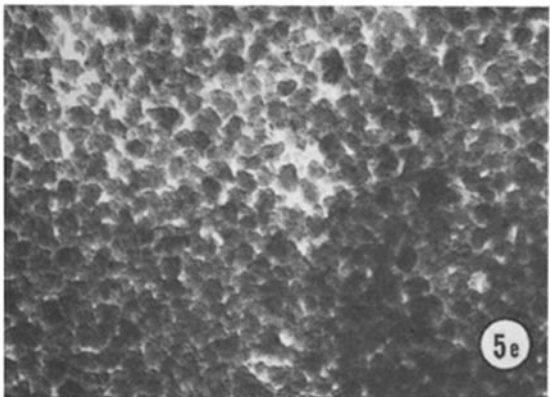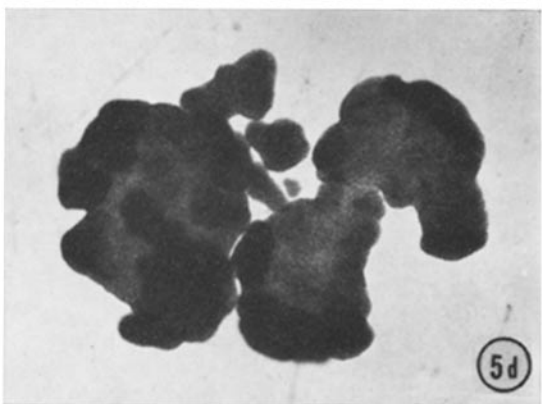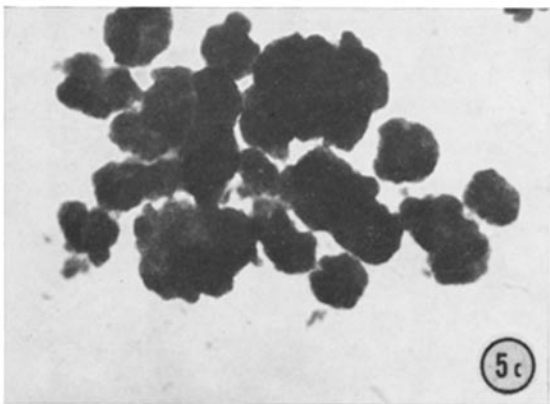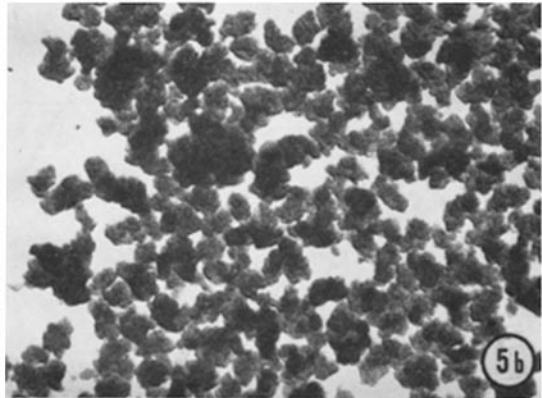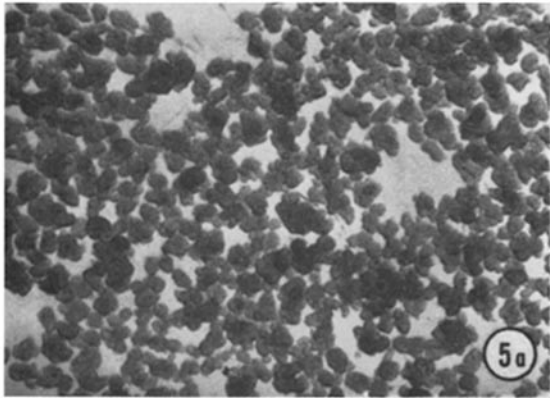
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