*DIFFERENTIATION IN CULTURE OF MIXED AGGREGATES OF DISSOCIATED TISSUE CELLS**

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Our concepts of the process of determination in embryonic development have been based largely on results obtained by extirpation, transplantation, and explantation of tissue masses consisting of many cells. These methods have revealed an apparent restriction of potencies during development. The varying results obtained with different methods, however, have led to serious questioning of the conclusions. For, as Harrison[1] pointed out, "there always may be new conditions, not yet tested, under which other potencies might be revealed."

An important factor influencing the degree and variety of differentiation in tissue culture is the size of the tissue mass involved.[2–5] Thus small pieces from the node region of the chick blastoderm (definitive primitive streak stage) fail to differentiate in culture, while larger pieces invariably form brain tissue.[4] Explants of presumptive head mesoderm from a single early gastrula of *Triton taeneatus* form only muscle *in vitro*, but larger masses consisting of identical cells, derived from the fusion of several of these same primordia, ofrm muscle, chorda brain sensory primordia, and ectoderm.[5] It is evident from these observations that a description of the potencies of a given tissue mass composed of many cells does not necessarily reveal the capacities of the individual constituent cells.

These studies raise the possibility that, when an intact tissue mass is judged to be determined by all the usual criteria, individual cells within the mass may retain wider potencies. Perhaps in transferring a mass of cells to a new site the immediate environment of the inner cells is but slightly altered. Such a change would be sufficient to cause a shift in the direction of cell differentiation in the earliest phases of development (e.g., early amphibian gastrula); but in more advanced stages, generally considered to be determined, a more acute change may be necessary. Accordingly, in the latter instance, fixity of the mass under the imposed conditions could not be taken as evidence of cell fixity.

With the discovery that it is possible to dissociate vertebrate tissue cells by treatment with high pH,[6] trypsin,[7, 8] or versene[9] and, after aggregation, to obtain characteristic differentiation *in vitro*, we have a promising method for attacking this problem. By mixing dissociated cells derived from different primordia (or even from differentiated tissues) and combining them into complex tissue masses by aggregation, we may drastically alter the immediate environment of embryonic tissue cells. The differentiation of such complex aggregates may then provide important information on the potencies of these cells.

It was with this hope that we began the present investigation. Our method has been to combine cells derived from two different organs at various advanced stages of organogenesis and study the differentiation *in vitro* of the complex aggregates.

METHODS

The organs used in this study were mesonephros and wing bud from $3^1/_2$- and 5-day chick embryos (stage 19–20 and stage 27).[10] White Wyandotte, Rhode Island

Red, and White Leghorn eggs were used, with no differences in result. To obtain an adequate mass of aggregated cells, large numbers of these organs were required (e.g., 30–40 mesonephroi and wing buds from stage 19 embryos).

Both trypsin and versene (disodium versenate) were used as dissociating agents during beginning phases of the investigation. Trypsin was soon found to be more satisfactory for our purposes, however, because after dissociation with this agent the cells tend to readhere to one another more readily. This yields a firmer aggregated mass. A 3 per cent trypsin solution was used (Difco 1:250 trypsin in Ca- and Mg-free Tyrode's solution, buffered with phosphate and bicarbonate). The trypsin solution, Tyrode's solution, and bicarbonate buffer were all sterilized by filtration through a fritted glass disk. This not only provides sterile media but also avoids the occasional cotton fibers that fall from plugs used in autoclaving, and which adhere firmly to dissociated tissues. Standard sterilization procedures were used for all other solutions and glassware. All solutions were prepared with demineralized water obtained by passing metal distilled water over an anionic-cationic exchange resin (Amberlite MB-1). In our experience this is quite as satisfactory for tissue cultures as Pyrex-distilled water.

Suspensions of dissociated cells of both mesonephros and wing bud were obtained by a modification of the procedures devised by Moscona.[7] Entire wing buds and mesonephroi, cleaned of most of the extraneous tissue, were placed in trypsin I (pH raised to 7.8 with 1 per cent KOH). The tissues were cut into small pieces in the enzyme solution and then incubated at 38° C. for about 15 minutes. Upon removal from the incubator, the tissues were drawn into an orally controlled pipette of small bore (0.08–0.1 mm. inner diameter). This causes the tissue to fragment into a mixture of free cells and cell clusters, composed of *ca.* 20–100 cells. The partly dissociated tissue was then forced out of the pipette into trypsin II (the same as trypsin I, except that the pH has been raised to 8.3 with the addition of 1 per cent KOH) and incubated at 38° C. for an additional 15 minutes. The suspension was then once again drawn into a very fine bore pipette (*ca.* 0.05 mm. inner diameter) and forced out of the pipette into a dish of Tyrode's solution. This procedure yields a suspension consisting almost entirely of individual cells (Fig. 1). However, a few pieces of ectoderm, consisting of 5–10 cells, always remain undissociated in suspensions derived wholly or partly from 5-day wing buds. The entire process of dissociation, from the time the tissue is placed in trypsin I until the dissociated cells are suspended in Tyrode's solution, requires approximately $1^1/_2$ hours.

The cells were washed free of trypsin and aggregated by the following procedure: centrifugation (2–3 minutes in a clinical centrifuge at 1610 × g), removal of the supernatant, replacement with fresh Tyrode's and centrifugation for an additional few minutes, removal of the supernatant, and replacement with a nutrient medium consisting of 2 parts horse serum, 2 parts Tyrode's solution, and 1 part embryo extract. The cells were centrifuged for 7–10 minutes in this medium, in order to force them into a more compact mass.

This aggregated mass of cells was removed intact from the centrifuge tube with the aid of a small spatula and cut into pieces of the desired size. One of these was fixed (Fig. 2), and the others were cultured overnight in the fluid nutrient medium in deep depression slides. The following day the explants were removed from this medium and cultured by the Fell method on the surface of a plasma-embryo extract

clot. During the sojourn in fluid medium the explants acquire a firm enough consistency to make culturing on the surface of the clot a feasible procedure. While this method of aggregation has been satisfactory in providing us with firm aggregates which differentiate in culture, it has one distinct drawback. There is considerable loss of peripheral cells, both in cutting the aggregated mass into explants and during the several hours in nutrient medium. Because of the possibility that differences in specific gravity may cause cells to separate during centrifugation, care was taken to cut the aggregated mass in such a manner as to insure inclusion of cells of varying specific gravity in each explant. The subsequent complex differentiation characteristic of larger explants suggests that representatives of all cell types are present in each explant (Figs. 6, 7, 9)

The clot was formed with 4 parts hen plasma, 1 part fresh embryo extract, and 0.5 part 0.001 per cent protamine sulfate (to achieve a firm clot). Embryo extract was made from embryos which, as far as possible, are the same age as the cells of the explant.[11] For example, aggregates from a 5-day embryo after one night in fluid medium were placed on a clot made with 7–8-day embryo extract, were transferred 4 days later to a clot made with 10-day extract (the same age as the tissue cells), and 4 days later to a clot made with 14-day extract. Explants were routinely shifted to a fresh place on the clot after 2 days and were transferred to a fresh clot every 4 days. In order to prevent bacterial growth, penicillin (Potassium Penicillin-G, Lilly; 125 units/cc) and streptomycin (CaCl complex, Merck; 0.875 mg/cc) were regularly added to the Tyrode's solution. These antibiotics have no detectable effect on the explants, as determined by comparison with parallel control cultures. Explants cultured by this method remain remarkably healthy for long periods in cultures (up to 16 days). Cytolysis, as judged by pycnosis, is at a minimum, being largely confined to the central area of the explant and to the first day or so in culture. Mitoses occur throughout the period *in vitro* but are most frequent during the first few days, prior to the differentiation of tissues. Explants were fixed in Bouin's solution after varying periods on the clot, from 1 to 12 days. Sections were cut at 6 μ and stained with Delafield's haematoxylin or Mallory's triple stain.

When mixed aggregates were desired, known quantities (by wet-weight determination) of mesonephros and wing bud tissue were disaggregated together in the same dish. Thus mixing begins in trypsin I, continues in trypsin II, and is completed in Tyrode's solution, where the cell suspension is drawn into a medium-bore pipette and flushed out several times. All other procedures were as described above.

RESULTS

Aggregates of Mesonephros Cells.—All cell aggregates of dissociated $3^1/_2$-day (stage 19–20) and 5-day (stage 27) mesonephros (a total of fifteen cases) formed characteristic mesonephric tubules after 3–6 days in watch-glass culture (Fig. 3). This confirms the results of Moscona and Moscona.[8]

Aggregates of Wing Bud Cells.—Aggregates of dissociated wing buds of embryos at stage 19–20 and stage 27 (a total of ten cases) do not differentiate during the first few days in culture. By the fifth day on the clot, large areas of procartilage differentiation appear (Fig. 4). In no case, however, did cartilage development proceed beyond this stage, even though explants were cultured for as long as 13 days. Furthermore, no other wing tissues appear (e.g., mesenchyme, muscle, or kerati-

nized epidermis), and all explants possessed large areas of undifferentiated cells. These results are at variance with those of Moscona and Moscona,[8] who obtained excellent cartilage and wing mesenchyme formation in aggregates of limb mesoderm cells. In view of the results presented in the next paragraphs, the method of culturing cannot be held responsible. The only differences that appear significant are a longer exposure to trypsin and the presence of ectoderm cells in our explants.

Mixed Aggregates of Mesonephros and Wing Bud Cells from Embryos of the Same Stage.—Aggregates composed of intermingled cells derived from dissociated mesonephroi and wing buds invariably form tissues when cultured by the watch-glass method; however, the degree of differentiation and the number of tissue types formed vary considerably.

The data on which the following observations are based are derived from study of twelve explants composed of dissociated cells from approximately equal quantities of mesonephros and wing bud tissue of $3^1/_2$-day embryos (stage 19–20). In one series 2.8 mg. of mesonephros and 2.0 mg. of wing buds yielded six explants. In another series equal quantities of mesonephros and wing bud tissue were used (5.5 mg. of each). This mass also yielded six explants. Comparable results were obtained in the two series. After 4 days in culture the beginnings of characteristic cartilage and mesonephric tissue are evident Areas of procartilage and of epithelial tissue appear (Fig. 5). Explants cultured for 7–9 days or more possess well-differentiated cartilage and mesonephric tubules (Fig. 6) and often sheets or whorls of keratinized material. In spite of the fact that the dissociated cells were thoroughly intermingled prior to aggregation, the individual tissues almost invariably occupy separate sectors of the explant. Moreover, the tissues generally have a characteristic regional relation to one another: cartilage in the central region of the explant, mesonephric tubules in a perichondrial position, and keratinized sheets and whorls near the periphery. No loose wing mesenchyme formed in

Abbbreviations: *c* = cartilage; *lm* = loose mesenchyme; *mt* = mesonephric tubule; *pc* = procartilage; *py* = pycnotic nuclei. All photomicrographs except Figure 1 are of sectioned material.

FIG. 1.—Dissociated cells of 5-day chick wing bud. Fixed directly on a slide immediately after dissociation. Stained with Delafield's haematoxylin. ×1,500.

FIG. 2.—Mixed aggregate of dissociated cells of $3^1/_2$-day mesonephroi and $3^1/_2$-day wing buds. Fixed immediately after centrifugation. Stained with Delafield's haematoxylin. ×160.

FIG. 3.—Explant derived from an aggregate of dissociated cells from $3^1/_2$-day mesonephroi. Note well-formed mesonephric tubules. Seven days in culture. Stained with Delafield's haematoxylin. ×160.

FIG. 4.—Explant derived from an aggregate of dissociated cells from 5-day wing buds. Note absence of characteristic cartilage and loose mesenchyme. Only procartilage areas are present. Thirteen days in culture. Stained with Delafield's haematoxylin. ×160.

FIG. 5.—Explant derived from a mixed aggregate of equal quantities of $3^1/_2$-day mesonephros and $3^1/_2$-day wing bud cells. Note the areas of beginning cartilage and mesonephric tubule formation. Four days in culture. Stained with Delafield's haematoxylin. ×160.

FIG. 6.—Explant derived from a mixed aggregate of equal quantities of $3^1/_2$-day mesonephros and $3^1/_2$-day wing bud cells. Note regional differentiation of cartilage and mesonephric tubules. Eight days in culture. Stained with Delafield's haematoxylin. ×160.

FIG. 7.—Explant derived from a mixed aggregate of equal quantities of 5-day mesonephros and 5-day wing bud cells. Note regional differentiation of cartilage and mesonephric tubules. Ten days in culture. Stained with Mallory's triple stain. ×160.

FIG. 8.—Explant derived from a mixed aggregate of dissociated cells from 5 mg. of $3^1/_2$-day mesonephroi and 8.6 mg. of 5-day wing buds. Note predominant differentiation of mesonephric tubules and absence of other tissues. Ten days in culture. Stained with Delafield's haematoxylin. ×160.

FIG. 9.—Explant derived from a mixed aggregate of dissociated cells from 8.3 mg. of 5-day mesonephroi and 3.6 mg. of $3^1/_2$-day wing buds. Note extensive formation of cartilage and loose mesenchyme, in contrast to the small number of mesonephric tubules. Ten days in culture. Stained with Delafield's haematoxylin. ×160.

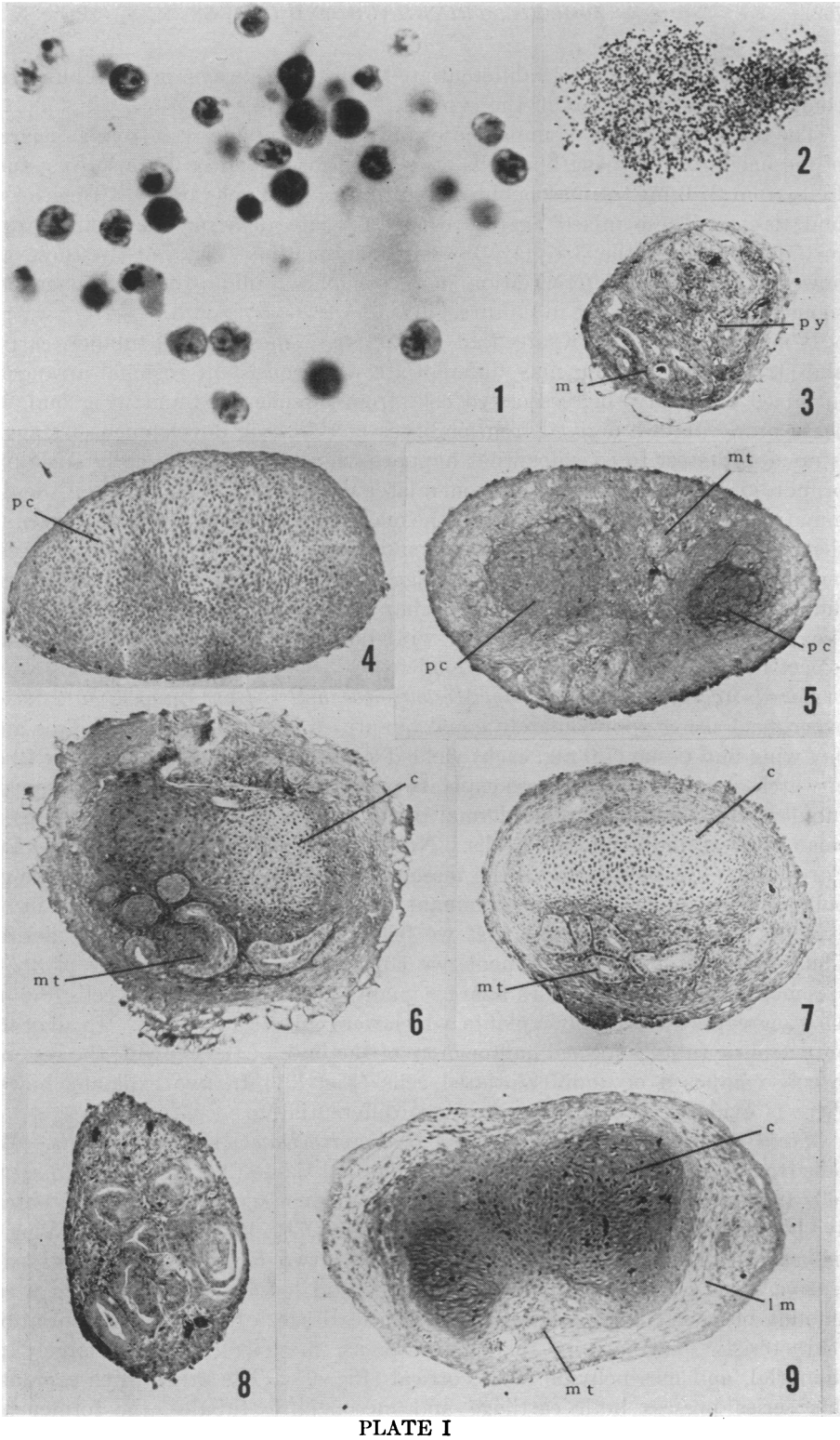

PLATE I

explants of this series. Undifferentiated cells are always present but usually comprise but a small part of the explant.

The differentiation of a similar series of four explants derived from an aggregate of mesonephros and wing bud cells of 5-day embryos (stage 27) also was studied. Cells from 21.6 mg. of mesonephros were mixed with cells from 22.3 mg. of wing bud tissue. These mixed aggregates differentiate in a manner similar to that just described for the $3^1/_2$-day–$3^1/_2$-day combination (Fig. 7). In these cases, however, advanced differentiation of mesonephric tubules and cartilage occurs sooner—by the fifth day in culture.

It is clear from these results that characteristic mesonephric tubules, cartilage, and stratified epithelium may differentiate with consistent regional arrangement in mixed aggregates of dissociated cells from mesonephros and wing bud, when these primordia are derived from embryos of the same developmental stage. It is now of interest to consider what happens when cells from an early stage of one primordium are mixed with cells from a later stage of the other. For, if the determination of the constituent cells and the mass of tissue are equal at any given time, then cells from a more advanced stage would have less capacity to regulate. Accordingly, if any tissues predominate in an aggregate composed of cells from embryos of different ages, they should be those characteristic of the more advanced organ (i.e., in a mixture of cells from early wing buds and later mesonephroi we should expect mesonephric tubules to predominate).

Mixed Aggregates of $3^1/_2$-Day Mesonephros and 5-Day Wing Bud Cells.—An experiment using approximately equal quantities of $3^1/_2$-day mesonephros and 5-day wing bud tissue (7.0 mg. each) yielded three mixed aggregates. After 12 days in watch-glass culture, all these explants possessed well-differentiated mesonephric tubules, a small amount of epidermal tissue (whorls of stratified epithelium), and small areas of undifferentiated cells. None formed cartilage or loose mesenchyme. Furthermore, in each instance the mesonephric tissue clearly comprises the great bulk of the explant! The predominant presence of mesonephric tissue in these explants was of such interest that we felt a more rigorous test to be desirable. Thus, in repeating the experiment, we combined a smaller quantity of $3^1/_2$-day mesonephros cells (5 mg.) with a larger quantity of 5-day wing bud cells (8.6 mg.). This aggregate yielded four explants, which were cultured 7–9 days. In all of these, mesonephric tubules formed half or more of the bulk of the explant, the rest being largely composed of undifferentiated cells (Fig. 8). In two explants, however, there is evidence of beginning cartilage differentiation.

Mixed Aggregates of $3^1/_2$-Day Wing Bud and 5-Day Mesonephros Cells.—When cells from a small quantity of $3^1/_2$-day wing bud tissue (3.6 mg.) were mixed with cells from a much larger quantity of 5-day mesonephros (8.3 mg.), we obtained five explants which became differentiated during 7–9 days in culture. Wing bud tissues predominated in all these cases. The two largest explants formed extensive areas of well-differentiated cartilage and loose mesenchyme and a small amount of poorly differentiated mesonephric tissue. Regional differentiation is characteristic—cartilage in the central areas, mesonephric tissue largely perichondrial, and mesenchyme most cortical (Fig. 9). The other three explants of this series possess both cartilage and mesonephric tubules, the former being definitely predominant.

From these results it is abundantly evident that the younger tissues predominate in mixed aggregates composed of mesonephros and wing bud cells from older and younger embryos. Not only do they comprise the greater portion of the differentiated explant, but they also differentiate to a more advanced degree. Moreover, these relationships persist even when a relatively smaller quantity of the $3^1/_2$-day tissue is used in forming the aggregate.

DISCUSSION

The remarkable differentiation of these mixed aggregates raises a number of interesting questions (e.g., why do cartilage and loose mesenchyme differentiate so much better in the presence of mesonephros cells?); but the central question concerns the cellular origin of the differentiated tissues. Basically, there are two possible answers to this question. Either each tissue is wholly derived from cells of the same tissue type (mesonephric tubules from mesonephric cells, cartilage from prospective cartilage cells, etc.), or each tissue is in part derived from cells of diverse origin, by a process of cell transformation (e.g., cartilage from mesonephros cells). We shall refer to these possibilities as the *redifferentiation* and *transformation* hypotheses, respectively. In both these hypotheses we must take into account the import of critical mass in tissue differentiation. Several lines of evidence indicate that with standard culture conditions a mass of cells must exceed a certain minimal size before differentiation may occur *in vitro*.[3,4] It therefore seems justifiable for us to assume that differentiation of any particular tissue in our mixed aggregates will not occur unless the required number of cells of that tissue type are clustered.

Redifferentiation Hypothesis.—There appear to be three possible mechanisms whereby the regionally differentiated tissues of our explants may originate by redifferentiation:

1. Redifferentiation *in situ* of the cells of each tissue type. This mechanism assumes there to be an occasional chance association of several cells of one tissue type, which increases in size by mitosis, if it is located in the proper region of the explant. Once the necessary size is reached, redifferentiation occurs. The cells of different tissue origin which are located in the midst of this differentiating mass will remain undifferentiated. In the case of an unmixed tissue, like cartilage, they must be eliminated. This may occur as a result of cytolysis or migration to another part of the explant or into the zone of outgrowth (negative cell movement).

2. Redifferentiation of the cells of each tissue type after they have been sorted out by *directed* cell movements. Thus procartilage cells would generally move toward the center of the explant, mesonephric cells to the perichondrial region, and wing mesenchyme and ectoderm cells to the periphery.

3. Redifferentiation of the cells of each tissue type after they become sorted out by *random* cell movements. This mechanism assumes that each tissue cell has a specific affinity for others of its type, so that upon contact they adhere.[12, 13] In this manner, masses of cells of each type will form. Redifferentiation will occur in those that meet the requirements of mass and regional differentiation (cartilage in the center, mesonephros in the perichondrial region, etc.). The remaining cells either will remain undifferentiated or will be eliminated by cytolysis or negative cell movement.

Cell Transformation Hypothesis.—This hypothesis also assumes that differentiation of a particular tissue begins by redifferentiation (differentiation in accord with prospective fate) of a small mass of cells derived from that tissue. The manner of formation of such a cell cluster may be by one or a combination of the mechanisms just discussed. When this cell mass reaches the necessary size, it will begin to differentiate, if it is located in the proper region of the explant. It is assumed that with the onset of differentiation this mass becomes an active center, specifically influencing surrounding cells to follow suit. As these surrounding cells begin to differentiate, they, in turn, will influence cells peripheral to them. This process continues until a large area composed of many cells is involved. It ceases when cells are reached which have already passed under the influence of another active center. This hypothetical phenomenon resembles an embryonic field. If most of the cells of the explant remain randomly distributed during this process, it follows that some cells derived from one tissue must become transformed into cells of another tissue. This hypothesis requires neither differential mitosis, differential cytolysis, nor negative cell movement; nor are extensive directional cell movements required.

In view of the comprehensive studies of Holtfreter[6] and Townes and Holtfreter[13] on mixed aggregates of amphibian gastrula and neurula cells, redifferentiation from cells of the same tissue type after directed cell movements emerges as a likely possibility. It must be emphasized, however, that even though there is a general tendency in the amphibian material for prospective epidermis cells to move outward and for mesoderm and neural plate cells to move inward, the specific fate of the inner mesodern cells of such aggregates is not known. Thus the possibility of cell transformation has not been eliminated.

A number of considerations suggest that cell transformations may occur in the differentiation of mixed aggregates of chick cells:

1. The striking differentiation of complex aggregates composed of mesonephros and wing bud cells from embryos of different ages into tissues that are almost entirely of one organ type (e.g., mesonephric tubules [Fig. 8]) is more readily explained by assuming a transformation of the cells of the older tissue. In addition, the predominance of tissues from the younger organ suggests that properties such as greater migratory activity and adhesiveness may be more significant in the differentiation of the aggregates than the presumed determination of the cells from the more advanced organ.

2. Prospective wing mesenchyme cells are present in abundance in all aggregates of wing bud and mesonephros cells, yet loose mesenchyme differentiates only in large explants (Fig. 9). The absence of this tissue in small explants is most directly explained by assuming that some prospective mesenchyme cells have been transformed.

3. Our evidence does not favor differential cytolysis. Pycnotic nuclei (our criterion of cytolysis[14]) are frequent during the first 2 days in culture, but their central location suggests that the cytolysis is due to general factors such as nutritional or respiratory deficiency and is not related to the regional differentiation of tissues. In general, explants are virtually free of cytolyzing cells after the first 3 days on the clot.

CONCLUSIONS

While it is clear from these observations that we must seriously consider the possibility that cell transformations are occurring during the differentiation of these mixed aggregates, the evidence is, in fact, equivocal at this time. We have as yet no information on cell movements in the differentiating aggregates and no direct information on cell origin. Evidence on this critical matter can be gained by marking one type of cells and following their fate in the explant. An effort to gain evidence of this sort, using S^{35} as a marker, is now in progress. A decision as to which of the proposed hypotheses, or other possible views, is correct must be deferred until the completion of these and other marking experiments.

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