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Intercellular Adhesion

I. A Quantitative Assay for Measuring the Rate of Adhesion*

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Summary. A quantitative procedure for determining the early kinetics of cell aggregation (adhesion) is described. The cells used for this study were obtained by dissociation of 8-day-old embryonic chicken neural retina with crude trypsin. The method is based on determining the decrease in single cells in an aggregating population with the Coulter electronic particle counter. A variety of experiments show that the method is reproducible and capable of detecting relatively small changes in the rate of aggregation. Using a number of criteria, the loss of single cells from the population with increasing time of incubation was shown to result from the formation of aggregates, and not from other phenomena such as cell death or changes in cell permeability. The intercellular adhesions formed under these conditions were stable to mechanical shear and to ethylenediaminetetraacetate, and were partially resistant to crude trypsin. The logarithm_{10} of the number of single cells in the population was found to be directly related to the time of incubation. The slope of the resultant straight lines could be used as a measure of the rate of aggregation. No lag in aggregation was demonstrable under the standard assay conditions. The rate was affected by the initial cell density, speed of rotation during aggregation, temperature, and by Ca^{2+} and Mg^{2+}. It was not affected by inhibitors of protein synthesis, metabolic inhibitors, ATP, ADP, cyclic-AMP, or horse serum at 37 °C. The quantitative method for determining the initial rate of adhesion should be applicable to studies on the chemistry of this process.

Intercellular adhesion or lack of adhesion in multicellular organisms plays a key role in a variety of biological phenomena, including morphogenesis, cell division, and metastasis [1, 29]. Although a universal definition cannot be given for intercellular adhesion [5], it is defined here as the ability of cells to form stable unions under the experimental conditions described below.

The long-range goal of the present investigation is the identification of the chemical substances responsible for intercellular adhesion which are presumably located on the cell surfaces. The studies presented are concerned with the development of a quantitative assay for measuring the *rate* of adherence (or "aggregation") of single cells to each other. The availability of such a method permits a quantitative evaluation of the effects on intercellular adhesion of environmental parameters such as

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temperature and of exogenous substances (antibodies, enzymes, haptens, ions, etc.). The method has been used as the basis for the isolation of a highly purified protein from horse serum [18] that promotes aggregation of embryonic neural retina cells, but which appears to have no effect on chick embryonic limb bud cells.

The quantitative kinetic assay described in this report stems from the observations of Townes and Holftreter [27], who showed that embryonic tissues can be dissociated, and that the aggregation or association of the resulting single cells could be studied in vitro. This method has been modified and extended by Moscona, Steinberg, and others (*see* review, [28]); it has provided important information and has resulted in a variety of novel theories to explain the adhesive process.

Recent work along these lines has attempted to quantitate cell adhesion. Thus, Roth and Weston [22] employed radioautographic techniques to measure and compare the specific adhesion of labeled cells to unlabeled aggregates of various cells types. A quantitative method was reported by Kemp, Jones, Cunningham, and Jones [10] who measured the change in light-scattering that occurs during the aggregation of stirred cell suspensions. Curtis and Greaves [7] determined the degree of aggregation by measuring the decrease in single cells with a hemocytometer; this procedure is quantitative, but it is time-consuming.

In the present studies, the rate of aggregation was determined with a Coulter electronic particle counter, a device that rapidly and reproducibly determines the absolute number of particles of different volumes in relatively large samples. The Coulter counter has been used previously in studies of cell aggregation [2, 3, 13], but the problem was not approached kinetically, and the parameters that influence aggregation were not described. As shown below, when the conditions for studying cell adhesion are carefully defined, reproducible rates are obtained.

Experimental Procedure and Results

Materials and Methods

Materials. All reagents were obtained commercially unless otherwise indicated. We wish to thank Drs. Heinrich Ursprung and Martin G. Larrabee for their gifts of cycloheximide and puromycin, respectively. The buffer Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) was purchased from Calbiochem. The trypsin used for dissociating cells was a crude 1:250 trypsin preparation obtained from Difco Company, and was prepared as a 2.5% stock solution in the Hanks CMF medium described below. White Leghorn eggs were obtained from Truslow Farms (Chestertown, Md.).

Media. Hanks balanced salt solution (Gibco Inc., Grand Island, N.Y.), containing 0.035% $NaHCO_3$, was buffered with 0.02 M Hepes and adjusted to pH 7.25 with NaOH; the resulting medium is designated HH. Calcium and magnesium-free Hanks medium (CMF),

and horse and chicken sera were obtained from Baltimore Biological Laboratories. All sera used in these studies were first heated at 56 °C for 30 min to inactivate complement.

The medium used for counting cell suspensions consisted of 0.01 M sodium potassium phosphate buffer, pH 7.4, containing 0.137 M NaCl and 0.0027 M KCl (PBS).

Preparation of Cell Suspensions. A number of experiments were conducted with liver, limb bud, and fore-brain from embryonic chickens, but the results presented below and most of the kinetic experiments were conducted with 8-day-old embryonic chicken neural retina cells. This tissue has often been used in this type of study [14] and is, in many ways, the tissue of choice. It is easily accessible, gives large numbers of cells from a single animal, and at this stage in development shows few histological signs of differentiation [30]; finally, it yields cell suspensions in which the individual cells are relatively homogeneous in size and shape. After dissociation, the cells appear spherical, measuring approximately 6 μ in diameter. Electron microscopic studies of the intact tissue, single cell suspensions, and aggregated cells will be reported elsewhere.

The dissociation technique is essentially that of Roth and Weston [26]. After dissection of the 8-day-old retinas from surrounding tissue, two retinas were suspended in 2 ml of CMF containing 10% chicken serum. The tissues were disintegrated into fragments by gentle aspiration into and out of a Pasteur pipette (tip bore, approximately 1 mm). The suspension was then incubated at 37 °C for 10 min; crude trypsin was added to a final concentration of 0.25%; and the mixture was maintained at room temperature for 30 min with aspiration at 5-min intervals as described above. The resulting suspension was diluted fivefold with fresh CMF-chicken serum medium, and filtered through a 20-μ mesh nylon screen (Nytex, from Tobler, Ernst and Traber, Inc., N.Y.) to remove large particles. Relatively homogeneous cell suspensions were finally obtained by centrifuging the filtrate for 10 min at $300 \times g$, and removing the top 7-ml portion of the supernatant fluid. The cells were harvested from the supernatant fluid by centrifuging at $900 \times g$ for 5 min, and the packed cells were resuspended in the appropriate volume of HH medium. Generally, two retinas gave 4×10^5 cells per ml when the packed cells were suspended in 30 ml of HH. Routine examination of the suspensions with a hemocytometer showed that $90 \pm 5\%$ of the cells in the population were single cells. The suspensions were used immediately for the experiments described below.

Measurement of Single Cells and of Aggregates. The experiments described in this report were conducted with a Coulter electronic particle counter (Coulter Electronics, Haileah, Fla.), equipped with an automatic recorder and a 100-μ aperture. The counter measures the change in conductivity of the buffer solution in the aperture when part of the solution is displaced by a particle impermeable to the buffer. The counter discriminates between particles of different volumes, and can determine the number of particles in the solution aspirated through the aperture. The two major experimental limitations are the size of the particles relative to the aperture, and the number of particles per unit volume of solution. The size limitation of the particles is that they must be less than two-thirds the size of the aperture. In the present case, the 100-μ aperture was used; it could therefore accurately measure particles up to 66 μ in diameter, which is 11 times the diameter of the average neural retina cell. Therefore, assuming that no change in cell volume occurred during aggregation, the apparatus could count a spherical aggregate of about 1,300 cells. Coincidence counting errors are obtained when the solution contains too many particles. In the present experiments, coincidence errors were avoided by diluting the suspensions with PBS medium to final concentrations of 3 to 4×10^4 cells per ml.

Theoretically, the rate of aggregation could best be measured by determining the rate of formation of aggregates, rather than by determining the rate of disappearance of single cells from the population; the latter method involves measuring differences between initial and final single cell densities. Attempts were therefore made to measure the rate of formation of aggregates, but the following experimental problems were encountered: (a) The aggregation process was random, leading to the rapid formation of aggregates containing different numbers

of cells when only a small fraction of the single cells had disappeared. Furthermore, since small aggregates apparently adhered to larger aggregates, the number of small aggregates did not increase in a predictable manner, but increased, remained constant, or decreased with time. (b) While the counter can be used to determine the volume of a particle, the important parameter was the number of cells per aggregate, and the conversion of volume to cell number involved a series of assumptions that could give misleading results. Therefore, in the experiments reported below, an easily and accurately determined parameter was used to measure the rate of aggregation, i.e., the rate of disappearance of single cells from the population. It was, of course, essential to show that the disappearance of single cells in each experiment resulted from aggregation, and not from some artifact such as lysis. As shown below, the Coulter counter easily distinguished between these possibilities.

The counter contains three major electronic controls, a "window setting", an amperage, and an amplification control. At a given amplification and amperage, the window can be set to discriminate between particles of slightly different volumes within one population (such as single cells), or it can be set to include all desired particles within a population. At a window setting and amperage that measures all the single cell population, changing the amplification permits measurement of particles that are 2, 4, 8, 16, 32, and 64 times the volume of the single cells; i.e., amplification is linearly related to cell volume.

As indicated above, the parameter of interest in the kinetic experiments was the number of single cells in a population, as opposed to particles of smaller volume (designated "cell debris"), or of aggregates. For reasons presented in detail below, single cells were routinely counted between window settings 20 and 48, at 1/amplification = 1, and at 1/amperage current = 0.707.

Size Spectrum of Cells. A typical profile of freshly dissociated cells is shown in Fig. 1. Particle number is presented as a function of window setting at the amplitude and amperage given above. The instrument response below a setting of 20 represents some small cells, debris, electrical noise, and perhaps "leaky" or permeable cells (*see* below). The majority of the single cell population was detected between window settings 20 and 48; this population contained approximately 60% of the total number of particles, and was defined as the single cell population for the experiments described below. The particles detected at window settings higher than 48 represented large single cells, some doublets (about 4% of the total population), and a small number of larger aggregates (approximately 2%). The range 20 to 48 was therefore selected to exclude the cell debris, large cells, and aggregates.

An essential requirement for the experiments described below was that the selected window settings, 20 and 48, include most of the single cell population, and exclude smaller and larger particles. This point, as well as the question of the reproducibility of the population from one preparation to another, was examined as illustrated in Fig. 1. Over the course of a year, 10 separate preparations gave remarkably consistent results. The curve in Fig. 1 represents the mean values of the 10 experiments, and the standard errors at each window setting are indicated. We therefore concluded that the method of preparation of the suspensions gave reproducible and consistent cell populations.

Assay of Aggregation. The assay involved measuring the decrease in single cells as a function of time. Standard conditions consisted of suspending cells at final concentrations of 1.0 to 1.33×10^5 cells per ml in 3 ml of HH medium in 25-ml screw-capped Erlenmeyer flasks (Bellco Glassware Co., Vineland, N.J.). The suspensions were rotated on a New Brunswick Gyratory Shaker Bath at 37 °C and 70 rpm. Flasks were removed at the indicated times (generally 0, 5, 10, 15, 20, and 30 min), usually in triplicate, diluted with 7 ml of PBS, and counted as described above. Replicate determinations, using three flasks per time point, showed that the maximum experimental variation in single cell number at a given time was less than ±6% (Fig. 2). This value includes the errors and variations introduced by differences in geometry of the flasks, pipetting, and in the Coulter counter (±3%, according to the manufacturer).

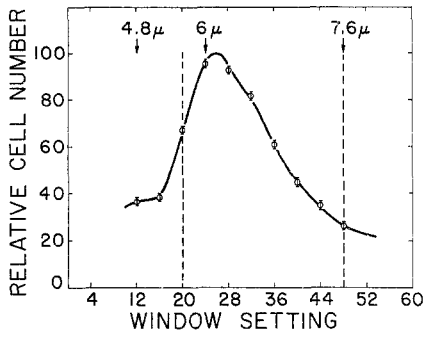

Fig.1

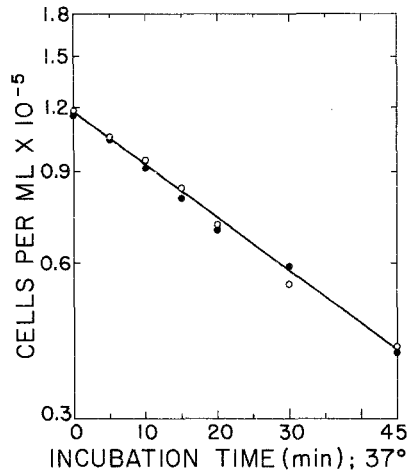

Fig. 2

Fig. 1. Distribution of cells with respect to size in populations obtained from 8-day-old embryonic chicken neural retina. Ten suspensions were prepared from different embryos, as described in the text and the cell number was determined at different window settings with the Coulter particle counter. Each point (○) represents the mean value of the 10 samples, and the range at each point (I) represents the range of the standard errors. The relationship between window setting, particle volume, and diameter was determined with both mouse lymphocytes and sheep red blood cells, and based on the known diameter and volumes of these cells. The diameters are shown at window settings 12, 24, and 48. The broken lines indicate the population included for all subsequent experiments (window settings 20 through 48), and defined as the "single cell population"

Fig. 2. Reproducibility of the method for measuring rate of disappearance of single cells. Cell suspensions were prepared by two investigators (○) and (•), each of whom started with one retina from the same 8-day-old embryo. The cells were permitted to aggregate under standard conditions (70 rpm, 37 °C). At the indicated times, three flasks were removed, the number of single cells remaining in the suspensions was determined, and the $\log_{10}$ of the mean of the three values was plotted against time of incubation

Validity of the Assay. The validity of the assay procedure was established by the following observations.

1. The number of single cells in the suspension decreased with time, whereas the number of larger particles increased.

2. Aggregates were visible, both macroscopically and microscopically.

3. Under normal conditions, there was no increase in the debris population as would be expected if single cell disappearance was the result of cell death and lysis. This held true unless conditions were specifically employed to increase the debris population. For example, suspension of the cells in distilled water resulted in a 300% increase in the debris in 10 min, with a concomitant decrease in the number of single cells.

4. A kinetic study of the change in single cells using the hemocytometer gave results comparable to those obtained with the Coulter counter (Fig. 3).

5. The dye nigrosin is excluded by healthy neural retina cells and taken up by leaky or moribund cells, a method that has long been used as a measure of the viability of a population [8]. Under standard assay conditions, nigrosin-positive cells were observed only rarely. However, nigrosin-positive cells were obtained by maintaining healthy cells for extended periods in CMF medium at 0 °C. For example, after 4 hr at 0 °C, the total number of single

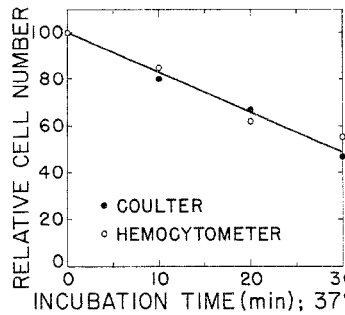

Fig. 3. Comparison of Coulter counter and hemocytometer methods for measuring single cells. A neural retina single cell suspension was permitted to aggregate under standard conditions, and the single cells remaining in the suspension were determined at the indicated times with the Coulter counter (•) and with the hemocytometer (◦)

cells determined with the Coulter counter decreased by 19%, whereas microscopic examination after treatment with nigrosin showed that 25% of the population had become nigrosin-positive. Direct examination of the cell population with the hemocytometer (without staining) showed no change in the number of single cells. Therefore, the important conclusion was reached that direct microscopic examination of the cells with the hemocytometer does not distinguish between healthy and "leaky" cells, whereas the Coulter counter does discriminate between the two cell types, and is therefore a more reliable procedure.

6. When the stable aggregates that formed in the standard assay were redissociated, most of the original population of single cells was recovered. In one such experiment, cells were allowed to aggregate for 60 min at 37 °C and 70 rpm, resulting in a reduction of the single cell population to 34% of its initial value. Aggregates were observed microscopically, and these were quite stable since they were not readily dissociated by repeated passage in a Pasteur pipette, or by incubation in 0.001 M ethylenediaminetetraacetate (EDTA) for 15 min at 37 °C. Dissociation was effected by repeating the trypsin treatment described above; this treatment resulted in an increase of the single cell population to 70% of its initial value, together with a significant increase in the debris population. The treatment with trypsin, in addition to the lysis it apparently effected in some cells, also was partially ineffective since a few small aggregates were visible under the microscope at the end of the incubation. The resistance of the aggregates to mechanical forces, to EDTA, and to trypsin, emphasized that the intercellular adhesions formed are remarkably stable.

The Kinetics of Aggregation. Under standard conditions of assay, single cells disappeared from suspension shaken at 37 °C and 70 rpm at the rate shown in Fig. 2.

To conveniently use such information, several mathematical relationships were tested to determine which would most easily yield the desired parameter, i.e., the rate of aggregation. Two such treatments proved useful: (a) The theory of flocculation kinetics [19] includes equations that describe the rate of aggregation of small particles. These equations were found to apply to the data shown in Fig. 2, despite the fact that there is no theoretical basis for the application of such equations to particles as large as the neural retina cells that aggregate under the conditions described above.

Table 1. *Effects of rotation speed, pH, and temperature on rates of aggregation of neural retina cells*[a]

Experiment number	Variable tested	Rate of aggregation (slope $\times 10^3$)	Standard error of slope ($\times 10^3$)	t
1.	Rotation speed		$n=6$	
	0 rpm	2.5	0.4	10.057[b]
	35 rpm	2.8	0.3	9.882[b]
	70 rpm	10.2	0.7	
	100 rpm	5.9	0.6	4.974[b]
2	pH			
	7.00	8.5	1.0	1.334
	7.25	10.6	1.2	
	7.50	9.0	1.4	0.863
3	Temperature			
	5 °C	0.9	0.3	7.459[b]
	26 °C	6.0	0.4	2.211
	37 °C	8.0	0.8	

[a] The standard conditions for aggregation, described in the text, were used unless otherwise indicated (70 rpm, pH 7.25, 37 °C). Single cell counts were obtained on replicate samples at 0, 5, 10, 15, 20, and 30 min ($n=6$). The best straight line, relating logarithm of cell number to time of aggregation, was obtained by computer analysis, and the slopes of these lines and their standard errors are presented above. Regression analysis was used to compare slopes obtained by varying rotation speed, pH, or temperature with the slope of the line obtained with the same cell suspension treated under standard conditions. The resulting correlation coefficients are presented as t values obtained by selecting $p=0.05$ where $n=6$. A value of t greater than 2.306 was interpreted to mean that the slope of the experimental line was significantly different from the slope obtained with the control in that experiment. Each experiment was performed with a different cell preparation.

[b] Significantly different, $p=0.05$.

(b) A much more convenient method resulted from recognition of the fact that the relationship between the logarithm of single cell number and time is linear (Fig. 2) over the first 30 min of aggregation (but generally not beyond this point). For present purposes, therefore, the slope of the line obtained in this type of plot will be designated "rate of aggregation" (or adhesion), and is used to compare cells treated under different conditions. A computer program was designed by Mr. Stefan Chipowsky to determine statistically the most accurate slope and the standard error of the slope from the raw data obtained in each experiment. When experimental parameters were varied, the slope of the line of the control set was compared with the experimental set by regression analysis and the correlation coefficients are presented as "t" values.

Reproducibility and Sensitivity of the Standard Assay. To determine whether the standard assay gave reproducible results, two investigators

started the procedure; each worked with one retina from the same embryo. Three flasks were used for each time point, and the rates of aggregation were found to be the following (slopes $\pm$ standard errors of the slopes of the lines determined as described above): 0.0103 ± 0.0004; 0.0099 ± 0.0004. It was therefore concluded that the standard assay procedure was reproducible.

The sensitivity of the assay method will, of course, determine how large an effect an experimental variable must have on the rate of aggregation before it can be detected. The sensitivity depends upon the number of samples used to determine the slopes, i.e., the number of samples taken for each time point where aggregation is permitted to occur in separate flasks, and the number of time points that are used to determine the slope. In the experiments reported in Tables 1, 2, and 3, a large number of experimental variables were tested, with six time points and two flasks per time point in each case. The standard errors are presented with each slope to give a measure of the accuracy for each value. However, it must be emphasized that a valid comparison between experimental and control values cannot be made simply by inspection of the slopes and their standard errors. The statistical methods outlined in Table 1 must be employed to compare the *slopes* of two lines.

The sensitivity of the assay method can be approximated by inspection of the tables. That is, application of student's "t" test showed that the experimental and control values were the same ($p = 0.05$) when the slopes of the lines agreed within 10 to 12% (with the exception of the pH experiment in Table 1), whereas they were different when they differed by 18% (Experiment 1, Table 2). Therefore, under the conditions defined above, differences in rates of aggregation of 15% or more should be detectable. Again, we note that the sensitivity can be increased by increasing the number of samples used for determining the slopes of the lines relating cell number and time of aggregation.

Effect of Initial Cell Density. A stock suspension of cells in HH medium was prepared by the standard procedure, and diluted in HH medium to give four suspensions containing the following initial densities of single cells per ml ($\times 10^5$): 2.0, 1.0, 0.82, and 0.55. As indicated in Fig. 4, the flasks were shaken at 37 °C and 70 rpm, and the rates of aggregation were determined by the usual procedure.

Three important results were obtained in this experiment: (a) Aggregation commenced without a lag period at all cell densities. In fact, under the standard assay conditions (at 37 °C), no lag period has ever been observed. (b) With the exception of the highest cell density, linear plots

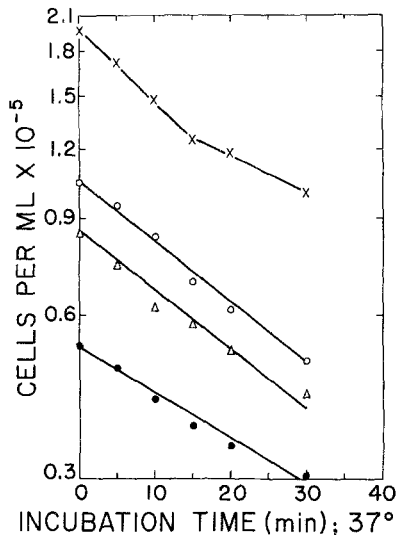

Fig. 4. Effect of initial cell density on the rate of aggregation of 8-day-old embryonic chicken neural retina cells. The tissue was dissociated and a cell suspension was prepared as described in the text. Initial cell densities were as follows (per ml): 2×10^5 (×–×–×); 1×10^5 (o–o–o); 0.82×10^5 (Δ–Δ–Δ); and 0.55×10^5 (•–•–•). Standard conditions were used for aggregation, and two flasks were removed at each time point to determine the number of single cells remaining in the population. The slopes of the lower three lines, the standard errors of the slopes, "t" values, and the conclusion that two of the slopes were the same (o–o–o, and Δ–Δ–Δ) whereas the slope of one (•–•–•) was different, was determined as indicated in Table 1. The values were as follows:

Line	Slope $\times 10^3$	Standard error $\times 10^3$	t
(o)	11.2	0.8	
(Δ)	9.7	0.7	1.407
(•)	8.3	0.7	2.621[a]

[a] Significantly different, $p = 0.05$.

were obtained in all cases when the results were treated as described above. At the highest cell density, a biphasic curve was obtained. (c) The slopes of the lines relating logarithm of cell number to time of aggregation decreased with decreasing initial cell density.

From these results, it is clear that linear kinetics were obtained only within a narrow range of cell densities and for a limited period of time (about 30 min). For all remaining experiments, the initial cell density was maintained between 1.0 and 1.33×10^5 cells per ml; in each experiment, the cell density was the same in each flask.

Effect of Rotation Speed. Earlier work [14] had shown that the size of aggregates formed over a period of hours depended upon the speed used

to rotate the flasks. The present experiments were designed to test the effect of varying the rotation speed. Table 1 shows the results obtained when suspensions were rotated at 0, 35, 70, and 100 rpm. The rate increased as the speed increased from 0 to 70 rpm, and decreased at 100 rpm. The simplest interpretation of these results is that increasing the rotation speed to 70 rpm results in increased frequency of collision between the cells, with resulting increase in the rate of aggregation. Presumably, at 100 rpm the shear forces are also increased to the point where aggregation is reduced despite the increased frequency of collision.

Based on these results, 70 rpm was selected as the standard rotation speed.

Effect of pH and Temperature. Since cells grown in tissue culture are seriously affected when the pH of the medium is not maintained close to 7, no attempt was made to study the effect of pH over a wide range. The effect of pH was studied at 7.00, 7.25, and 7.50, and (as shown in Table 1) there was no significant difference in the rates of aggregation at these pH values. The pH selected for standard conditions was 7.25.

The effect of temperature on aggregation has been studied [6, 16, 24], but apparently conflicting results were obtained. Moscona [16] detected no aggregation with neural retina cells at temperatures below 5 °C. Similar results were obtained by Steinberg [24] when the cells were shaken at 6.5 °C, although some aggregation occurred in the absence of shaking. In contrast, Curtis [6] used the hemocytometer method with limb bud cells, and found that aggregation occurred at 1, 6, or 37 °C, although the rate was decreased at the lower temperatures.

The results (Table 1) clearly show that the rate of aggregation has a marked temperature dependence; at 5 °C, the rate was very low.

Effect of Calcium and Magnesium. These cations have been reported to be required for cell adhesion [23]. An experiment was therefore performed in which dissociated cells prepared in the standard manner were resuspended in either CMF or HH medium. The results presented in Table 2 show that the cells did aggregate at a significant rate in the CMF medium, but the rate was less than that obtained in the HH medium. In view of the fact that the cells were not washed exhaustively, traces of Ca^{++} and Mg^{++} may have been present in the experimental suspension, and at concentrations sufficient for some aggregation to occur.

Biochemical Aspects of Cell Aggregation

Presumably, the process of dissociation of cells results from the removal of some material from the cell surfaces. If this hypothesis is correct, then aggregation or adhesion would require either the secretion of stored

material or of de novo synthesis to replace the material that has been removed, assuming, of course, that the new contacts made between the cells are identical with those in the undissociated tissue. The chemical nature of the material (or materials) released by treatment of cells with crude trypsin has not been extensively investigated. Cook, Heard, and Seaman [4] found that such treatment of erythrocytes released a sialic acid-containing mucopeptide as the predominant species. Recently, Kemp et al. [10] reported that both EDTA and trypsin released similar types of substances from 5-day-old chick fibroblasts.

The effects of puromycin, actinomycin, and temperature on aggregation have been studied in Moscona's laboratory [17]; these workers concluded that de novo protein synthesis is a prerequisite for adhesion after dissociation with trypsin. Kemp et al. [10] also found that puromycin inhibited aggregation but that the effect could only be detected after about 1 hr. However, this view has been challenged because horse serum is frequently used in the basic salts solution employed for studying aggregation [7]. According to this interpretation, horse serum contains a protein that inhibits aggregation, and the adhesive process can only occur when the cells produce a component, such as a proteinase, that destroys the inhibitor. Thus, Curtis and Greaves [7] concluded that the effect of puromycin, actinomycin, etc., on aggregation is to prevent the synthesis of the factor that destroys the inhibitor in the aggregation medium. It should be noted that Moscona [15] was able to duplicate his earlier results in serum-free medium.

A novel basis for cell adhesion has been proposed by Jones [9], who claimed that ADP and ATP exerted opposing effects on the process. ADP stimulated aggregation, whereas ATP had the opposite effect. By analogy with similar studies on platelets [21], Jones [9] interpreted the nucleotide effects as follows. A protein of the actomyosin type is present on cell surfaces, and whether or not a cell will adhere depends on the state of contraction of this protein. In the presence of ATP, adhesiveness is lost, whereas it is restored by ADP.

The availability of the assay described in this report made it possible to determine quantitatively the effects of some of the substances mentioned above on the initial kinetics of cell aggregation.

Effects of DNAase, Nucleotides, and Metabolic Inhibitors. The results of experiments with these substances are presented in Tables 2 and 3.

DNAase at final concentrations of 30 μg/ml (bovine pancreatic DNAase, once crystallized, Sigma Chemical Co.) was added to solutions used to suspend the packed dissociated cell pellet. No effect on the rate of aggregation was detected.

Table 2. *Effects of calcium-magnesium-free medium, DNAase, and horse serum on rates of aggregation of neural retina cells*[a]

Experiment number	Conditions[b]	Rate of aggregation (slope $\times 10^3$)	Standard error of slope ($\times 10^3$)	t
1	HH (control)	11.4	1.2	
	CMF	9.3	0.9	3.809[c]
2	HH (control)	9.6	1.0	
	HH + 30 μg/ml DNAase	9.3	1.0	0.236
3	HH (control)	8.9	0.6	
	HH + 0.01 HS	9.4	0.5	0.531
	HH + 0.10 HS	9.4	1.0	0.364
	HH + 0.50 HS	9.0	0.8	0.080
	HH + 1.0 HS	9.2	0.7	0.276

[a] The controls for each experiment were performed under the standard conditions described in Table 1 and in the text, and the results evaluated as described in Table 1. Each experiment was performed with a different cell preparation. The concentrations of horse serum (first treated at 56 °C for 30 min to destroy complement) are given as ml of serum, per flask.

[b] HH = Hanks solution buffered at pH 7.25 with 0.02 M Hepes (N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid). CMF = calcium and magnesium-free Hanks solution. HS = horse serum.

[c] Significantly different, $p = 0.05$.

The effects of ATP, ADP, and cyclic 2′, 3′-AMP at final concentrations of 1 μmole/ml were examined in the usual medium, with or without 2 μmoles of $CaCl_2$ per ml. Control suspensions contained cells in the HH medium, or in the HH medium supplemented with 2 μmoles of $CaCl_2$ per ml. No differences in the rates of aggregation were detected between the control and experimental cell suspensions.

Two types of metabolic inhibitors were studied, those that affect energy metabolism, and those that inhibit protein synthesis (results in Table 3). Each experiment is divided into three parts: (a) a set of control flasks without inhibitor; (b) a set of flasks in which the inhibitor was present only during the aggregation process; and (c) a set of flasks in which the inhibitor was present during trypsinization and all subsequent steps. The latter group was included since approximately 20 min was required between the treatment with trypsin and the initiation of aggregation; presumably, protein synthesis and other metabolic events could occur during this period.

The results showed that the metabolic inhibitors did not affect the rate of aggregation. Therefore, it seems reasonable to conclude that the early kinetics of aggregation of neural retina cells, prepared and studied under *these specific conditions*, is independent of energy-linked processes, or of de novo protein synthesis.

Table 3. *Effects of ATP, ADP, cyclic-AMP, metabolic inhibitors, and inhibitors of protein synthesis on rates of aggregation of neural retina cells*

Experiment number	Treatment[a]			Rate of aggregation (slope $\times 10^3$)	Standard error ($\times 10^3$)	t
	compound added[b]	final concentration per ml	added at step no.			
1	HH (control)			7.5	0.7	
	+ATP	1.0 µmole	2	7.2	1.0	0.212
	+ATP+0.002 M Ca^{2+}	1.0 µmole	2	7.4	0.9	1.324
	+0.002 M Ca^{2+}		2	7.1	1.6	0.115
2	HH (control)			8.0	0.8	
	+ADP	1.0 µmole	2	8.7	0.9	0.592
	+2′,3′-AMP	1.0 µmole	2	7.2	1.5	0.478
3	HH (control)			8.8	0.7	
	+KF	1.0 µmole	1	7.7	0.6	1.249
	+KF	1.0 µmole	2	8.0	0.3	1.070
4	HH (control)			7.9	0.8	
	+DNP	0.5 µmole	1	8.3	0.5	0.442
	+DNP	0.5 µmole	2	8.7	0.7	0.777
5	HH (control)			7.1	0.7	
	+Azide (NaN_3)	0.5 µmole	1	6.7	1.0	0.333
	+Azide (NaN_3)	0.5 µmole	2	6.6	0.8	0.414
6	HH (control)			9.5	1.2	
	+Cycloheximide	2 µg	1	10.3	0.4	0.605
	+Cycloheximide	2 µg	2	10.6	0.9	0.690
7	HH (control)			9.2	0.8	
	+Puromycin	10 µg	1	9.8	1.1	0.417
	+Puromycin	10 µg	2	9.0	0.8	0.917

[a] Each experiment was conducted with a different cell preparation. Controls were incubated under standard conditions as described in Table 1 and the text, and the results of each experiment were statistically evaluated and are presented as described in Table 1. Compounds were added at one of the following stages in each experiment: *Step 1*, prior to dissociation of the cells with trypsin; they were maintained at the indicated concentrations during all subsequent steps; *Step 2*, to the single cell suspension prior to measuring the rate of aggregation.

[b] HH = Hanks solution buffered at pH 7.25 with 0.02 M Hepes (N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid). All compounds were dissolved in HH.

Effect of Horse Serum. As noted above, Curtis and Greaves [7] reported that horse serum contains an inhibitor of cell adhesion which exerts its optimum effect at 1 °C, while showing only a limited effect at 37 °C. The reduction in the inhibitory activity at 37 °C was explained by degradation of the inhibitory protein by factors (enzymes?) produced by the cells during the aggregating process. Recently, two reports appeared indicating that these findings could not be corroborated [3, 16], but it is

important to note that the more recent work was not performed under conditions identical to those of Curtis and Greaves [7].

The effect of horse serum was therefore reinvestigated by using the kinetic approach described in this report. As shown in Table 2, at 37 °C, concentrations of horse serum varying from 0.0033 to 0.33 ml of serum per ml of suspension showed no effect on the rate of aggregation. However, an important effect was observed at 5 °C, that is, the horse serum promoted aggregation. The details of the experiments at 5 °C are reported in the accompanying communication [18].

Discussion

Cell adhesion is a process of fundamental biological significance, and has been the subject of intensive investigation in many laboratories [5]. The mechanism of this process remains entirely obscure. The difficulty in attacking the problem is emphasized by the many diverse, and sometimes conflicting, reports in the literature. For example, there is no general agreement even on such basic points as a definition for the process of cell adhesion.

The major problem to investigators in this area is that the formation of a stable aggregate from a suspension of single cells involves not one process, but many. Thus, conflicting results could be obtained unless each study was conducted under identical conditions. These processes may even be sequential, although not necessarily synchronous among all the cells in one aggregate, or among different aggregates in the same suspension. For example, when cells from a mixture of two different tissues are mixed, an aggregate forms which contains both types, but the cells eventually "sort out" [25]. Thus, sorting out may well involve mechanisms different from those involved in early aggregate formation. Similarly, it is possible that the last of a series of steps in the formation of tissues, or of extremely stable aggregates, involves the secretion of a "ground substance" [20] common to all cells in the aggregate.

In addition to the possibility that more than one step is involved in the formation of stable aggregates from single cells, earlier studies have been seriously hampered by the lack of a quantitative assay for measuring cell adhesion, particularly for measuring the *rate* of formation of cell aggregates. The lack of such a method has made it difficult to determine quantitatively the effects of exogenous substances, such as metabolic inhibitors, on the process. This report presents a simple reliable method for determining the *initial* rate of cell aggregation. Whether the early process described here involves the formation of specific or nonspecific cell-cell bonds, or both, remains to be determined.

The present method involves determining the rate of disappearance of single cells from a suspension maintained under defined conditions. The results are readily amenable to analysis by standard statistical procedures. We wish to emphasize that under the conditions used for these studies, at least with embryonic chicken neural retina cells, stable aggregates are formed, not merely loose collections of cells. The tight unions formed in the aggregates are resistant to shear forces and to rupture by EDTA; the bonds behave similarly to those in intact neural retina in that they are partially dissociated by crude trypsin.

The important parameters that affected the initial rate of aggregation were found to be the initial cell density, the rate of rotation of the flasks containing the suspensions, the temperature, and the presence of divalent cations.

The rate of aggregation was not affected by a number of exogenous substances, including ATP, ADP, cyclic-AMP, metabolic (energy) inhibitors, inhibitors of protein synthesis, and DNAase. The *tentative* conclusion from these results is that neither energy-dependent processes nor de novo protein synthesis are required for the early steps in the adhesion of embryonic neural retina cells when such cells have been dissociated with crude trypsin under the conditions defined above. The mechanism by which crude trypsin dissociates the tissue to single cells remains to be elucidated, and, of course, may have little or nothing to do with the early steps in aggregation; for example, trypsin may hydrolyze the final products of cell adhesion (e.g., the "ground substance").

The assay method presented in this report is the basis for the isolation of a purified protein from horse serum that promotes aggregation of embryonic neural retina cells at 5 °C [18]. It seems reasonable to suppose that this quantitative procedure will have wide applicability in studying the general problem of cell adhesion.

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