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On the Recovery of Adhesiveness by Trypsin-Dissociated Cells

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Summary. A sensitive method for assaying aggregation of dissociated cells has been developed which allows the determination of the mean number of cells per aggregate of a cell population. We have demonstrated that exposure of dissociated 6- or 7-day chick embryo neural retinal cells to trypsin in calcium-free solution renders them unable to aggregate for a half hour in stirred cell suspensions. Aggregation was noticeable first at 30 to 40 minutes and progressed to the formation of massive compact aggregates. Because the half-hour aggregation lag occurred both in the absence of serum and in medium reclaimed from aggregated preparations, the possibilities were excluded that it was due either to an inhibitor of aggregation in the serum, or was the time required for release into the medium of soluble aggregation-promoting materials emanating from the cells themselves. Cells dissociated by divalent cation withdrawal (Ca^{++}, Mg^{++}-free saline with EDTA) aggregated without a lag. The trypsin-induced lag does not appear to be the result of trypsin adsorbed to the surfaces of dissociated cells, as the lag is not abolished by addition of trypsin inhibitors to the aggregation medium. Microelectrophoresis of dissociated cells did not reveal changes in surface charge density during recovery from trypsinization. A variety of proteins and calcium ion, if present during trypsinization, protect the cells against the trypsin-induced aggregation lag. If the temperature was reduced from 37 to 6 °C, aggregation of fully adhesive cell populations came to a complete halt within 2 to 3 minutes. Aggregation resumed with a 5 to 10 minute delay when the temperature was returned to 37 °C. The rapidity of onset and reversal of inhibition of aggregation by low temperature treatment militates against the hypothesis that the low-temperature inhibition of aggregation acts by suppressing the synthesis of cell surface components necessary for adhesion. The abolition of the aggregation lag in trypsinized cells was also shown to be temperature-dependent; a 20-minute cold pulse administered in the middle of the lag period extended the length of the lag by exactly 20 minutes.

Adhesiveness of cell surfaces is required so that cells can construct coherent tissues. A problem basic both to cell biology and to developmental

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biology is the mechanism of intercellular adhesion. The following study is a report on a method for studying mechanisms of cellular adhesion which employs aggregation of dissociated cells as an assay for adhesiveness. In this study, two procedures are described whereby dissociated cells can be rendered reversibly nonadhesive. The purpose of the present study is to describe these procedures in detail and to present the results of initial experiments utilizing these procedures to study basic mechanisms of cellular adhesion.

Two different treatments are commonly used to effect dissociation of animal tissues into suspensions of isolated cells. One method involves incubation of the tissue in a medium from which divalent cations have been removed, while the other involves treatment of the tissue with a proteolytic enzyme such as trypsin. For many tissues, the most effective method for tissue dissociation involves combination of the two treatments (Steinberg, 1967).

If placed in the proper environment, disaggregated cells are usually able to aggregate with each other to establish masses of adhering cells (Holtfreter, 1943; Moscona & Moscona, 1952; Townes & Holtfreter, 1955; Steinberg, 1958; Armstrong, 1966). In the following study, a comparison is made between the rates of aggregation of embryonic chicken cells dissociated either by removal of divalent cations from the environment by ethylene-diamine tetra acetate (EDTA) or by the proteolytic enzyme trypsin. It is observed that EDTA-dissociated cells are able to aggregate as soon as they are placed under the appropriate environmental conditions while trypsin-dissociated cells show a pronounced half-hour lag period before aggregation begins. The first portion of the study that follows represents an analysis of the aggregation lag of trypsin-treated cells.

The second portion of the present report details studies of a second method for rendering cells reversibly nonadhesive. Trypsin-dissociated cells maintained at 37° C for 70 to 100 min (a period of time adequate to overcome the trypsin-induced aggregation lag) aggregate rapidly. Aggregation of these same cells does not occur at low temperatures. The low temperature-induced prevention of aggregation shows a rapid onset (2 to 3 min) and is completely reversible.

Hopefully, if one knew why cells are not adhesive during the first half hour following exposure to trypsin and the nature of the changes that take place during this period that allow cells to recover adhesiveness, the information would be directly applicable to problems of the mechanisms of adhesion. Similarly, it is desirable to know why cells are nonadhesive at low temperatures because this knowledge should shed further light on fundamental mechanisms of adhesion.

Materials and Methods

Cell Dissociation

Neural retinas were dissected from 6- or 7-day chicken embryos in cold Hanks' saline. CMF-dissociated cells were prepared by incubating neural retinas in CMF Hanks' solution, pH 7.4, containing 0.1 % Na_2EDTA at room temperature. Trypsin dissociation was carried out in a 0.1 % or 0.25 % crude trypsin (Difco 1:250) solution or a 3 % crude trypsin (Difco 1:250)-1 % pancreatin (Difco) mixture made up in CMF Hanks' (with or without 0.1 % Na_2EDTA) (Steinberg, 1962). Trypsin solutions were at a pH of 7.8 to 8.0 and the tissues were treated for 25 min at 37 °C. DNase plus Mg^{++} was added to digest any slime formed during the course of trypsin treatment (Steinberg, 1963). In all cases, the actual dissociation was accomplished using the shear produced by stirring with a mechanical test tube stirrer ("Cyclomixer", Clay Adams Co., New York).

Cell Aggregation

Following dissociation, the cells were pelleted, washed, and resuspended in Eagle's minimal essential medium (Eagle, 1959). In most cases, the culture medium contained 10 % horse serum ("MEM + HS") but in other cases, serum was omitted ("MEM") or was replaced by soybean trypsin incubator (type 1-S, 2 × cryst-Sigma) ("MEM + STI"). Aggregation was carried out at 37 °C in 10 or 25 ml Erlenmeyer flasks. Cell suspensions were stirred on a gyratory shaker at 70 rpm. Cell concentrations in the range of 1 to 4 × 10^6/ml were employed.

In most experiments, aggregation was quantitated by measuring the average number of cells per aggregate using a technique employing membrane filters (Armstrong, 1966; Steinberg & Granger, 1966). At the beginning of aggregation and at various times afterwards, 0.1-ml samples of culture medium and cells were withdrawn from the aggregation vessels. The cells in these aliquots were filtered onto the surface of a Millipore filter and were then fixed with Bouin's solution. The filter with cells was then stained with hematoxylin and eosin or azure A-naphthol yellow, dehydrated in ethyl alcohol, cleared in toluene and mounted on a microscope slide with Permount (Millipore Filter Corp., 1964). Loss of cells and cell aggregates from the filter was negligible. Cell aggregation was assayed by counting the number of cells in each of at least 1,000 aggregates per slide, sampled at random. (An "aggregate" was defined as a single cell or a cohering group of cells.) The parameter used to express the degree of cell aggregation was the average number of cells per aggregate. In other cases, the degree of aggregation was ascertained by measuring with an ocular micrometer the sizes of aggregates within flasks.

Cell Electrophoresis

The electrophoretic mobility of dispersed cells was determined in a Bangham microscopic electrophoresis apparatus (Bangham, Flemans, Heard & Seaman, 1958; Armstrong 1966). In most cases, the suspending medium was 0.145 M NaCl, buffered to pH 7.2 to 7.3 with 3 mM $NaHCO_3$. Cells were washed free of tissue culture medium by centrifugation and resuspension in the 0.145 M NaCl solution. Temperature was 25 °C.

Estimation of Protease Activity

The trypsin-inhibiting activity of various cell culture media was tested using a photographic film assay system. Trypsin solutions were serially diluted in the various culture media and several 10-μliter aliquots of each dilution were placed on the emulsion side of

premoistened pieces of exposed Kodak Plus X Pan photographic film. These were incubated for 12 hr at 37 °C in a moist chamber. Trypsin activity was shown by dissolution of the gelatin of the developed photographic emulsion. Although the assay system is only semi-quantitative, reproducible differences in trypsin inhibiting activities of the various culture media could readily be detected.

Results and Conclusions

Reversible Nonadhesiveness Following Treatment with Proteolytic Enzymes

Temporary Nonadhesiveness of Trypsin-Dissociated Cells. The kinetics of aggregation of trypsin-dissociated cells from the neural retina of the chick embryo are presented in Fig. 1. The trypsin-dissociated cells show a half-hour

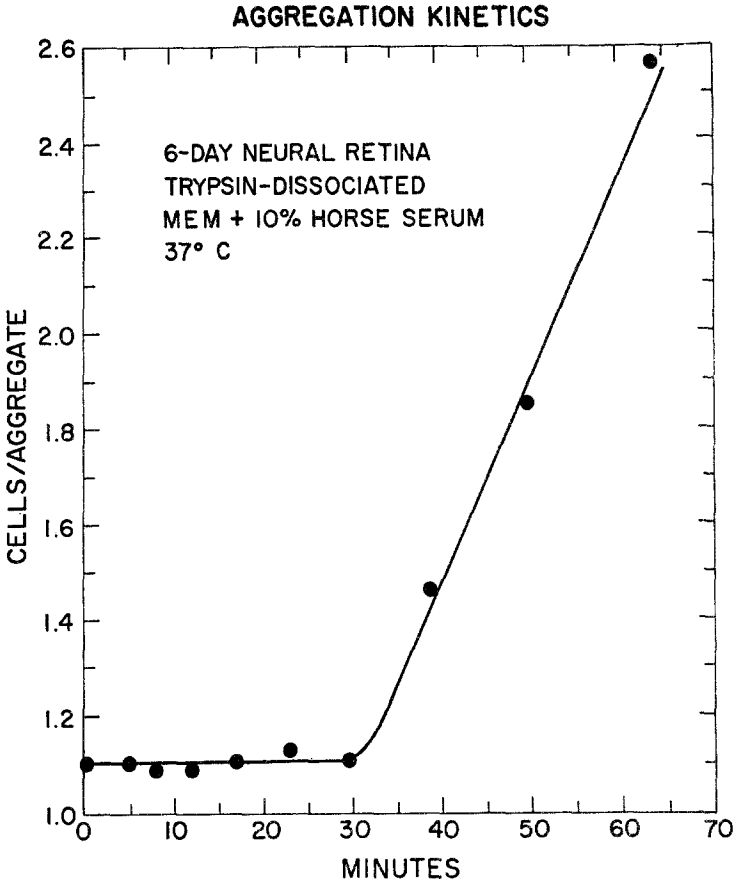

Fig. 1. Aggregation kinetics of trypsin-dissociated, 6-day neural retinal cells. Cell suspensions in Eagle's MEM+10% horse serum at 37 °C were stirred (70 rpm) on a gyratory water-bath shaker. The degree of aggregation in the cell suspension is expressed as the average number of cells per aggregate. Trypsin-dissociated cells began aggregating only after a half hour in culture

refractory period, followed by the onset of rapid aggregation. Addition of small, pre-formed aggregates to the cell suspension to serve as nucleation sites did not reduce the refractory period. Although the results presented in Fig. 1 represent a single experiment, this experiment has been repeated many times with the same results: trypsin-dissociated cells invariably show a re-aggregation lag of about a half hour.[1]

In Fig. 1, and in most of the aggregation data that follow, the parameter employed to express the degree of aggregation is the mean number of cells per aggregate (determined by counting the number of cells in each of a thousand or more aggregates chosen at random). The raw data summarized in Fig. 1 are presented in Table 1. As is clear from Table 1, aggregation in stirred cell suspension involves the formation of quite large aggregates even at times when a majority of "aggregates" contain only one cell. Thus, by the time the mean number of cells per aggregate has risen to 1.5 or 2.0, a substantial amount of aggregation visible to the eye has occurred.

Is the Aggregation Lag Due to a Temporary Inadequacy of the Culture Medium? In an initial attempt to account for the aggregation lag, the possibility was examined that the lag represents the time required for the dissociated cells to "condition" the culture medium to a degree sufficient to enable it to support cell aggregation. Conditioning could be of two sorts: (1) *aggregation inhibitors* present in fresh medium might need to be removed or inactivated, or (2) the dissociated cells might need to add *aggregation-promoting substances* to the culture medium. As an example of the first case, certain preparations of blood serum have been shown to contain high levels of aggregation-inhibiting proteins (Curtis & Greaves, 1965). If this is true in the present case, then the lag might represent the time necessary for the dissociated cells to inactivate or remove these inhibitory molecules. However, in the present case, the lag does not appear to be caused by inhibitory factors in the serum since it also occurs if trypsin-dissociated cells are allowed to aggregate in serum-free medium (Fig. 2). The extent of the aggregation lag is the same (30 to 35 min) in serum-free medium as in complete medium.

In addition, trypsin-dissociated cells allowed to aggregate in medium that has previously supported aggregation (and which can thus be presumed both to be devoid of aggregation-preventing factors present in fresh medium and to contain any required aggregation-promoting materials contributed by the cells themselves) show the same aggregation lag as cells aggregated in fresh

1 A similar lag has been observed with trypsin-dissociated 4-day limb bud, 7-day heart and 7-day liver. Dissociation with crystalline trypsin, crystalline alpha-chymotrypsin or pronase results in a lag similar to that produced by crude trypsin.

Table 1. Number of aggregates contained in each aggregate class (data for Fig. 1)

Minutes in culture	Aggregate class[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	>20[b]	Average no. cells per aggregate	Number of aggregates examined
0	903	85	10	2																		1.111	1,000
5	910	77	12	1																		1.104	1,000
8	929	66	4	1		Number of aggregates tallied for each aggregate class																1.077	1,000
12	935	61	5																			1.071	1,001
17	908	77	13	2																		1.109	1,000
23	895	84	18	1	3																	1.135	1,001
30	927	61	6	1	2			2					1									1.110	1,000
39	840	80	29	16	8	5	4	1	4	2	1	1	2	1	1	2		1		1		1.462	999
50	715	138	56	24	12	10	9	5	6	4	1	1	1	1	3	2	1	3	1		23 (2) 24	1.866	996
64	708	128	50	22	18	8	6	9	3	7	5	5	5	6	2	2	2	1		2	22 23 (2) 24 25 26 29 (2) 30 32 38 42 77 276	2.585	1,000
80[c]	637	117	64	41	29	13	11	13	5	7	10	1	2	6	1	6	4	5	2	4	21 (3) 22 (3) 23 24 31 (3) 32 (2) 33 34 35 (2) 36 39 40 43 45 47 68 357	3.613	1,007

[a] Aggregate class "1" designates single cells; class "2" designates pairs; class "3" designates triplets, etc.

[b] In this column, the first member of each number-pair designates the aggregate-class (i. e., the number of cells in the aggregate) and the second member (enclosed by parentheses) is the number of aggregates tallied for that aggregate-class. If there is no number in parentheses following the aggregate-class designation, then the number of aggregates tallied for that class is one.

[c] The data-point for 80 min of aggregation was not included in Fig. 1 since the reliability of the method is reduced at higher levels of aggregation (for an explanation of this, see Discussion).

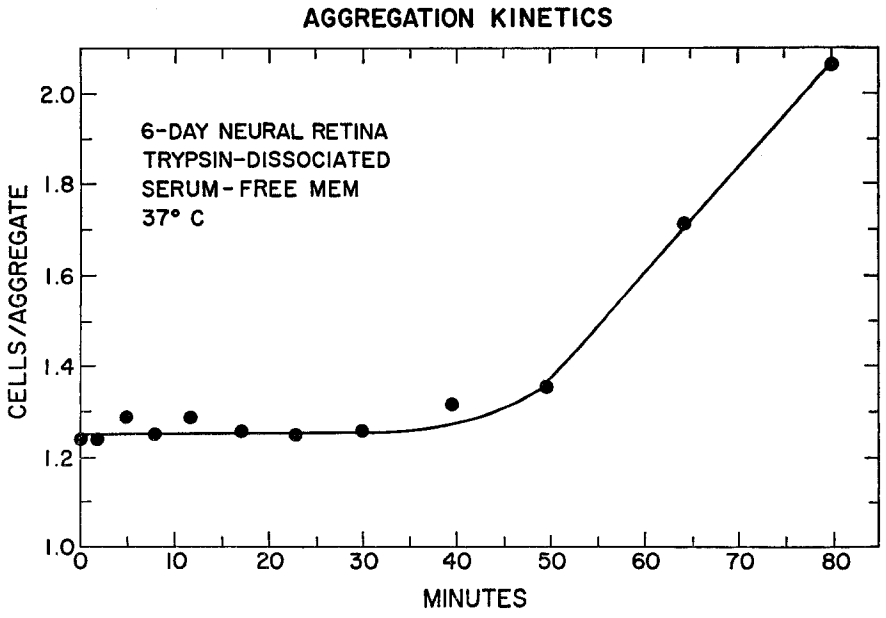

Fig. 2. Trypsin-dissociated, 6-day neural retinal cells showed an aggregation lag in serum-free culture medium similar to that shown in medium containing horse serum (compare with Fig. 1)

medium (Fig. 3). Thus, the aggregation lag cannot be explained as a consequence of a requirement for conditioning of either the first kind (removal of aggregation inhibitors) or of the second kind (addition of aggregation-promoting materials to the medium by dissociated cells).

Is the Aggregation Lag Due to an Effect of Trypsin upon the Cells? If the aggregation lag is not a result of alteration of the culture medium, then it may be a direct result of the procedure used to effect disaggregation of the tissue. To examine this possibility, 6-day chick embryo neural retina was dissociated in the absence of trypsin, using CMF treatment. As shown in Fig. 4, CMF-dissociated cells show no detectable aggregation lag: cells aggregate as soon as they are placed in culture. It appears, then, that the aggregation lag is induced by treatment with proteolytic enzymes.

Time-Course of Recovery of Adhesiveness by Trypsinized Cells. While the evidence presented so far demonstrates the *onset* of adhesiveness in trypsinized cells at about a half hour after the cells are cultured, it does not reveal how much time is required for the cells to develop their full adhesive potentials as determined by measurement of rates of cell aggregation. In an effort to answer this question, a procedure was developed in which trypsin-disso-

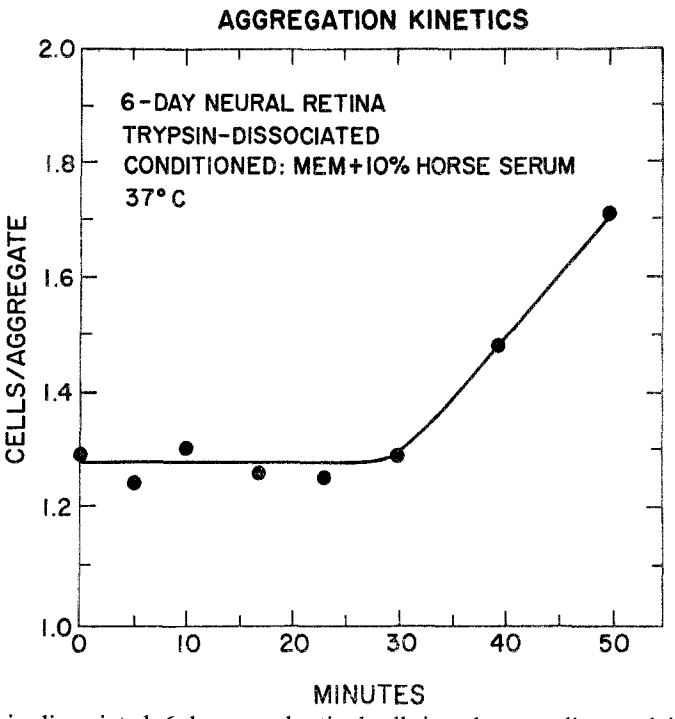

Fig. 3. Trypsin-dissociated, 6-day neural retinal cells in culture medium reclaimed from a preparation of cells which had already undergone aggregation showed an aggregation lag similar to that shown when cells are aggregated in fresh culture medium (compare with Fig. 1)

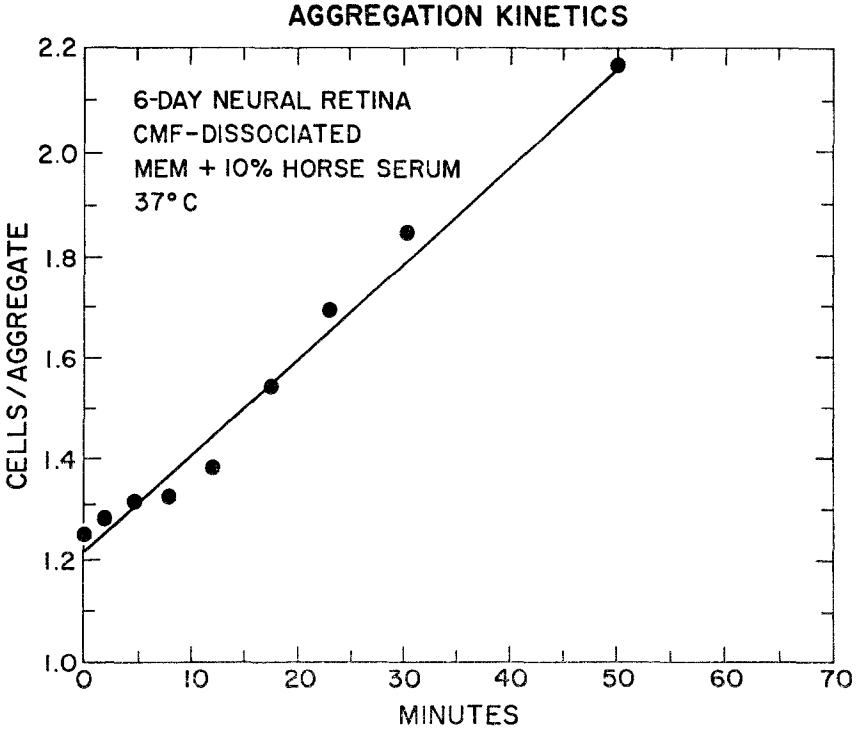

Fig. 4. Six-day neural retinal cells dissociated by withdrawal of divalent cations (in Ca^{++}, Mg^{++}-free saline) aggregate without a detectable aggregation lag (compare with Fig. 1)

ciated cells were maintained in culture, but were prevented from aggregating by the application of strong shearing forces administered at regular intervals (every 5 min). The shearing forces were produced by stirring the stock cell suspension vigorously with a vortex-type test tube stirrer. Most adhesions formed in the interval between successive stirrings were broken. The aggregative ability of these "preincubated cells" was then tested at regular intervals. Aliquots of preincubated cells were removed from the stock suspension, placed in shaker flasks and allowed to aggregate for 13 min. At the end of this period, the cells were removed from culture and the degree of aggregation in the 13-min interval was assayed using the standard procedures.

The results of such a study are presented in Fig. 5. The time when aggregation is begun with each aliquot is plotted on the abscissa and the extent of aggregation of the aliquot during the 13-min test interval is plotted on the ordinate. The latter value is a measure of the extent to which cells have recovered their ability to aggregate during the preincubation and test periods. Cell aliquots preincubated for zero minutes or 10 min and assayed at 13 and 23 min, respectively, did not aggregate. Cell aliquots preincubated for 20 to 60 min (assayed from 33 to 73 min, respectively) showed increasing but submaximal levels of aggregation during the 13-min test period. Cell populations preincubated for 70 min aggregated at the same rate as cells prewarmed for 80 or 90 min and presumably had fully recovered from the effects of trypsinization, at least insofar as their ability to initiate mutual adhesions is concerned. From these studies, and from those presented in Fig. 1, it can be concluded that these trypsinized cell populations showed the first signs of recovery of adhesiveness at about 30 min and had recovered completely by about 70 to 80 min. Trypsinized cells preincubated until recovery was complete aggregated rapidly without any sign of an aggregation lag (Fig. 6).

CMF-dissociated cells showed no detectable aggregation lag (Fig. 4). However, simple kinetic curves, as shown in Fig. 4, do not show whether freshly dissociated CMF-treated cells are maximally adhesive (i. e., are able to aggregate as rapidly as cells maintained for a period of time in culture). The same preincubation procedure applied to trypsinized cells detailed above was used to examine this question: in marked contrast to trypsin-dissociated cells, CMF-dissociated cells aggregated as rapidly at time zero as at any later time (Fig. 7). Moreover, although aggregation rates in different experiments are not as comparable as those within a single experiment, their aggregation rate was found to approximate the maximal rate attained by trypsinized cells after preincubation to maximal adhesiveness (Fig. 8).

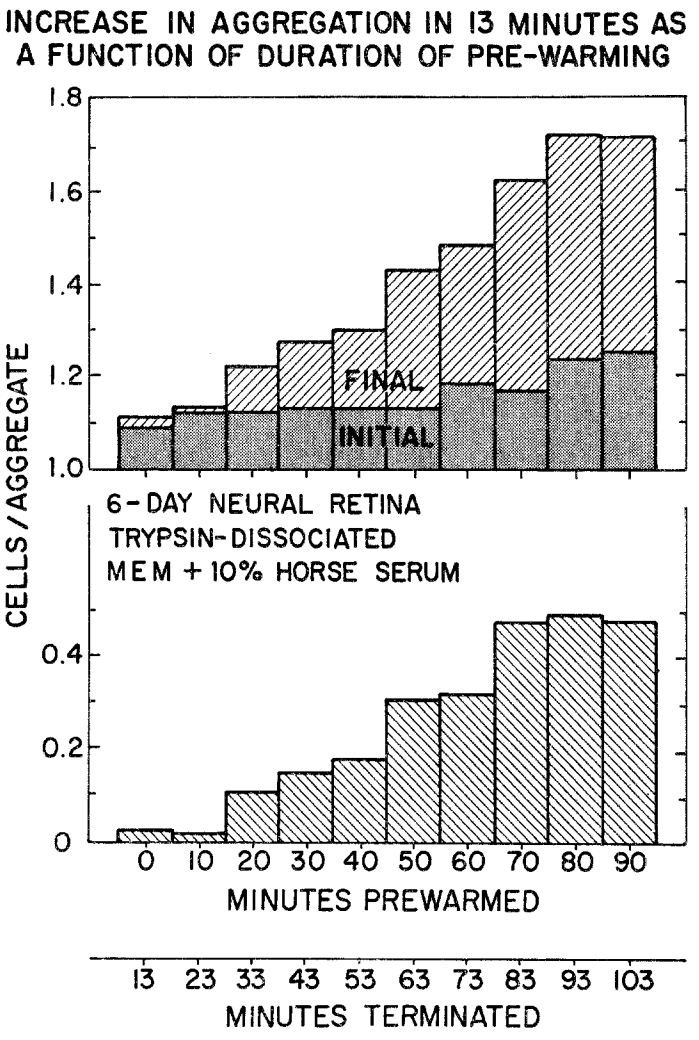

Fig. 5. Time-course of recovery of adhesiveness by trypsinized cells. A stock population of suspended, trypsin-dissociated cells at 37 °C was discouraged from aggregating by the periodic application of shearing forces to break whatever adhesions had been made. At regular intervals, aliquots of this cell suspension were removed and tested for their ability to aggregate in conventional gyratory culture for 13 min. The top graph shows degree of aggregation of each aliquot at the beginning ("INITIAL") and end ("FINAL") of the 13-min test period. Note that the prevention of aggregation in the stock cell suspension is not complete. The bottom graph shows the degree of aggregation achieved during the test period (FINAL minus INITIAL) as a function of time in culture. Cells are maximally adhesive after a 70-min prewarming period

Is the Aggregation Lag Caused by Trypsin Adsorbed onto Cell Surfaces? Since the aggregation lag has been shown to be caused by trypsin treatment, and since the lag cannot be explained as the time necessary for a conditioning

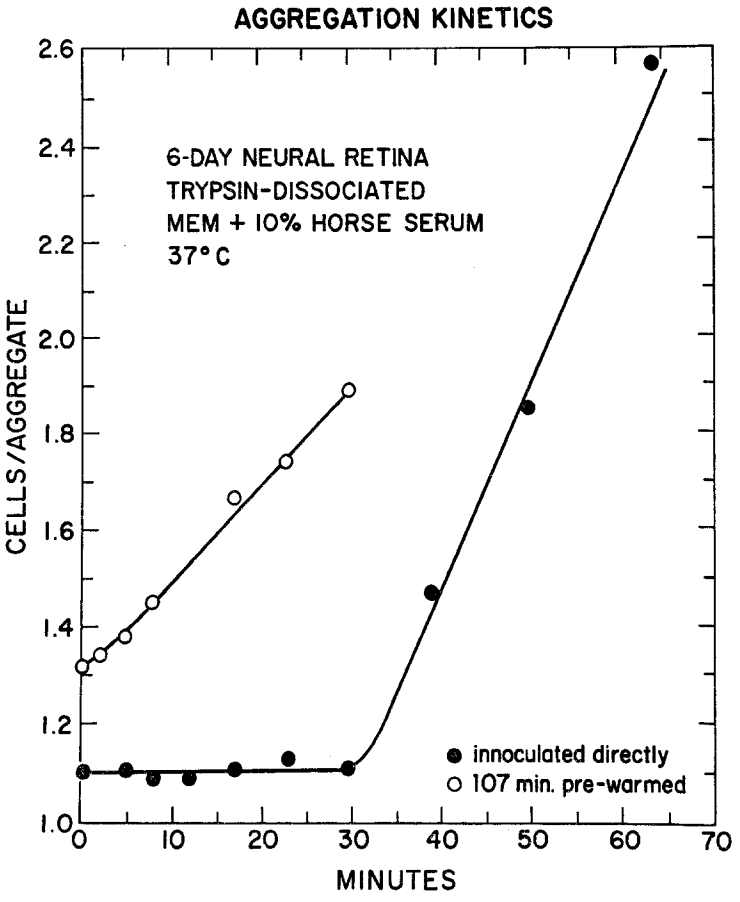

Fig. 6. Trypsinized cells preincubated for a sufficient period aggregate without an aggregation lag. A population of trypsin-dissociated cells was divided into two aliquots. One was allowed to aggregate and the other was preincubated (with periodic shearing) for 107 min before being allowed to aggregate under standard conditions. The directly cultured portion of the cell population aggregated only after the usual half-hour lag period (same data as for Fig. 1), whereas the 107-min preincubated cells aggregated without a lag

of the culture medium, then it must be supposed that the lag represents the time necessary to repair some alteration of the cell produced by protease treatment. One plausible possibility is that enzymatically active trypsin adsorbs to the surfaces of dissociated cells and, by virtue of its proteolytic activity, prevents their subsequent adhesion in tissue culture. This hypothesis would account for the aggregation lag as the time necessary for deadsorption or inactivation of this surface-adsorbed trypsin. There is evidence that cells can adsorb enzymatically active trypsin onto their surfaces. Trypsin adsorbs to a variety of substances (Kobamoto, Löfroth, Camp, Van Amburg &

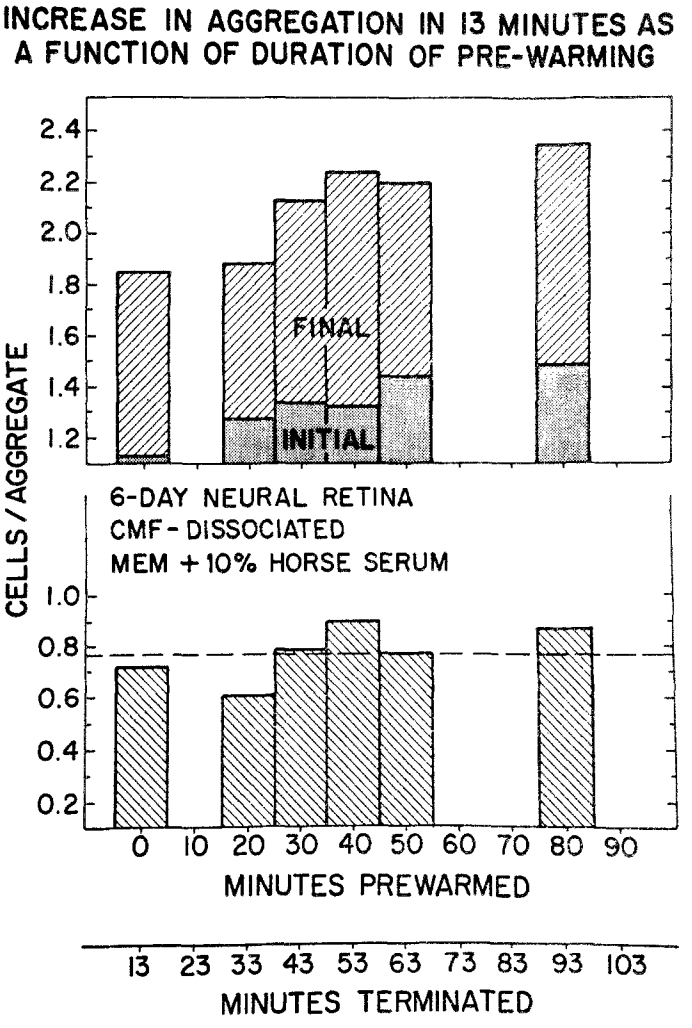

Fig. 7. CMF-dissociated cells are maximally adhesive even without preincubation. The same procedure employed in the experiment shown in Fig. 5 was applied to CMF-dissociated cells. Whereas trypsin-dissociated cells only gradually acquire the capacity to establish adhesions at the maximal rate, CMF-dissociated cells initiate adhesions at their maximal rate from the very start of aggregation

Augenstein, 1966), and cells treated with trypsin are subsequently able to digest extracellular proteins (Rosenberg, 1960; Moscona, Trowell & Willmer, 1965, p. 55; Poste, 1971) presumably as a result of surface-adsorbed trypsin. In the last case cited, this ability to digest extracellular protein was abolished by treatment with trypsin inhibitor. Barnard, Weiss and Ratcliffe (1969) have reported that a substantial reduction in the electrophoretic mobility of iso-

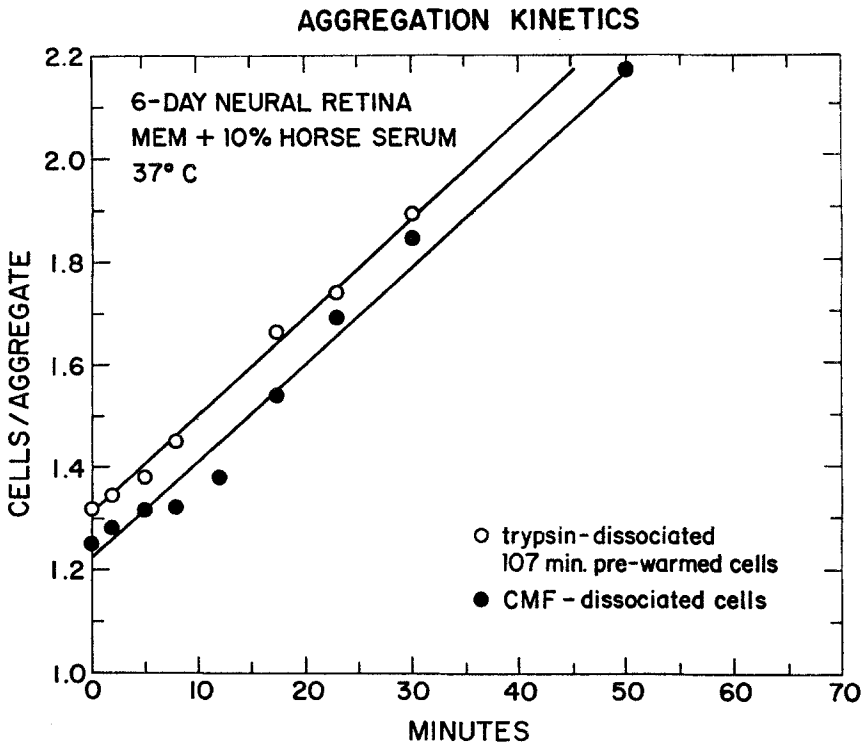

Fig. 8. The rate of aggregation of maximally adhesive preincubated trypsinized cells (taken from Fig. 6) was the same as the rate of aggregation of CMF-dissociated cells (taken from Fig. 4)

lated neural retinal cells follows treatment with diisopropylfluorophosphate-inactivated trypsin, and attribute this reduction in mobility to adsorption of trypsin to the cell surfaces. In addition, adhesion of trypsin-treated L-cells to glass does not occur unless these cells are treated with a trypsin inhibitor (Hebb & Chu, 1960). Both soybean trypsin inhibitor and serum trypsin inhibitor were effective in the latter system.

To test the role of surface-adsorbed trypsin, we examined the effect of various trypsin inhibitors on the trypsin-induced reaggregation lag (Table 2). There was no consistent difference in the aggregation kinetics of cells incubated in MEM alone, MEM + horse serum or MEM + soybean trypsin inhibitor. Both of the latter solutions are strongly inhibitory to the proteolytic activity of trypsin (*see* Table 3). There was no detectable protease activity in MEM + HS even at 4000 μg/ml added trypsin. In the presence of 0.5 mg/ml soybean trypsin inhibitor, detectable proteolytic activity was lacking at 10 μg/ml trypsin. With MEM or Sorensen's phosphate buffer, protease activity was

Table 2. Reaggregation of trypsin-dissociated 6-day chick embryo neural retinal cells: Effect of trypsin inhibitors on the aggregation lag

Culture medium	Increase in average number of cells per aggregate				
	20 min[a]	40 min	60 min	80 min	100 min
Experiment #1: Average number of cells per aggregate at zero time = 1.047					
MEM[c]		0.052[b]	0.102	0.150	0.519
MEM + 10% HS		0.069	0.116	0.222	0.423
MEM + STI	0.009	0.026	0.121	0.272	
Experiment #2: Average number of cells per aggregate at zero time = 1.063					
MEM		0.060	0.147	0.367	0.276
MEM + 10% HS		0.014	0.103	0.154	0.209
MEM + STI	−0.030	0.018	0.069	0.170	0.338
Experiment #3: Average number of cells per aggregate at zero time = 1.052					
MEM		0.028	0.043	0.223	0.342
MEM + 10% HS		0.005	0.072	0.129	0.276
MEM + STI	−0.009	0.018	0.040	0.174	0.488

[a] The time in culture at 37 °C. Cell suspensions (2×10^6 cells/ml; 3 ml of medium per flask) were stirred on a gyratory shaker at 70 rpm.

[b] Degree of aggregation is expressed as increase in the average number of cells per aggregate over that observed at the start of the experiment.

[c] MEM = Eagle's minimal essential medium. HS = horse serum. STI = soybean trypsin inhibitor. Inhibitor concentration in Experiments #1 and #2 is 0.5 mg/ml and in experiment #3 is 1 mg/ml.

detectable by the assay system even at dilutions of 0.04 to 0.01 μg/ml trypsin. The protease assay system used revealed at least a 10^4-fold difference in trypsin inhibiting ability of MEM + HS as compared with MEM, yet the cellular aggregation kinetics were the same in these two tissue culture media. We conclude that the lag does not represent time necessary for the inactivation of cell surface bound trypsin.

Is the Aggregation Lag a Result of Altered Pseudopodial Activity? Aggregation of dissociated cells may depend on an ability to make pseudopodial projections (Pethica, 1961; Lesseps, 1963). Thus, the trypsin-induced aggregation lag may be the result of a temporary (i. e., half-hour) loss in the ability of cells to protrude pseudopods. Time-lapse movies were made of trypsin-dissociated 6-day neural retinal cells as a means to test this hypothesis. Trypsinized cells suspended in MEM + 10% HS were placed on detergent- and aicohol-cleaned microscope slides. Coverslips were sealed on with Vaseline and the assembly was placed at 37° C in a Wild Hot-and-Cold Microscope Stage fitted to a Wild M 20 microscope. Filming was initiated immediately

Table 3. Relative trypsin-inhibitor activities of various culture media[a]

Protease[b] (in μg/ml)	Amount of protease activity[c] in:			
	Sorensen's phosphate buffer, pH 7	MEM	MEM + STI[d]	MEM + 10% HS
4,000	4[e]			
400	3	$2^1/_2$	2	–
100	3	3	2	–
40	2	2	1	–
10	2	2	0	
4	2	$1^1/_2$	–	–
1	$1^1/_2$	2	–	
0.4	1	0	–	–
0.1	0	0	–	
0.04	0	–	–	–
0.01	–	0	–	
0.001	–	–	–	

[a] *See* footnote c of Table 2.

[b] Protease was 3% Difco 1:250 Trypsin + 1% 1:75 Pangestrin dissolved in CMF Hanks + 2 mM EDTA and diluted first 1:10 in Sorensen's buffer, and then in the various media to give the indicated concentration.

[c] Conditions of incubation: (1) Photographic film was soaked overnight in Sorensen's buffer and blotted dry; (2) 10 λ of solution was used for each assay; (3) Incubation was in a humid chamber at 37 °C for 12 hr. At the end of incubation, the film was washed in water with rubbing, dried, and the results were tabulated.

[d] STI is soybean inhibitor (cryst.) at 0.5 mg/ml.

[e] Intensity of reaction of protease with film emulsion: 4: Very strong protease activity; emulsion digested through to cellulose backing; area of emulsion digested greatly exceeded the area of the initial drop. 3: Strong activity; area of emulsion digestion somewhat greater than area of initial drop. 2: Moderate activity; area of emulsion digested about that of initial drop. 1: Weak activity; emulsion was digested to cellulose backing in only small portions of the area covered by the initial drop. 0: Very weak activity; surface of emulsion merely etched. – indicates no reaction.

and continued for 2 hr at 6 or 10 frames/min, with magnifications of 24 or 49 ×. Films were analyzed with an L/W stop-action projector. Each cell in the field was assigned a number, and its behavior was analyzed throughout the period of culture. Neural retinal cells stuck to the glass very weakly or not at all during the observation period, so it is believed that their behavior in the present situation is similar or identical to their behavior in shaker-flask culture. Sixty cells were studied. Behavior was as follows:

19 cells showed moderate to vigorous pseudopodial activity at all times. Pseudopods were large blunt lobopods; 35 cells were relatively quiescent at all times. These cells appeared to be alive and well but formed very fine short pseudopods rather than large ones; 1 cell was quiescent early and then devel-

oped pseudopodial activity later; 1 cell showed active pseudopods initially and later became quiescent; 1 cell showed pseudopodial activity early, and then became bipolar, apparently due to adhesion to the substrate via filopods at each end of the cell; 3 cells were quiescent early and became bipolar later.

These observations do not support the hypothesis that recovery of adhesiveness depends on a recovery of pseudopodial activity. A majority of cells (54/60) showed no change in surface activity over the period of culture.

It is likely that trypsinization alters the composition of the cell surface, either by inactivation or removal of materials necessary for adhesion or by the induction of a rearrangement of surface components. Cells so altered might well be nonadhesive during the period necessary for replacement of lost or inactivated surface components or for re-ordering of the disarranged components. This hypothesis is attractive since it raises the possibility that further study of the aggregation lag might allow one to correlate changes in the composition or structure of the cell surface with the reacquisition of adhesiveness. The lag could thus be used as a tool for exploration of the nature of intercellular adhesion itself. In what follows, we report some initial attempts to understand the nature of possible cell surface changes occurring during the lag period.

Is the Aggregation Lag Due to an Altered Cell Surface Charge? Electrophoresis is a means for studying the composition of surfaces, and in particular, surface net electrostatic charge (Abramson, Moyer & Gorin, 1942; Seaman & Heard, 1960; Haydon, 1961; Glaeser, 1963). We hoped by this means to correlate possible changes in the composition of the cell surface with the observed adhesive changes, thus gaining information bearing upon the chemistry of cellular adhesion.

To examine the possibility that the cell surface during the lag period is characterized by an altered net surface charge, the electrokinetic mobilities of freshly dissociated, trypsinized cells were compared to those of cells which had been incubated in culture medium ("preincubated") for a period of time sufficient to allow cellular adhesiveness to be re-established (using the same procedure for "preincubating" cells as was described previously). Only two of five trials showed a statistically significant difference, by Student's t test, between mean values of mobility of unincubated cells and of cells incubated for 90 to 130 min (Table 4). Thus, there was no consistent difference between the electrokinetic mobility of the nonadhesive, unincubated cells and that of the adhesive, incubated cells.

We recognized the possibility that labile surface changes occurring during the course of incubation could be reversed or obscured by the 0.145 M NaCl suspending medium used in the above electrophoresis experiments. Conse-

Table 4. Electrophoretic mobility[a] of trypsin-dissociated chick embryo neural retinal cells immediately following trypsin treatment and after varying periods of incubation at 37 °C in minimum essential medium, Eagle, containing 10% horse serum

Suspension medium	Anodal mobility immediately following trypsinization (μ/sec/V/cm)	Mobility difference between incubated and unincubated trypsinized cells (incubated minus unincubated)		Statistical significance of 90 to 130 min difference
		40 to 50 min of incubation	90 to 130 min of incubation	
0.145 M NaCl	0.919	−0.030	−0.006	$p>0.2$
	0.878	−0.021	+0.017	$p>0.2$
	0.841	−0.002	+0.145	$p<0.01$
	0.902	+0.018	+0.040	$p>0.2$
	0.861	+0.087	+0.094	$p<0.02$
MEM+HS	0.913	—	+0.041	$p<0.1$

[a] Conditions for electrophoresis: 25 °C, pH 7.3.

quently, MEM+HS was employed as the suspending medium for electrophoresis in one trial (Table 4). The change in electrophoretic mobility upon incubation in this case was on the borderline of statistical significance and was small at best. On the other hand, the mobility difference between EDTA-dissociated and trypsin-dissociated (nonincubated) neural retinal cells is rather considerable. In our hands, the former had a mobility (towards the anode) of 1.36 ± 0.03 (SE)[2] μ/sec/volt/cm and the latter a mobility of 1.03 ± 0.03 (SE). This is an easily detectable difference and is much greater than any observed in the comparison between incubated and nonincubated trypsin-dissociated cells. In summary, the recovery of adhesiveness by trypsinized cells is not accompanied by marked changes in net surface charge; moreover, EDTA-dissociated cells and prewarmed, trypsin-dissociated cells differ markedly in net surface charge but initiate adhesions at identical rates.

Reduction of the Aggregation Lag by Modification of the Trypsinization Procedure. In a second attempt to investigate the possibility that the restoration of adhesiveness occurs by resynthesis or rearrangement of lost or disordered surface components, the effects of alterations in the trypsinization procedure were studied. In these experiments, a semi-quantitative visual assay for degree of aggregation was substituted for the more quantitative Millipore filter assay technique. Rather than enumerating the number of cells per aggregate, aggregation was followed by measuring the sizes of aggregates

2 Our value of −1.36 μ/sec/V/cm for EDTA-dissociated cells is smaller than the value of −1.70 reported by Barnard *et al.* (1969). The reasons for this discrepancy are not clear.

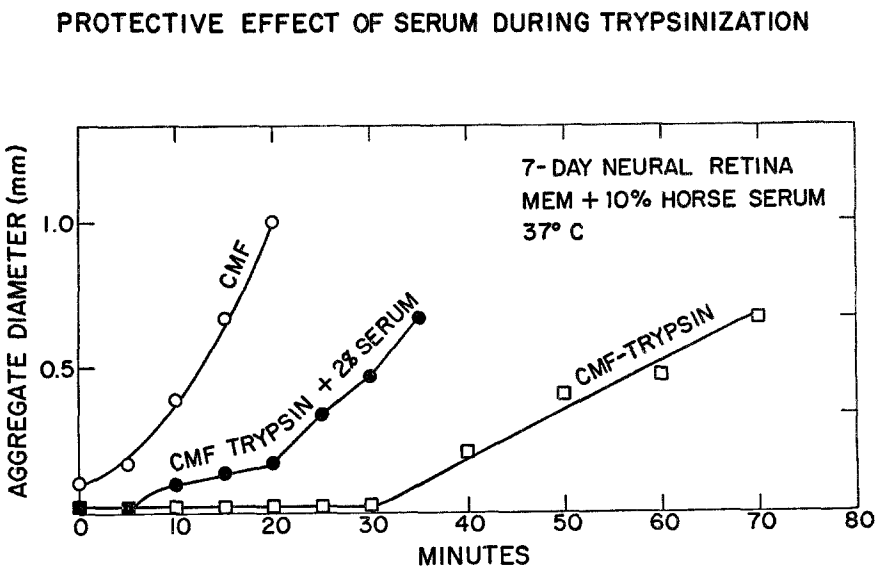

Fig. 9. Chicken serum present during trypsinization partially protects cells from the trypsin-induced aggregation lag. The aggregation kinetics of CMF-dissociated retinal cells (no lag) are compared with the aggregation kinetics of cells dissociated in 0.25% trypsin (half-hour lag) or in 0.25% trypsin + 2% chicken serum. In the last case, the aggregation lag was considerably shortened. A period of no aggregation (between 0 and 5 min) was followed first by a period of slow aggregation (between 5 and 20 min) and then by a period of more rapid aggregation. In the experiments illustrated here and in Fig. 9, the extent of aggregation was recorded as the diameter of the largest prominent class of aggregates present in the population, rather than as the average number of cells per aggregate

using an ocular micrometer. This method was sufficiently accurate for the purpose of these experiments. For example, using this technique, the half-hour aggregation lag of trypsinized cells and the immediate onset of aggregation of CMF-dissociated cells could both be readily demonstrated. The results of these experiments show that the addition of certain *proteins* (*see* Coon, 1966; Roth & Weston, 1967; Roth, 1968; Orr & Roseman, 1969) or *calcium ion* to the trypsin-containing dissociation medium interferes little with the ability of trypsin to dissociate neural retinal tissue but does result in the abolition or reduction in length of the reaggregation lag once the dissociated cells are placed in culture.

The effect of chicken serum added to the trypsin solution used to effect dissociation is presented in Fig. 9. The aggregation lag has been shortened but not altogether abolished in treated cells. It was found that to exert any protective effect, chicken serum must be present during the period of exposure to trypsin; pretreatment or post-treatment of trypsinized cells with serum is ineffective in reducing the lag. Serum at a concentration as low as (but not much lower than) 0.5% reduced the length of the lag induced by

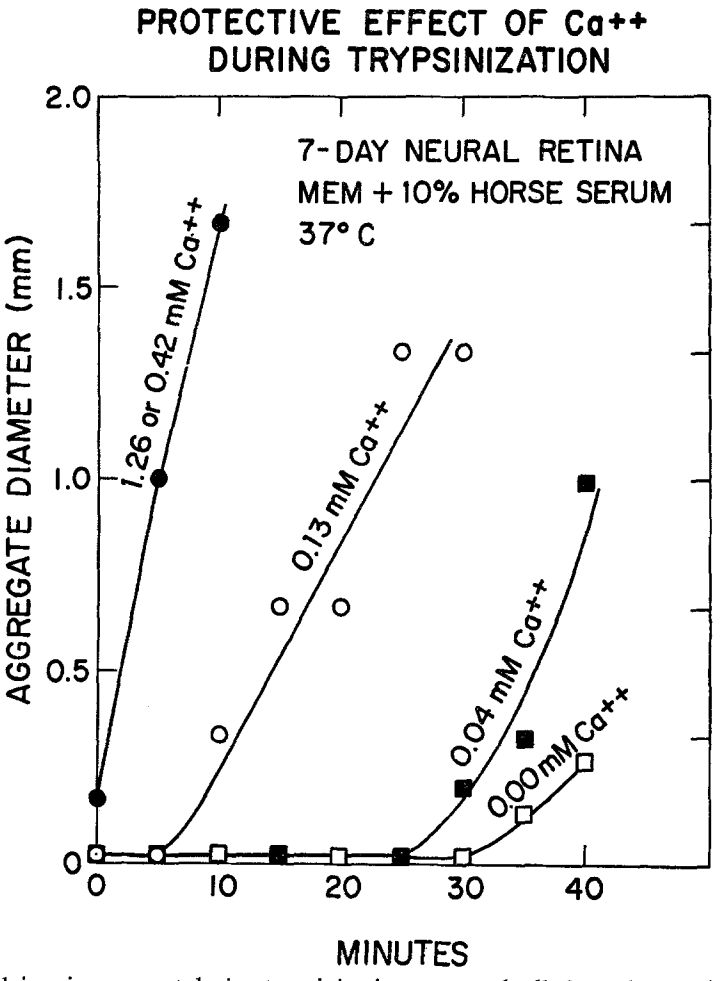

Fig. 10 Calcium ion present during trypsinization protected cells from the trypsin-induced aggregation lag. Each curve represents the aggregation kinetics of a retinal cell population dissociated with trypsin within the presence of a given concentration of Ca^{++}. At 1.26 or 0.42 mM Ca^{++} (•), the aggregation lag is not present. In the presence of lower concentrations of Ca^{++} [0.13 mM (○) or 0.042 mM (■)], the aggregation lag is shortened. In the absence of Ca^{++}, the usual half-hour aggregation lag was evident. At 0.04 or 0.13 mM Ca^{++}, the lag was shortened. At 0.42 or 1.26 mM Ca^{++}, the lag was abolished

0.1% 1:250 trypsin in CMF saline. Horse serum, as well as various proteins added to the trypsin solution, had an effect similar to that of chicken serum. Protection has been demonstrated also with fibrinogen, serum albumin and casein. It was found that chicken serum and fibrinogen, at least, can protect cells dissociated in chymotrypsin or pronase as well as in trypsin.

Another agent which can protect cells against a trypsin-induced lag is Ca^{++} (Fig. 10). When present during trypsinization (0.1% 1:250 trypsin), Ca^{++} at 1.3 mM or 0.42 mM totally prevented the aggregation lag. At 0.13 mM,

partial protection was afforded. Mg^{++} did not substitute for Ca^{++}. It appears that the protection afforded by proteins is not due solely to Ca^{++}-contaminants, since fibrogen dialyzed against an EDTA-containing solution was almost as effective as the undialyzed protein.

Temperature Dependence of Adhesiveness and of its Recovery by Trypsinized Cells

In the prewarming experiments cited before, the culture temperature was always 37 °C. It has previously been demonstrated that cell aggregation is inhibited at reduced temperatures (Moscona, 1961*b*; Steinberg, 1962; Moscona & Moscona, 1966; Jones & Morrison, 1969). It was concluded earlier that the effect of warmth in promoting aggregation "... persists only as long as the warm temperature is maintained" (Steinberg, 1962). The sensitive techniques of the present study permit this conclusion to be examined more closely than has heretofore been possible (Fig. 11). Trypsinized cells, pre-

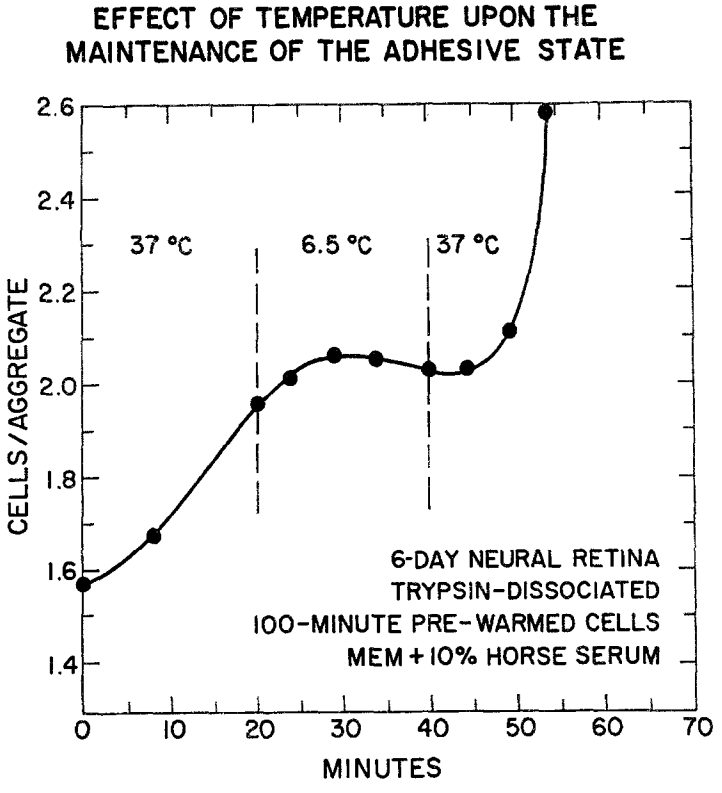

Fig. 11. The effect of temperature on cell aggregation. When a maximally adhesive cell population (trypsinized cells preincubated 100 min) was cooled to 6.5 °C during the course of aggregation, aggregation ceased within a few minutes. The cessation of aggregation was reversible after rewarming to 37 °C, with a 5 to 10 min delay

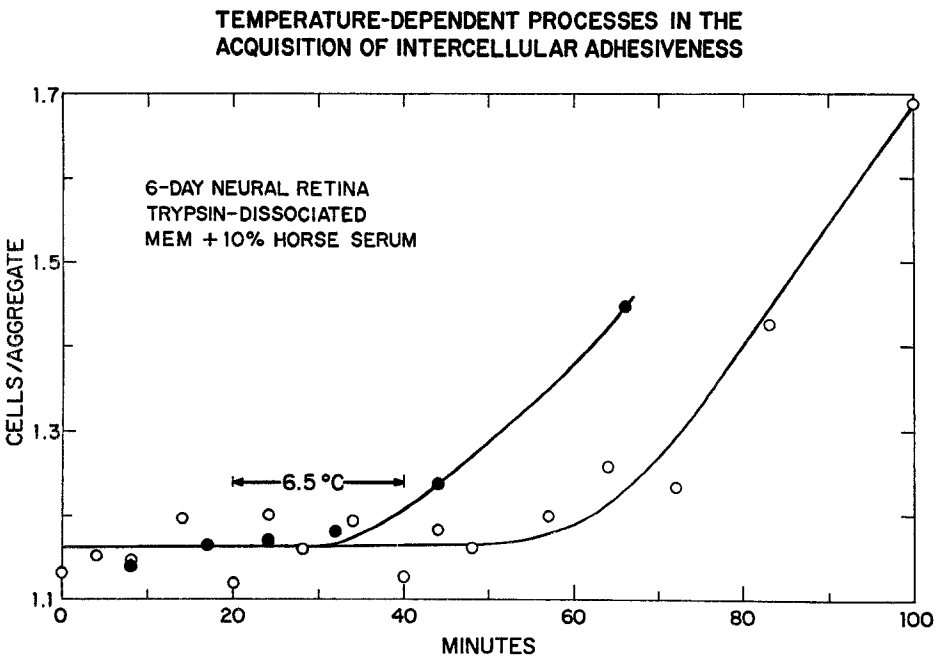

Fig. 12. Low temperature treatment administered during the lag period postponed the reacquisition of adhesiveness in trypsinized cells. A population of trypsinized cells was divided into two aliquots. One was maintained at 37 °C throughout (•) while the other was maintained at 37 °C for 20 min, then cooled to 6.5 °C for the next 20 min, and finally rewarmed to 37 °C (○). The 20-min cold pulse during the lag period prolonged the lag by 20 min

incubated with periodic dispersal for 100 min to ensure maximal adhesiveness, were allowed to aggregate for 20 min at 37 °C and were then rapidly cooled to 6.5 °C in a refrigerated water bath shaker.[3] Aggregation ceased within a few minutes. The cessation of aggregation was reversible: when the flask was quickly rewarmed at 40 min to 37 °C, aggregation again became noticeable after a lag of a few minutes.[4] This result shows that the aggregation of adhesive cells can be turned on and off by manipulation of the temperature, with a delay in response of a few minutes. Reduced temperature inhibits not only the ability of already adhesive cells to aggregate, but also, as shown in the next experiment, the recovery of adhesive properties of cells rendered temporarily nonadhesive through trypsinization. In this experiment (Fig. 12), the population of trypsinized cells was divided into two subpopula-

3 Using a sensitive thermistor inserted into the shaker flask, we have determined that cooling or rewarming takes about 2 min.

4 This lag was observed every time chilled cell suspensions were rewarmed.

tions. The control subpopulation (solid circles) was allowed to aggregate under standard conditions and displayed the usual half-hour aggregation lag. The experimental subpopulation (open circles) was maintained at 37 °C for 20 min and then, while still in the lag phase, was rapidly cooled to 6.5 °C for another 20 min before being warmed again to 37 °C. The 20-min cold-pulse administered in the middle of the lag phase postponed the initiation of aggregation by just 20 min.

Discussion

The Trypsin-Induced Aggregation Lag

In the present study, a number of characteristics of aggregating cell populations have been established. Although dissociated cells prepared from embryonic chick neural retina by withdrawal of divalent cations are able to aggregate as soon as they are placed in the appropriate conditions, cells treated with trypsin in the absence of divalent cations exhibit a half-hour aggregation lag which precedes the period of aggregation. If Ca^{++} or protein (chicken or horse serum, serum albumin, casein, fibrinogen) are present during trypsinization, dissociation is not markedly impaired but the dissociated cells are partially or even completely protected from the trypsin-induced lag. Although trypsin-dissociated retinal cell populations show the first sign of aggregation in about a half hour, maximal rates of aggregation are not achieved by these populations until approximately 70 to 80 min of incubation. Since the duration of the lag period is not affected by inclusion of potent trypsin inhibitors in the culture medium, it appears that the lag is not due to the adsorption of active enzyme onto cell surfaces. Electrophoretic studies of trypsinized cells were conducted in an attempt to gain information regarding changes in cell surface composition (i. e., changes in the density of ionogenic groups on cell surfaces). Reproducible differences in net negative surface charge density were not detected between freshly trypsinized, non-adhesive cells and trypsinized cells that had been maintained in culture for a sufficient time to allow full recovery of adhesiveness; but these latter cells exhibited a distinctly lower net negative surface charge density than EDTA-dissociated cells that aggregate at an identical rate. Clearly, the changes in cell surface composition that these electrophoretic measurements reflect are not in the constituents directly responsible for initiating intercellular adhesions. Barnard *et al.* (1969) report a marked increase in electrophoretic mobility (from -1.09 to -2.06 μ/sec/V/cm) that occurs over a period of 24 hr when trypsin-dissociated chick embryo neural retinal cells are placed in culture. However, the increase they observed over the first 2 hr in culture (i. e., the period of time of interest in the present study) was only 0.1 unit.

Using the sensitive assay system devised to study the kinetics of cell aggregation, we have demonstrated that low temperature very rapidly (2 to 3 min) and reversibly inhibits aggregation of *fully adhesive* cell populations. The rapidity of onset of this effect militates against the idea proposed by Moscona (Moscona, 1961*b*; Moscona & Moscona, 1966) that the low-temperature inhibition of aggregation acts by suppressing the synthesis of cell-surface components necessary for adhesion. We propose that the initiation of adhesions between cells requires a particular configuration of surface molecules, and that low temperature alters this configuration.

In addition to suppressing aggregation of already-adhesive cell populations, a pulse of low temperature treatment during the lag-period extends the lag-period by an equal length of time. We conclude that the lag-period is a time during which a temperature-sensitive process necessary for the re-establishment of adhesiveness is carried out, and that low temperature arrests this process but does not reverse it.

Comparison with Other Studies

Cell Aggregation Studies. Studies of cell aggregation in stirred cell suspensions have employed a variety of procedures to quantify rates and extents of aggregation under various conditions. Techniques used include the determination of final sizes of aggregates (Moscona, 1961*b*; Humphreys, 1963; Kuroda, 1963; Glaeser, Richmond & Todd, 1968; Lilien, 1968; Gershman, 1970); the determination of the rate of disappearance of single cells from cell suspensions (utilizing direct enumeration: Curtis & Greaves, 1965; Ede & Agerbak, 1968; Dunn, Owen & Kemp, 1970; or Coulter-counter determination: Orr & Roseman, 1969); direct enumeration of number of particles in suspension (Curtis, 1970); determination of particle size using turbidometric methods (Knight, Jones & Jones, 1966; Kemp, Jones, Cunningham & James, 1967; Kemp, 1970); determination of the rate of adhesion of dissociated cells to "collecting aggregates" (Roth & Weston, 1967; Roth, 1968; Roth, McGuire & Roseman, 1971); and determination of the mean number of cells per aggregate by direct counting (Armstrong, 1966; Steinberg & Granger, 1966; the present study). Of these, the last-mentioned gives the most direct, complete and readily interpretable data for study of the early stages of aggregation, because only this method presents and utilizes all of the data on the distribution of cells in the various aggregate size classes (*see* Table 1). This procedure, however, is very tedious in practice and is most accurate in the early stages of aggregation. Its decreasing accuracy in later stages of aggregation is due to the manner in which aggregation proceeds in stirred suspension:

since large aggregates have a larger collision-radius than small aggregates, they increase in size very rapidly (Steinberg & Roth, 1964). In practice, once the average number of cells per aggregate exceeds about 2.5, the particle distribution is one with a large number of single cells, a few small aggregates, and a small number of very large aggregates (*see* Table 1). Under these conditions, the determination of the degree of aggregation by the direct counting procedures is markedly affected by the number of these rare, large aggregates that happen to be included in the sample that is counted. [5]

Published figures of Jones, Kemp and their co-workers, depicting the kinetics of early aggregation of trypsinized cells, reveal what appears to be an aggregation lag (Jones, 1966; Kemp, 1969; Jones & Kemp, 1970; Jones, Kemp & Groschel-Stewart, 1970). These authors, however, interpret this observation in a different way. Orr and Roseman (1969) observed immediate aggregation after trypsinization. In this case, however, 2% chicken serum was present in the trypsin solution. In our experiments, inclusion of 2% chicken serum reduced the lag from a half hour to 5 min. Where calcium removal was employed to effect dissociation, an aggregation lag was absent under most (Giudice, 1965; Armstrong, 1966; Kleinschuster & Moscona, 1972) but not all (Curtis & Greaves, 1965) conditions. Amphibian embryo cells aggregating in stationary culture showed no lag when citrate was used to dissociate tissues but showed an infinite lag (i. e., cells never aggregate) following trypsin treatment (Townes, 1953; Feldman, 1955).

Treatment of cells with trypsin or other proteases affects not only their ability to adhere to each other in culture, but also their ability to adhere to solid substrates. It has been reported (Weiss & Kapes, 1966) that RPMI#41 cells (an established cell line derived from a human osteogenic sarcoma) do not make firm adhesions to glass for up to 4 to 6 hr following treatment with crude trypsin (Difco 1:250) or alpha-chymotrypsin. Crystalline trypsin or elastase pretreatment decreased the strength of adhesion to glass for shorter periods (1/2 to 1 hr). Neuraminidase had no effect. Hebb and Chu (1960) and Fischer, Puck & Sato (1958) report that trypsinized cells are unable to attach to glass unless trypsin inhibitors are included in the culture medium.

5 Accurate data for the later stages of aggregation could, in principle, be obtained by a variant of the membrane filter method. If equal volumes of an aggregating cell suspension are deposited on filters of equal area, the number of cells per unit area of the filter will remain constant as the number of cells per aggregate increase. Therefore, number of cells per aggregate will be directly proportional to area per aggregate, a parameter that can be accurately measured during the late stages of aggregation, and should be unaffected by the size distribution of the particular aggregates that happen to fall within the area chosen for measurement. Due to inequalities in cell settling, sampling and cell distribution on the filters, we were never able to perfect this method.

However, Kolodny (1972) has reported that trypsinized 3T3 cells adhere to plastic dishes as rapidly in the absence of serum as in its presence.

Effects of Proteolytic Enzymes on Living Cells. The cause of the aggregation lag is still uncertain. Our data indicate that the lag does not represent the time necessary for dissociated cells to condition the culture medium before aggregation can begin (as has been reported, for example, by Curtis and Greaves, 1965). Rather, the lag seems to represent the time necessary to repair changes in dissociated cells caused by their exposure to proteases. The nature of the changes induced by trypsin and the nature of the cellular repair processes are both questions of considerable interest. Knowledge of these processes might provide insight into the basic process of cellular adhesion.

Since cells contact one another at their surfaces during the establishment and maintenance of intercellular adhesions, it is generally assumed that the composition and activities of cell surfaces are of the most direct bearing upon the mechanisms of intercellular adhesion. Consequently, attempts to elucidate the biochemistry of intercellular adhesion through a study of the aggregation lag would hold the greatest promise if it could be demonstrated that the changes responsible for the initial inability to aggregate were caused by an effect of trypsin directly upon the cell surface. It is generally assumed that trypsin is prevented by the plasma membrane from entering the cytoplasm of intact cells (Northrop, 1926). Certainly trypsin and other proteases are capable of altering the chemical composition of cell surfaces. Effects include removal of plasma membrane sialomucopeptides (Cook, Heard & Seaman, 1960; Mäkelä, Miettinen & Pesola, 1960; Langley & Ambrose, 1964; Shea & Ginsburg, 1968; Kemp, 1969; Codington, Sanford & Jeanlog, 1970; Price, 1970; Forstner, 1971), alteration of the distribution of receptor sites for lectins (Burger, 1969; Inbar & Sachs, 1969; Sela, Lis, Sharon & Sachs, 1970; Moscona, 1971; Kleinschuster & Moscona, 1972; Nicolson, 1972; Nicolson & Blaustein, 1972) and anti-glycolipid antibodies (Hakomori, Teather & Andrews, 1968), and a reduction in electrophoretic mobility (Ponder, 1951; Engel, Pumper & Joseph, 1968; Kemp, 1969; Barnard *et al.*, 1969; the present study). The last effect is due, at least in erythrocytes, to a removal of cell surface sialic acid (Cook *et al.*, 1960). Treatment with EDTA causes a release of cell surface materials (Edidin, 1966; Kemp *et al.*, 1967; Neville, 1968; Allen & Snow, 1970), but no change in electrophoretic mobility (Häyry, Penttinen & Saxén, 1965; Brent & Forrester, 1967; Barnard *et al.*, 1969) and does not detectably alter the distribution of lectin-binding sites of nontransformed cells (Inbar & Sachs, 1969).

In parallel with observed changes in cell-surface composition, trypsin-treated cells show altered behavioral characteristics. Included in these are reduced adhesion of cells to solid substrates (Easty, Easty & Ambrose, 1960; Weiss, 1963) and to each other (Moscona, 1952; 1963; Steinberg & Granger, 1966; Steinberg, 1967; Wiseman, Steinberg & Phillips, 1972); more rapid emigration of cells from explants (Simms & Stillman, 1937; Lefford, 1965); reduced binding of cytophilic antibodies (Kossard & Nelson, 1968), viruses (Zajac & Crowell, 1965), and sperm (Aketa, Onitake & Tsuzuki, 1972); altered carbohydrate uptake and metabolism (Kuo, Holmlund & Dill, 1966; Phillips, 1967); a release from postconfluence inhibition (Martz & Steinberg, 1972) of growth *in vitro* (Burger, 1970; Sefton & Rubin, 1970; Poste, 1971); increased deformability of the cell surface (Weiss, 1966); and animalization (Moore, 1952) and activation (Jensen, 1948) of unfertilized sea urchin eggs.

Significance and Expectations

Our initial attempt to look for surface changes accompanying reacquisition of adhesiveness is represented in the electrophoretic studies, used to measure densities of ionogenic groups at cell surfaces. Since ionogenic groups may be important in intercellular adhesiveness (Steinberg, 1958; Weiss, 1960; Pethica, 1961; Curtis, 1962; Armstrong, 1966; Collins, 1966; Kiremidjian & Kopac, 1972), we hoped to detect changes in cellular surface charge during the recovery of adhesiveness following trypsinization. However, meaningful correlations between electrophoretic mobility and cellular adhesiveness were not found.

A problem with electrophoretic measurements is that they do not reveal the *distribution* of ionogenic groups over the surface of cells (Weiss, 1963). It has been demonstrated recently that certain cell-surface components may occur in clusters (Nicolson, Hyman & Singer, 1971; Nicolson & Singer, 1971; Smith & Revel, 1972). Adhesion of the cells used in the present study may occur only between specialized surface patches displaying high densities of the surface components responsible for cell adhesion. Fine-structural studies of cell aggregates show that cells are closely approximated over only limited areas of their surfaces (Armstrong, 1970, 1971). If adhesion does require a particular arrangement of surface components in the plane of the cell surface. then the aggregation lag-period may be not a period for replacement of materials lost from cell surfaces during trypsinization but rather a period for rearrangement of materials in the plane of the membrane. Trypsinization may redistribute the molecules required for adhesion, and the lag may

represent the time required for their return to a normal arrangement. Similarly, other conditions that promote reversible loss of adhesiveness [i. e., low temperature (the present study) and metabolic inhibitors (Kemp *et al.*, 1967)] may act by causing a reversible redistribution of surface components incompatible with the establishment of new adhesive junctions. The rapidity of onset and reversal of the loss of adhesion under conditions of low-temperature treatment reported in the present study argues in favor of a redistribution process and against a mechanism involving a loss and resynthesis of surface components necessary for adhesion, such as has been proposed by Moscona (1961*b*).

Direct evidence consistent with the hypothesis that low temperature and protease treatment cause a reversible loss of adhesiveness via an effect on the distribution of components in the plane of the cell's surface is provided by electron-microscopic studies of the distribution of components in the plasma membrane and in the cell surface. Protease treatment alters the distribution of surface lectin receptors (Nicolson, 1972) and intramembranous particles revealed by the freeze-etch technique (Engstrom, 1970, cited in Branton & Deamer, 1972). Following protease treatment, lectin receptor sites are clustered rather than dispersed. Intramembranous particles are initially caused to cluster, but with prolonged treatment they subsequently disperse and are eventually lost. The intramembranous particles revealed by the freeze-etch technique protrude onto the cell surface as receptor sites for influenza virus, wheat germ agglutinin and A- and B-blood group antibodies (Pinto da Silva, Douglas & Branton, 1971*a*, *b*; Tillack, Scott & Marchesi, 1971). Similarly, alteration of the distribution of antibody and lectin receptor sites in the plane of the cell surface is produced by low temperature treatment (Smith & Revel, 1972).

The cell surface is a fluid entity capable of rapid rearrangement (Frye & Edidin, 1970; Pinto da Silva, 1972; Singer & Nicolson, 1972). If the reacquisition of adhesiveness depends upon a rearrangement of ionized groups, then this would not be detectable by cell electrophoresis. If the temporary non-adhesiveness induced by trypsin is the result of disarrangement of components within the cell surface, then the protecting activity of calcium or proteins introduced into the trypsin preparations used for dissociation might well depend upon their ability to stabilize the surface against disarrangement. In the present context, it would be of considerable interest to attempt to correlate conditions which affect cellular adhesiveness [divalent cation removal (Armstrong, 1966); trypsinization and low temperature, as detailed in the present paper] with their effects upon the fine-structural arrangement of cell surface components. It would also be of interest to study the effect on cellular adhe-

siveness of external conditions known to alter the arrangement of cell surface components (i. e., low pH; Pinto da Silva, 1972).

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References

Abramson, H. A., Moyer, L. S., Gorin, M. H. 1942. Electrophoresis of Proteins. pp. 13–37. Reinhold, New York

Aketa, K., Onitake, K., Tsuzuki, H. 1972. Tryptic disruption of sperm-binding site of sea urchin egg surface. *Exp. Cell Res.* **71**:27

Allen, A., Snow, C. 1970. The effect of trypsin and ethylenediamene-tetraacetate on the surface of cells in tissue culture. *Biochem. J.* **117**:32 *P*

Armstrong, P. B. 1966. On the role of metal cations in cellular adhesion: Effect on cell surface charge. *J. Exp. Zool.* **163**:99

Armstrong, P. B. 1970. A fine structural study of adhesive cell junctions in heterotypic cell aggregates. *J. Cell Biol.* **47**:197

Armstrong, P. B. 1971. Light and electron microscope studies of cell sorting in combinations of chick embryo neural retina and retinal pigment epithelium. *Wilhelm Roux' Archiv* **168**:125

Bangham, A. D., Flemans, R., Heard, D. H., Seaman, G. V. F. 1958. An apparatus for microelectrophoresis of small particles. *Nature* **182**:642

Barnard, P. J., Weiss, L., Ratcliff, T. 1969. Changes in the surface properties of embryonic chick neural retinal cells after dissociation. *Exp. Cell Res.* **54**:293

Boeryd, B., Lundin, P. M., Norrby, K., Schelin, U. 1967. Ultrastructure of a mouse sarcoma in its solid, enzymatically dissociated, and ascitic forms. *Acta Path. Microbiol. Scand.*, suppl. **187**:7

Branton, D., Deamer, D. W. 1972. Membrane structure. *Protoplasmatologia* II.E.**1**.:1

Brent, T. P., Forrester, J. A. 1967. Changes in surface charge of HeLa cells during the cell cycle. *Nature* **215**:92

Burger, M. M. 1969. A difference in the architecture of the surface membrane of normal and virally transformed cells. *Proc. Nat. Acad. Sci.* **62**:994

Burger, M. M. 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature* **227**:170

Codington, J. F., Sanford, B. H., Jeanlog, R. W. 1970. Glycoprotein coat of the TA_3 cell. I. Removal of carbohydrate and protein material from viable cells. *J.. Nat. Cancer Inst.* **45**:637

Collins, M. F. 1966. Electrokinetic properties of dissociated chick embryo cells. I. pH-surface charge relationships and the effect of calcium ions. *J. Exp. Zool.* **163**:23

Cook, G. M. W., Heard, D. H., Seaman, G. V. F. 1960. A sialomucopeptide liberated by trypsin from the human erythrocyte. *Nature* **188**:1011

Coon, H. G. 1966. Clonal stability and phenotypic expression of chick cartilage cells in vitro. *Proc. Nat. Acad. Sci.* **55**:66

Curtis, A. S. G. 1962. Cell contact and adhesion. *Biol. Rev.* **37**:82

Curtis, A. S. G. 1969. The measurement of cell adhesiveness by an absolute method. *J. Embryol.* **22**:305

Curtis, A. S. G. 1970. On the occurrence of specific adhesion between cells. *J. Embryol.* **23**:253

Curtis, A. S. G., Greaves, M. F. 1965. The inhibition of cell aggregation by a pure serum protein. *J. Embryol.* **13**:309

Dalen, H., Todd, P. W. 1971. Surface morphology of trypsinized human cells *in vitro*. *Exp. Cell Res.* **66**:353

Dunn, M. J., Owen, E., Kemp, R. B. 1970. Studies on the mechanism underlying the inhibition by puromycin of cell aggregation *in vitro*. *J. Cell Sci.* **7**:557

Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* **130**:432

Easty, G. C., Easty, D. M., Ambrose, E. J. 1960. Studies of cellular adhesiveness. *Exp. Cell Res.* **19**:539

Easty, G. C., Mutolo, V. 1960. The nature of the intercellular material of adult mammalian tissues. *Exp. Cell Res.* **21**:371

Ede, D. A., Agerbak, G. S. 1968. Cell adhesion and movement in relation to the developing limb pattern in normal and *talpid*^{3} mutant chick embryo. *J. Embryol.* **20**:81

Edidin, M. 1966. The release of soluble H-2 alloantigens during disaggregation of mouse embryo tissue by a chelating agent. *J. Embryol.* **16**:519

Edwards, G. A., Fogh, J. 1959. Micromorphologic changes in human amnion cells during trypsinization. *Cancer Res.* **19**:608

Engel, M. B., Pumper, R. W., Joseph, N. R. 1968. Electrometric determination of surface charge of cultured mammalian cells. *Proc. Soc. Exp. Biol., N. Y.* **128**:990

Feldman, M. 1955. Dissociation and reaggregation of embryonic cells of *Triturus alpestris*. *J. Embryol.* **3**:251

Fischer, H. W., Puck, T. T., Sato, G. 1958. Molecular growth requirements of single mammalian cells: The action of fetuin in promoting cell attachment to glass. *Proc. Nat. Acad. Sci.* **44**:4

Forstner, G. G. 1971. Release of intestinal surface-membrane glycoproteins associated with enzyme activity by brief digestion with papain. *Biochem. J.* **121**:781

Frye, L. D., Edidin, M. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7**:319

Gershman, H. 1970. On the measurement of cell adhesiveness. *J. Exp. Zool.* **174**:391

Giudice, G. 1965. The mechanism of aggregation of embryonic sea urchin cells; a biochemical approach. *Devel. Biol.* **12**:233

Glaeser, R. M. 1963. The Electrical Charge and Surface Properties of Intact Cells. Ph. D. Thesis. University of California. *Also*: Office of Technical Services, U. S. Dept. Commerce, Washington

Glaeser, R. M., Richmond, J. E., Todd, P. W. 1968. Histotypic self-organization by trypsin-dissociated and EDTA-dissociated chick embryo cells. *Exp. Cell Res.* **52**:71

Hakomori, S., Teather, C., Andrews, H. 1968. Organizational differences of cell surface "hematoside" in normal and virally transformed cells. *Biochem. Biophys. Res. Commun.* **33**:563

Haydon, D. A. 1961. The surface charge of cells and some other small particles as indicated by electrophoresis. I. The zeta potential-surface charge relationships. *Biochim. Biophys. Acta* **50**:450

Häyry, P., Penttinen, K., Saxén, L. 1965. The different effects of some methods of disaggregation on the electrophoretic mobility of the HeLa-cell. *Ann. Med. Exp. Fenn.* **43**:91

Hebb, C. R., Chu, M. Y. W. 1960. Reversible injury of L-strain mouse cells by trypsin. *Exp. Cell Res.* **20**:453

Holtfreter, J. 1943. A study of the mechanics of gastrulation. Part I. *J. Exp. Zool.* **94**:261

Humphreys, T. 1963. Chemical dissolution and *in vitro* reconstruction of sponge cell adhesions: Isolation and functional demonstration of the components involved. *Devel. Biol.* **8**:27

Inbar, M., Sachs, L. 1969. Interaction of the carbohydrate-binding protein concanavalin A with normal and transformed cells. *Proc. Nat. Acad. Sci.* **63**:1418

Jensen, A. B. 1948. Activation of unfertilized sea urchin eggs by trypsin. *Nature* **162**:931

Johnson, C. F. 1966. Disaccharidase: Localization in hamster intestine brush borders. *Science* **155**:1670

Jones, B. M. 1965. Inhibitory effect of *p*-benzoquinone on the aggregation behavior of embryo-chick fibroblast cells. *Nature* **206**:1280

Jones, B. M., Kemp, R. B. 1970. Aggregation and electrophoretic mobility studies on dissociated cells (2). The effects of ATP and ADP. *Exp. Cell Res.* **63**:301

Jones, B. M., Kemp, R. B., Groschel-Stewart, U. 1970. Inhibition of cell aggregation by antibodies directed against actomyosin. *Nature* **226**:261

Jones, B. M., Morrison, G. A. 1969. A molecular basis for indiscriminate and selective cell adhesion. *J. Cell Sci.* **4**:799

Jones, P. C. T. 1966. A contractile protein model for cell adhesion. *Nature* **211**:365

Kemp, R. B. 1969. Studies on the effect of dissociating agents on the electrophoretic mobility and aggregative competence of embryonic chick muscle cells. *Cytobios* **1**:187

Kemp, R. B. 1970. The effect of neuraminidase (3:2:1:19) on the aggregation of cells dissociated from embryonic chick muscle tissue. *J. Cell Sci.* **6**:751

Kemp, R. B., Jones, B. M. 1970. Aggregation and electrophoretic mobility studies of cells. I. The effects of *p*-benzoquinone and tannic acid. *Exp. Cell Res.* **63**:293

Kemp, R. B., Jones, B. M., Cunningham, I., James, M. C. M. 1967. Quantitative investigation on the effect of puromycin on the aggregation of trypsin- and versene-dissociated chick fibroblast cells. *J. Cell Sci.* **2**:323

Kiremidjian, L., Kopac, M. J. 1972. Changes in cell adhesiveness associated with the development of *Rana pipiens* pronephros. *Devel. Biol.* **27**:116

Kleinschuster, S. J., Moscona, A. A. 1972. Interactions of embryonic and fetal neural retinal cells with carbohydrate-binding phytohemagglutinins: Cell surface changes with differentiation. *Exp. Cell Res.* **70**:397

Knight, V. A., Jones, B. M., Jones, P. C. T. 1966. Inhibition of the aggregation of dissociated embryo-chick fibroblast cells by adenosine triphosphate. *Nature* **210**:1008

Kobamoto, N., Löfroth, G., Camp, P., Van Amburg, G., Augenstein, L. 1966. Specificity of trypsin adsorption onto cellulose, glass, and quartz. *Biochem. Biophys. Res. Commun.* **24**:622

Kolodny, G. M. 1972. Effect of various inhibitors on readhesion of trypsinized cells in culture. *Exp. Cell Res.* **70**:196

Kossard, S., Nelson, D. S. 1968. Studies on cytophilic antibodies. IV. The effects of proteolytic enzymes (trypsin and papain) on the attachment to macrophages of cytophilic antibodies. *Aust. J. Exp. Biol. Sci.* **46**:63

Kuo, J. F., Holmlund, C. E., Dill, I. K. 1966. The effect of proteolytic enzymes in isolated adipose cells. *Life Sci.* **5**:2257

Kuroda, Y. 1963. Changes in aggregation and differentiation of cartilage cells grown in monolayer cultures. *Exp. Cell Res.* **30**:446

Langley, O. K., Ambrose, E. J. 1964. Isolation of a mucopeptide from the surface of Ehrlich ascites tumor cells. *Nature* **204**:53

Lefford, F. 1965. Pretreatment with enzymes on explant outgrowth *in vitro*. *Exp. Cell Res.* **38**:285

Lesseps. R. J. 1963. Cell surface projections: Their role in the aggregation of embryonic chick cells as revealed by electron microscopy. *J. Exp. Zool.* **153**:171

Lilien, J. E. 1968. Specific enhancement of cell aggregation *in vitro*. *Devel. Biol.* **17**:657

Mäkelä, O., Miettinen, T., Pesola, R. 1960. Release of sialic acid and carbohydrates from human red cells by trypsin treatment. *Vox Sang.* **5**:492

Martz, E., Steinberg, M. S. 1972. The role of cell-cell contact in "contact" inhibition of cell division: A review and new evidence. *J. Cell Physiol.* **79**:189

Millipore Filter Corp. 1964. Techniques for Exfoliative Cytology. Application Data Manual – 50

Moore, A. R. 1952. An animalizing effect of trypsin and its inhibition by lithium in the developing eggs of *Strongylocentrotus droebachiensis*. *J. Exp. Zool.* **121**:99

Moscona, A. 1952. Cell suspensions from organ rudiments of chick embryos. *Exp. Cell Res.* **3**:535

Moscona, A. 1961*a*. Rotation-mediated histogenic aggregation of dissociated cells. A quantifiable approach to cell interaction *in vitro*. *Exp. Cell Res.* **22**:455

Moscona, A. 1961*b*. Effect of temperature on adhesion to glass and histogenic cohesion of dissociated cells. *Nature* **190**:408

Moscona, A. A. 1963. Inhibition by trypsin inhibitors of dissociation of embryonic tissue by trypsin. *Nature* **199**:379

Moscona, A. A. 1971. Embryonic and neoplastic cell surfaces: Availability of receptors for concanavalin A and wheat germ agglutinin. *Science* **171**:905

Moscona, A., Moscona, H. 1952. The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J. Anat.* **86**:287

Moscona, A., Moscona, H. 1966. Aggregation of embryonic cells in a serum-free medium and its inhibition at suboptimal temperatures. *Exp. Cell Res.* **41**:697

Moscona, A., Trowell, O. A., Willmer, E. N. 1965. Methods. *In*: Cells and Tissues in Culture. E. N. Willmer, editor. Vol. I, p. 19. Academic Press Inc., New York

Neville, D. M. 1968. Isolation of an organ specific protein antigen from cell-surface membrane of rat liver. *Biochim. Biophys. Acta* **154**:540

Nicolson, G. L. 1972. Topography of membrane concanavalin A sites modified by proteolysis. *Nature, New Biol.* **239**:193

Nicolson, G. L., Blaustein, J. 1972. The interaction of *Ricinus communis* agglutinin with normal and tumor cell surfaces. *Biochim. Biophys. Acta* **226**:543

Nicolson, G. L., Hyman, R., Singer, S. J. 1971. The two dimensional topographic distribution of H-2 histocompatibility alloantigens on mouse red blood cell membranes. *J. Cell Biol.* **50**:905

Nicolson, G. L., Singer, S. J. 1971. Ferritin-conjugated plant agglutinins as specific saccharide stains for electron microscopy. Application to saccharides bound to cell membranes. *Proc. Nat. Acad. Sci.* **68**:942

Northrop, J. H. 1926. The resistance of living organisms to digestion by pepsin or trypsin. *J. Gen. Physiol.* **9**:497

Ohkuma, S., Ikemoto, S. 1965. Release of a sialomucopeptide from human red cells and destruction of the I blood group receptor of their cells by crystalline trypsin treatment *Proc. Japan Acad.* **41**:482

Orr, C. W., Roseman, S. 1969. Intercellular adhesion. I. A quantitative assay for measuring the rate of adhesion. *J. Membrane Biol.* **1**:109

Pethica, B. A. 1961. The physical chemistry of cell adhesion. *Exp. Cell Res.*, suppl. **8**:123

Phillips, H. J. 1967. Some metabolic changes resulting from treating kidney tissue with trypsin. *Canad. J. Biochem.* **45**:1495

Pinto da Silva, P. 1972. Translational mobility of the membrane intercalated particles of human erythrocyte ghosts: pH-dependent, reversible aggregation. *J. Cell Biol.* **53**:777

Pinto da Silva, P., Douglas, S. D., Branton, D. 1971*a*. Localization of A antigen sites on human erythrocyte ghosts. *Nature* **232**:194

Pinto da Silva, P., Douglas, S. D., Branton, D. 1971*b*. Location of A_1 antigens on the human erythrocyte membrane. *J. Cell Biol.* **47**:159 a.

Ponder, E. 1951. Effects produced by trypsin on certain properties of the human red cell. *Blood* **6**:350

Poste, G. 1971. Tissue dissociation with proteolytic enzymes. Adsorption and activity of enzymes at the cell surface. *Exp. Cell Res.* **65**:359

Price, P. G. 1970. Electron microscopic observations of the surface of L-cells in culture. *J. Membrane Biol.* **2**:300

Rosenberg, M. D. 1960. Microexudates from cells grown in tissue culture. *Biophys. J.* **1**: 137

Roth, S. A. 1968. Studies on intercellular adhesive selectivity. *Devel. Biol.* **18**:602

Roth, S. A., Weston, J. A. 1967. The measurement of intercellular adhesion. *Proc. Nat. Acad. Sci.* **58**:974

Seaman, G. V. F., Heard, D. H. 1960. The surface of the washed human erythrocyte as a polyanion. *J. Gen. Physiol.* **44** (1):251

Sefton, B. M., Rubin, H. 1970. Release from density dependent growth inhibition by proteolytic enzymes. *Nature* **227**:843

Sela, B.-A., Lis, H., Sharon, N., Sachs, L. 1970. Different locations of carbohydrate-containing sites in the surface membrane of normal and transformed mammalian cells. *J. Membrane Biol.* **3**:267

Shea, L., Ginsburg, V. 1968. Release of sugars from HeLa cells by trypsin. *In*: Biological Properties of the Mammalian Surface Membrane. A. Manson, editor. p. 67. Wistar Inst. Symp. #8, Wistar Institute Press, Philadelphia, Pa.

Simms, H. S., Stillman, N. P. 1937. Substances affecting adult tissue *in vitro*. I. The stimulating action of trypsin on fresh adult tissue. *J. Gen Physiol.* **20**:603

Singer, S. J., Nicolson, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720

Smith, S. B., Revel, J.-P. 1972. Mapping of concanavalin A binding sites on the surface of several cell types. *Devel. Biol.* **27**:434

Steinberg, M. S. 1958. On the chemical bonds between animal cells. A mechanism for type-specific association. *Amer. Naturalist* **92**:65

Steinberg, M. S. 1962. Role of temperature in the control of aggregation of dissociated embryonic cells. *Exp. Cell Res.* **28**:1

Steinberg, M. S. 1963. ECM: Its nature, origin, and function in cell aggregation. *Exp. Cell Res.* **30**:257

Steinberg, M. S. 1967. Avian and mammalian cell dissociation. *In*: Methods in Developmental Biology. F. Wilt and N. K. Wessells, editors. p. 565. T. Y. Crowell Co., New York

Steinberg, M. S., Granger, R. E. 1966. The re-acquisition of adhesiveness of trypsinized chick embryonic cells *in vitro*. *Amer. Zool.* **6**:579 (abstr).

Steinberg, M. S., Roth, S. A. 1964. Phases in cell aggregation and tissue reconstruction. An approach to the kinetics of cell aggregation. *J. Exp. Zool.* **157**:327

Tillack, T. W., Scott, R. E., Marchesi, V. T. 1971. Further studies on the association between glycoprotein receptors and the intramembranous particles of the red cell membrane. *Abstr. 11th Ann. Mtg. Amer. Soc. Cell Biology*, New Orleans, p. 305

Townes, P. L. 1953. Effects of proteolytic enzymes on the fertilization membrane and jelly layers of the amphibian embryo. *Exp. Cell Res.* **4**:96

Townes, P. L., Holtfreter, J. 1955. Directed movements and selective adhesion of embryonic amphibian cells. *J. Exp. Zool.* **128**:53

Weiss, L. 1960. Studies on cellular adhesion in tissue culture. III. Some effects of calcium. *Exp. Cell Res.* **21**:71

Weiss, L. 1963. The pH value at the surface of *Bacillus subtilis*. *J. Gen. Microbiol.* **32**:331

Weiss, L. 1966. Studies on cell deformability. II. Effects of some proteolytic enzymes. *J. Cell Biol.* **30**:39

Weiss, L., Kapes, D. L. 1966. Observations on cell adhesion and separation following enzyme treatment. *Exp. Cell Res.* **41**:601

Wiseman, L. L., Steinberg, M. S., Phillips, H. M. 1972. Experimental modulation of intercellular cohesiveness: Reversal of tissue assembly patterns. *Devel. Biol.* **28**:498

Zajac, I., Crowell, R. J. 1965. Location and regeneration of enterovirus receptors of HeLa cells. *J. Bacteriol.* **89**:1097