

## On the Role of Metal Cations in Cellular Adhesion: Effect on Cell Surface Charge<sup>1</sup>

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**ABSTRACT** An examination has been made of the hypothesis that the role of divalent cations in promoting reaggregation of dissociated cells is principally to reduce the negative electrostatic charge at the cell surface. This hypothesis would suggest that cells in the presence of different divalent cations would reaggregate at identical rates if the concentrations of the different cations were so adjusted that in all cases the cells possess the same charge. This expectation has not been fulfilled. Magnesium ion was most effective in promoting reaggregation, calcium somewhat less so, and strontium and barium had little or no effect.

It seems well established that the ability of individual animal cells to adhere to one another normally requires the presence of some divalent cation in the external milieu. In a now classic study, Herbst ('00) showed that the cells of the sea-urchin larva separated from one another when the larva was placed in sea water which was lacking in calcium and magnesium ions. Subsequent findings that a variety of other tissues could also be dissociated upon removal of calcium and magnesium reinforced the notion that these ions play an essential role in the process of cell adhesion.

A variety of hypotheses have been proposed to account for this dependence of cell adhesion upon the presence of divalent cations in the medium (Gray, '31; Schmitt, '41; Steinberg, '58). One of the most appealing of these arises as a consequence of a more general discussion recently presented by Curtis ('60, '62, '66) concerning the question of just what physical forces are of significance in the phenomenon of cellular adhesion.

Curtis has proposed that the forces operative in cell adhesion are the same as those which are of greatest importance in determining the properties of lyophobic colloids. Considerable success has been achieved in interpreting the behavior of these latter systems if it is assumed that the most important forces between the colloid particles are van der Waals forces of attraction and electrostatic forces of repulsion (Derjaguin

and Landau, '41; Verwey and Overbeek, '48). The latter forces result from an electrical charge carried at the surfaces of the colloid particles. The particles which make up a lyophobic colloid system can be caused to adhere to one another by addition of electrolyte to the medium. The added electrolyte exerts its role primarily by reducing the electrostatic forces of repulsion.

Like colloid particles, cells bear an electrical charge at their surfaces. Under physiological conditions, the net surface charge of most investigated cells is negative in sign (Abramson et al., '42; Bangham and Pethica, '60; Collins, '66; Dan, '34; Nevo et al., '61). This charge apparently is due mainly to ionogenic groups which are a part of the surface structure. The hypothesis proposed by Curtis suggests that the primary role of the positively charged divalent cations in promoting cell adhesion is to lower the cells' net negative surface charge (Curtis, '66). This would reduce the electrostatic repulsive forces between individual cells. Adhesion could occur upon sufficient reduction of these repulsive forces.

The present study is an attempt to test the hypothesis that  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  are required for cells to adhere mainly because these ions lower surface charge. If it could be shown that other cations were also able

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to bind to the cells, and thus lower charge, and if the hypothesis above is correct, then these other cations should also be able to promote cell adhesion.

As a test of this hypothesis, a comparison was made of the rates of reaggregation of populations of dissociated tissue cells in the presence of different divalent cations. The surface charge of the cells of each population was reduced to the same extent, but the populations differed in regard to the divalent cation used to effect this charge reduction. Marked differences were noted in the capacities of the different cations to promote reaggregation under these circumstances.

Only the four common alkaline earth cations were employed because at physiological pH values many other metal cations form large polynuclear complexes which often possess a very high charge (Ahrland et al., '54; Hietanen, '54). By virtue of their large size and high charge, these molecules might be able to cross-bridge cells, much in the manner that cationic polyelectrolytes do (Katchalsky et al., '59), a mode of action which might be quite different from that of the alkaline earths, which do not form polynuclear complexes (Sillén and Martell, '64).

#### MATERIALS AND METHODS

In the studies reported in this paper, the principal techniques used were dissociation of tissues into individual cells; microelectrophoresis, and measurement of the degree of reaggregation achieved under standard conditions by dissociated cells.

*Cell dissociation.* The source of cells used in all experiments was the limb bud from the four to four and one-fourth day chick embryo. Limb buds were dissociated into individual cells in a solution of ethylene-diamine tetra-acetate (EDTA) using a method similar to one described by Curtis and Greaves ('65). Limb buds were removed and each was chopped into several pieces using needle-knives. The pieces were then soaked in the dissociating medium: 2 mM EDTA solution, pH 8.0 made up in Hank's solution buffered at pH 8.0 with 40 mM Tris at 8°C. Dissociation was accomplished by shearing with a mechanical test tube stirrer ("Cyclo-mixer," Clay Adams Co., New York, N. Y.).

Cell clumps and red blood cells were removed by means of a brief low speed centrifugation. The single cells and small clumps remaining in suspension were freed of dissociation medium by centrifugation, and were washed twice with resuspension and centrifugation in 0.145 M NaCl. The final pellet of cells was suspended in the solution of interest. The duration and rates of centrifugation ( $220 \times$  gravity; 3 minutes) were adjusted so that most of the cells could be collected into firm pellets while most of the cell debris resulting from dissociation was left in suspension. The most critical step in this procedure is that of cell dispersion itself. If the "cyclomixing" is done correctly, a large yield of viable cells can be obtained. Cell viability, as judged by visual examination and exclusion of nigrosin, was high; 85–95% of the cells looked healthy and excluded dye.

*Microelectrophoresis.* The microelectrophoresis apparatus used is similar to that devised by Bangham et al. ('58) and has been described in detail by Collins ('66). Location of the stationary layer (the region where there is no electroendosmosis) was calculated and then checked empirically using human red blood cells, which are of known mobility (Bangham et al., '58). In our hands, the mobility of red cells suspended in 1/15 M phosphate buffer was 1.27  $\mu$ /sec/volt/cm. This calibration was repeated at least once a month to check the proper alignment of the apparatus.

In all studies on limb bud cells, the ionic strength was maintained constant at 0.145 M. Osmolarity was not held constant in those solutions containing divalent cations, as all non-ionized compounds which might have been used for this purpose caused a marked drop in pH, presumably due to oxidation at the platinum-black electrodes of the electrophoresis apparatus.

The only buffer present was sodium bicarbonate. The potential drop across the electrophoresis cell was 5.3 volts/cm. Temperature was 25°C. The pH was 7.0–7.3. Cell viability and pH were assayed both before and after each electrophoresis run. All reagents were Baker "Analyzed"

grade. Water was doubly distilled, the second distillation being from Pyrex glass.

**Cell reaggregation.** Reaggregation was performed in saline solutions identical with those used for electrophoresis. The cell suspensions were stirred during the course of aggregation so that individual cells were kept in suspension and were continually contacting one another. In favorable environments, a fraction of these contacts resulted in the formation of stable adhesions between cells. Reaggregation vessels were 0.8 (I.D.)  $\times$  2 cm test tubes capped with serum bottle stoppers. Stirring was accomplished by placing these on a tissue culture roller tube rotator (Scientific Industries Inc., Springfield, Mass.) which revolved 60 times per minute. The large end of the serum bottle stopper was fitted over the mouth of the reaggregation vessel. The small end fitted snugly into the mouth of a 16 ml screw-cap test tube which could then be inserted into the drum of the roller tube rotator. Temperature was 37°C. All glassware used in both the dissociation and reaggregation steps was acid washed and then treated with horse serum and rinsed before use to prevent cells from sticking to the glass. The volume of fluid in each reaggregation vessel was 0.2 ml.

Degree of reaggregation was assayed at 15 minutes employing the method of Steinberg and Granger (in preparation). The cells in each reaggregation vessel were sucked down onto the surface of a Millipore filter, where they were then fixed with Bouin's fixative modified to contain 4% glutaraldehyde in place of formaldehyde. The cells adhere very strongly to the filter, which is then processed in toto according to standard histological methods and mounted whole on a microscope slide without loss of cells (Millipore Filter Corp., '64). A count was made of the number of cells in each of at least 1,000 aggregates per slide, sampled at random. The average number of cells per aggregate was used to express the degree of aggregation.

Limb bud cells do not remain viable for prolonged periods in the simple saline solutions used. However aggregation in the short times employed was no less than when the cells were suspended in a more complete medium (Eagle's Minimal Essen-

tial Medium, supplemented with 10% horse serum). Since statistically significant differences in aggregation were achieved by different cell populations in as short a time as 15 minutes, this was chosen as the standard length of time for reaggregation experiments.

#### RESULTS AND CONCLUSIONS

*Surface charges of limb bud cells as a function of divalent cation concentration.* The net electrical surface charge was measured by means of cell electrophoresis. The results are summarized in figures 1-5. The parameter used in these figures and

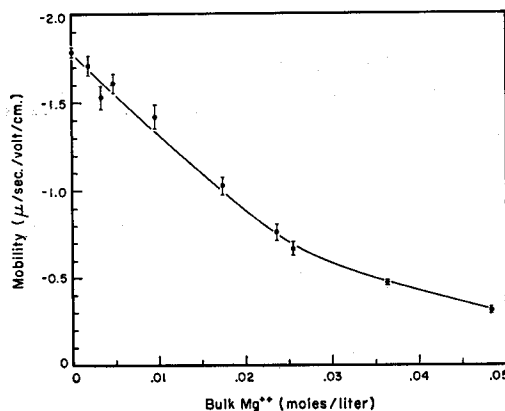


Fig. 1 Electrophoretic mobility of limb bud cells as a function of bulk magnesium ion concentration. Ionic strength was held constant at 0.145 M. The pH is 7.0-7.3. Error of the mean is indicated by the 95% confidence interval.

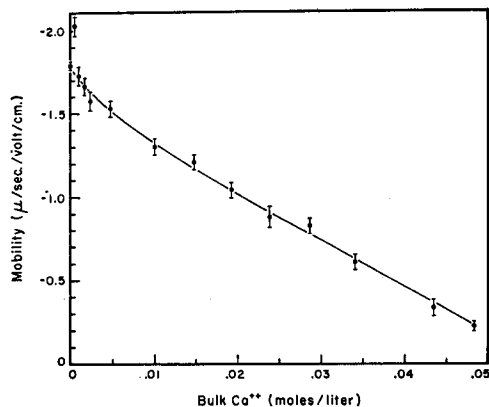


Fig. 2 Electrophoretic mobility of limb bud cells as a function of bulk calcium ion concentration. Ionic strength is 0.145 M. The pH is 7.0-7.3.

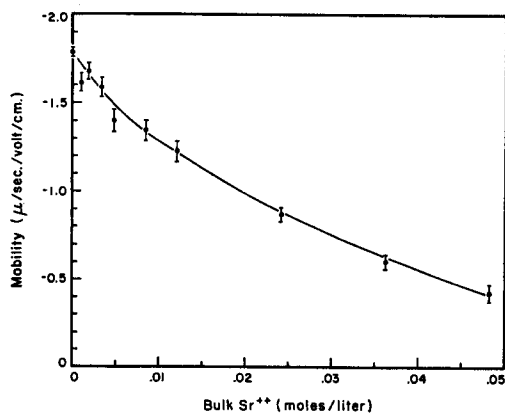


Fig. 3 Electrophoretic mobility of limb bud cells as a function of bulk strontium ion concentration. Ionic strength is 0.145 M. The pH is 7.0-7.3.

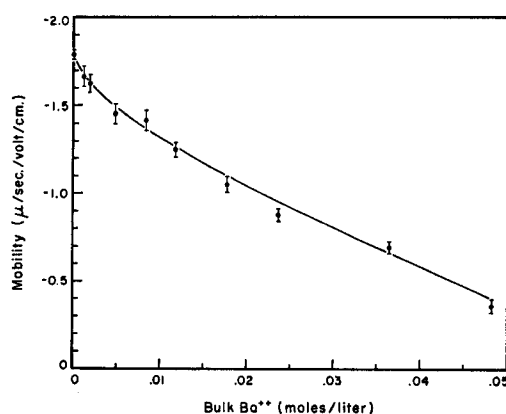


Fig. 4 Electrophoretic mobility of limb bud cells as a function of bulk barium ion concentration. Ionic strength is 0.145 M. The pH is 7.0-7.3.

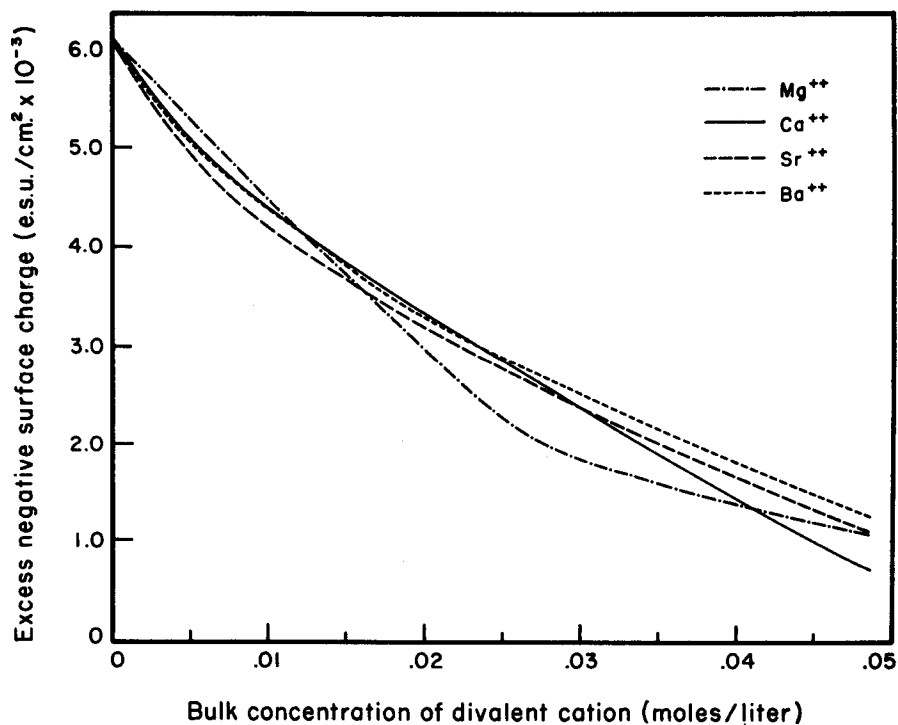


Fig. 5 Net surface charge of limb bud cells as a function of bulk alkaline earth cation concentration. Surface charge was calculated from the data presented in figures 1-4 by means of the Gouy-Gorin equation.

in all other places in the text for expression of error of the mean is the 95% confidence limit. In all cases ionic strength was 0.145 M and pH was 7.0–7.3. Each point represents the arithmetic mean of mobilities determined for between 20 and 50 individual cells.

Surface charges ( $\sigma$ ) were computed from electrophoretic mobility using the Gouy-Gorin equation (fig. 5):

$$\sigma^2 = \frac{NDkT}{2000\pi} \sum_i \left( C_i \exp \left( \frac{-1200\pi enZ_i u}{DkT} \right) - 1 \right),$$

where N (Avogadro's number) =  $6.03 \times 10^{23}$  moles<sup>-1</sup>, D (dielectric constant — water at 25°C) = 78.54, k (Boltzmann's constant) =  $1.38 \times 10^{-16}$  ergs/degree, T = 298.16°K,  $C_i$  and  $Z_i$  are the concentration and charge of ion species  $i$ ,  $e$  (electron charge) =  $4.8025 \times 10^{-10}$  electrostatic units,  $n$  (viscosity — water at 25°C) =  $0.8937 \times 10^{-2}$  poise, and  $u$  is the electrophoretic mobility in cm/sec/volt/cm (Abramson et al., '42). This equation assumes that the surface in question is smooth and of large radius of curvature (Abramson et al., '42); that the particle is rigid and nonconducting (Overbeek, '50); that the charge is uniformly distributed over the surface of the particle (Davies and Rideal, '63); that values of D and  $n$  for the bulk solution can be used (Bolt, '55; Chatteraj and Bull, '59; Haydon, '60); that the applied field can be vectorially added to the electrostatic field of the particle (Overbeek, '50); and that the surface is impenetrable to counter-ions (Davies and Rideal, '54; Haydon, '61). Correction for finite size of counter-ions was not applied (Overbeek, '50).

It is obvious that divalent cations do decrease net cell surface charge, as is required for the hypothesis being examined to be true. It is clear in figure 5 that the different alkaline earths differ little in degree of binding to limb bud cells.

The reversibility of divalent cation binding to limb bud cells (table 1) was evaluated by comparing the mobilities in 0.145 M NaCl of cells which had previously been incubated in concentrated solutions of divalent cations with the mobilities to cells which had never been exposed to divalent cation solutions. Since cells treated in these two different fashions show quite

similar mobilities, the conclusion seems justified that binding of divalent cations is almost completely reversible.

*Effect of temperature on surface charge.* Surface charge was investigated at both 25°C and 37°C. The data are presented in table 2. Surface charge and cation binding seem largely independent of temperature, at least between 25° and 37°C.

*Cell reaggregation.* Knowing the surface charge — cation concentration relationships from the previous experiments, it was possible to test the hypothesis that the major role of divalent cations in pro-

TABLE 1  
Reversibility of cation binding to chick embryo limb bud cells

Divalent cation	Electrophoretic mobility in $\mu$ /sec/volt/cm	
	With divalent cation <sup>1</sup>	With divalent cation removed from suspension medium <sup>2</sup>
None <sup>3</sup>		1.788 ± 0.026
Ca <sup>++</sup>	0.228 ± 0.030	1.732 ± 0.077
Mg <sup>++</sup>	0.315 ± 0.017	1.615 ± 0.074
Ba <sup>++</sup>	0.359 ± 0.039	1.652 ± 0.084

<sup>1</sup> Mobility values taken from figures 1–4.

<sup>2</sup> Cells were first suspended in a solution of the divalent cation (for the length of time usually necessary to complete an electrophoretic determination). They were then washed and resuspended in 0.145 M NaCl at pH 7.0–7.3, and their mobilities were determined.

<sup>3</sup> Solution is 0.145 M NaCl.

<sup>4</sup> Solutions are 0.048 molar in divalent cation (ionic strength = 0.145 M).

TABLE 2  
Effect of temperature on surface charge of chick embryo limb bud cells

Medium <sup>1</sup>	Surface charge in esu/cm <sup>2</sup> × 10 <sup>-3</sup>	
	25°C	37°C
0.145 M NaCl	6.14 ± 0.10	6.05 ± 0.29
0.0048 M Ca <sup>++</sup> <sup>2</sup>	5.15 ± 0.17	4.59 ± 0.32
0.0048 M Ba <sup>++</sup> <sup>2</sup>	4.90 ± 0.20	4.97 ± 0.19

<sup>1</sup> pH = 7.0–7.3.

<sup>2</sup> Ionic strength was maintained at 0.145 M by addition of NaCl (to 0.13 M).

TABLE 3  
Surface charges and divalent cation concentrations used in reaggregation experiments

Surface charge (esu/cm <sup>2</sup> × 10 <sup>-3</sup> )	Amount of cation (moles/liter)			
	Mg <sup>++</sup>	Ca <sup>++</sup>	Sr <sup>++</sup>	Ba <sup>++</sup>
5.51	0.0035	0.0022	0.0022	0.0019
5.12	0.0058	0.0046	0.0039	0.0044

moting cell reaggregation is to reduce the negative surface charge of the cells. To test the hypothesis, two values of surface charge were selected and the amounts of each of the alkaline earth cations necessary to bring the cells to those charges were read from the graph of surface charge as a function of cation content of the medium (fig. 5). The surface charges selected and the cation concentrations which give these values are summarized in table 3.

If the hypothesis is correct it would seem that rates of reaggregation at a given surface charge in each of the ionic environments should be the same. The data

presented in figure 6 and table 4 show that this is far from the case. Magnesium is most effective in promoting reaggregation; calcium is moderately effective; and barium and strontium have little or no effect.

Although a concentration of  $Mg^{++}$  higher than the concentrations of the other divalent cations was employed in any given trial, this cannot account for the greater effectiveness of  $Mg^{++}$  in promoting reaggregation. In this connection it should be noted that although the concentration of  $Mg^{++}$  in the first trial ( $\sigma = 5.51 \times 10^3$  esu/cm<sup>2</sup>) is lower than the concentrations of  $Ca^{++}$ ,  $Sr^{++}$  or  $Ba^{++}$  in the second and third trials ( $\sigma = 5.12 \times 10^3$  esu/cm<sup>2</sup>), the

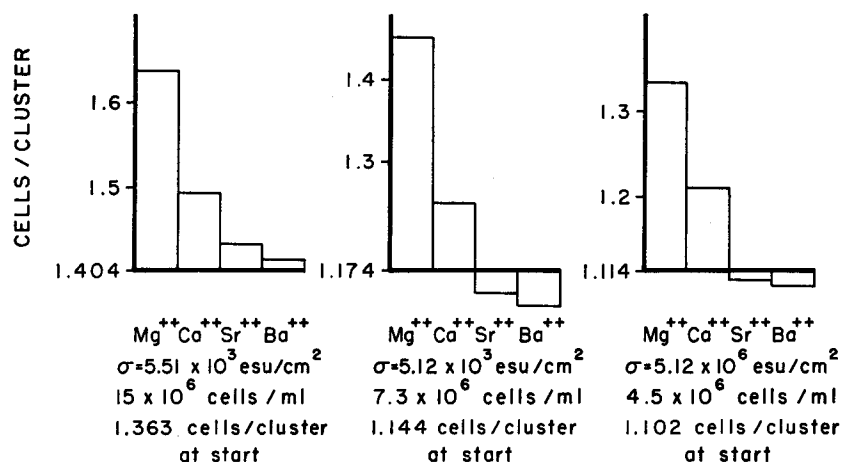


Fig. 6 Reaggregation of dissociated limb bud cells in the presence of different alkaline earth cations. Degree of reaggregation is expressed as average number of cells per cluster. Reaggregation was allowed to proceed for 15 minutes at 36–37°C. The cell suspensions were stirred continuously during the experiment. The horizontal base line is the degree of reaggregation achieved in the absence of any divalent cation (i.e.: in 0.145 M NaCl). Except for this control sample, the cells in each population of a given experiment were at the same value of surface charge, but in each case were brought to that charge by a different divalent cation. Ionic strength was 0.145 M. The pH was 7.1–7.2. The results of three separate experiments are presented.

TABLE 4  
Aggregation of EDTA — dissociated chick embryo limb bud cells in the presence of different alkaline earth cations

Cation	Increase in average number of cells/cluster			Arithmetic mean for the three trials	95% confidence limits for the mean
	Trial 1	Trial 2	Trial 3		
$Mg^{++}$	+0.234	+0.272	+0.218	+0.241	±0.070
$Ca^{++}$	+0.090	+0.078	+0.096	+0.088	±0.007
$Sr^{++}$	+0.030	-0.027	-0.012	-0.003	±0.074
$Ba^{++}$	+0.011	-0.042	-0.019	-0.017	±0.067

lower concentration of  $Mg^{++}$  is still more effective in promoting reaggregation than the higher concentrations of  $Ca^{++}$ ,  $Sr^{++}$  and  $Ba^{++}$ . In fact, preliminary experiments indicate that concentrations of  $Mg^{++}$  at least as low as 0.0001 M will support reaggregation (Armstrong — unpublished).

In order to arrive at some indication of statistical significance for the data, the increase in degree of aggregation induced by the presence of each divalent cation was determined for the three experiments summarized in figure 6. This increase was taken to be the value for the degree of aggregation in the presence of the given cation minus that in its absence. Values obtained in the three experiments were averaged, and the significance of the differences between means was evaluated by means of the t-test. The results are shown in table 4. All differences between arithmetic means are significant in excess of the 99.5% level of confidence with the exception of the difference between the arithmetic means for  $Sr^{++}$  and  $Ba^{++}$ . The latter do not differ significantly from each other, nor do they appear to differ from the controls to which divalent cations had not been added.

Some comprehension of the progress of reaggregation at 15 minutes can be gained from an examination of primary data as presented in table 5. It can be seen that there is both a decrease in the fraction of "clusters" containing only one cell and an increase in the fraction of clusters containing two or more cells. As aggregation proceeds, an increasing number of clus-

ters larger than any observed in the absence of aggregation make their appearance.

*Cell viability.* When performing experiments on processes such as cell reaggregation, it is necessary to be able to check the physiological condition of the cells. Three different approaches were used: observations on dye exclusion; time lapse cinematography; and determination of the capacity for reaggregation in mixtures of  $Mg^{++}$  and  $Ba^{++}$ .

Each time an electrophoresis run was performed, the condition of the cells was examined using the criteria of microscopic appearance and dye (nigrosin) exclusion. Many (but not all) inviable cells are unable to exclude nigrosin and are stained blue as a consequence. High concentrations of divalent cations killed the cells in appreciable numbers, but these effects were not apparent until concentrations of 0.01–0.02 M were reached. Calcium seemed to be the least toxic; strontium the most.

Time-lapse cinematography was performed on dissociated limb bud cells suspended in the solutions listed in table 3 for  $\sigma = 5.12 \times 10^3$  esu/cm<sup>2</sup>. Cells in 0.145 M NaCl were also studied. Frames were taken every two seconds at a relatively low magnification so that a large number of cells could be included in the visual field. The cells were placed on an ordinary soft glass microscope slide which had previously been cleaned with abrasive ( $CeO_2$ ) and were photographed at 37°C with Nomarski differential interference contrast optics (Nachet, Paris). Each preparation was observed for at least an hour.

TABLE 5  
Fraction of the total number of cells represented in each cluster class (trial no. 3 of fig. 6)

Cation	Fraction of cells in each cluster class									Average no. of cells per cluster	No. of clusters examined
	1	2	3	4	5	6	7	8	9		
Start	0.837	0.110	0.027	0.022	0.004					1.102	1009
No divalent cation	0.818	0.127	0.037	0.014	0.004					1.114	1021
$Ba^{++}$	0.840	0.123	0.025	0.007	0.005					1.095	1006
$Sr^{++}$	0.836	0.113	0.034	0.011			0.006			1.102	1026
$Ca^{++}$	0.712	0.147	0.097	0.028	0.019				0.007	1.210	1060
$Mg^{++}$	0.588	0.228	0.094	0.039	0.011	0.013	0.021		0.007	1.332	1009

Cell death was not noticeably different in the different ionic environments. The only cells that died within 30–60 minutes were those that appeared injured at the beginning of filming, these probably having been damaged during dissociation. The moribund cells were characterized by being very swollen and having a lowered refractility. Brownian motion of internal particles was often evident in them.

In solutions of all ions except magnesium, general cell appearance and behavior were the same. The cells were rounded and not strongly adherent to the glass. They showed considerable pseudopodial activity. When magnesium was present, however, about half of the cells flattened onto the glass of the microscope slide within minutes. The degree of flattening achieved by some cells was remarkable, with the cell diameter in the plane of the slide increasing three to four fold. Even cells which were this flattened appeared to be alive, as a limited amount of membrane ruffling was often visible. It is of interest that cells show the greatest adhesion for glass in the presence of  $Mg^{++}$  — the same ion which is most effective in promoting cell reaggregation.

A mixing experiment was also performed. Limb bud cells were preincubated in a solution containing  $Ba^{++}$  (at 0.0019 M; see table 3) under conditions normally used for reaggregation. After 15 minutes, enough  $MgCl_2$  solution was added to achieve a  $Mg^{++}$  concentration of 0.0035 M (see table 3) with ionic strength being maintained at 0.145 M. Reaggregation was allowed to proceed for another 15 minutes. A second population was treated in an identical manner except that  $Ba^{++}$  was lacking. A control population was also set up to which no divalent cations were added at any time.

The results of this experiment are presented in table 5. The contention that the absence of aggregation in the presence of  $Ba^{++}$  might be attributable solely to toxic effects of this ion seems unlikely in view of the considerable amount of aggregation achieved by cells in mixtures of  $Ba^{++}$  and  $Mg^{++}$ . Reaggregation achieved in the presence of the mixture is markedly less than in the presence of  $Mg^{++}$  alone. The reason for this is not clear since cell via-

TABLE 6  
Reaggregation of EDTA dissociated chick embryo limb bud cells in mixtures of  $Mg^{++}$  and  $Ba^{++}$

Divalent cation	Average number of cells per cluster	
	Trial 1	Trial 2
None	1.245	1.385
$Mg^{++}$	1.695	2.055
$Ba^{++} + Mg^{++}$	1.472	1.795

bility as assayed by dye exclusion was not impaired in the mixture. If reaggregation is somehow dependent upon binding of  $Mg^{++}$  to a small number of cell surface sites (Steinberg, '58), this reduction might be due to a competition between  $Ba^{++}$  and  $Mg^{++}$  for these sites. Aggregation in the presence of  $Mg^{++}$  was more extensive than in the experiments presented in figure 6.

#### CRITICISM OF THE EXPERIMENTAL METHOD

In this section four criticisms which can be leveled against the design of these experiments will be discussed.

*Ambiguity of surface charge calculations.* The most useful parameter to be obtained from the electrophoretic studies would be net surface charge. The equation which has been employed to relate electrophoretic mobility to charge is that of Gorin and Gouy (Abramson et al., '42). This probably somewhat underestimates the amount of charge (Haydon, '61). The objection that the calculations of surface charge are probably not exact would seem not too severe since changes in the values of surface charge upon addition of divalent cation are of greater interest than the absolute values themselves.

*Specialized regions of adhesion.* If adhesions are initiated only at certain regions of the cell surface, then the information of greatest usefulness would be reduction of electrical charge by divalent cations at these special regions alone. Electrophoresis of suspended cells measures net charge of the cell as a whole and thus does not supply this information if the special regions provide only a fraction of the total cell surface charge. Cells apparently initiate adhesions at points of sharp curvature (Lesseps, '63). If binding of the various cations to these regions should prove to be very different from

binding to the rest of the cell, then the experiments described in this report would not be adequate to rule out charge reduction as the primary function of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in cell adhesion. In certain tissues, desmosomes and similar ultrastructural specializations may be regions of heightened adhesiveness. A study of electron micrographs of aggregates of limb bud cells kindly prepared for me by Dr. Roland Lesseps, S. J., revealed no desmosomes or similar cell surface specializations.

*Binding of ions to two cells at once.* It is probable that initial adhesions are made over very small proportions of the total areas of the cells' surfaces (Lesseps, '63). It seems not unlikely that many of these initial adhesions might be quickly broken by the shear produced as the cell suspension is stirred, since the rate of aggregation decreases with increased stirring (Moscona, '61). Whether or not initial contacts result in stable adhesions may depend on whether the areas of adhesion can be broadened quickly enough to prevent the contact from being broken.

The relative strengths of binding of the different alkaline earth cations to the pair of apposed cell surfaces at these edges of advancing cell adhesion formed during the broadening of the area of contact may be very different from those measured by electrophoresis. This is because in this region of the cell's surface, the cations may bind not to single cells, but to two cells at once.

In the light of this objection, to preserve the hypothesis under investigation, two assumptions would seem to be required. (1) The rate of cell aggregation would have to be strongly dependent upon the rate of increase in area of adhesion once an initial contact is made; and (2) the binding strengths of the *already-bound* alkaline earth cations to the *second cell* at the region of advancing contact would have to be in the order  $\text{Mg}^{++} > \text{Ca}^{++} > \text{Ba}^{++} \approx \text{Sr}^{++}$ . No means for testing this last assumption is known to the author.

*Ambiguity of kinetic measurements.* Possibly the most interesting criticism is that the kinetic measurements, which supply the *rates* of cell reaggregation, do not provide the sort of information that is of greatest usefulness, that is, the *strengths* of cell to cell adhesions. Strength of cell

adhesion is the thermodynamic work performed when the two cells originally distant from each other are brought together and an adhesion of unit area is made between them (Steinberg, '64). Although rate of cell reaggregation will depend on strength of cell adhesion, it may also depend on other factors, such as cell shape or cell rigidity. No differences in either of these or any other visually ascertainable parameters in the presences of the different cations was noted in the time-lapse cinematographic studies. To the author's knowledge, no one has ever succeeded in unambiguously and directly measuring the strength of adhesion of one cell to another in either absolute or even relative terms (Steinberg, '64).

#### DISCUSSION

In the years that have elapsed since Herbst ('00) performed his classical experiment of dissociating sea urchin embryos into individual cells through removal of calcium ions, considerable evidence has accumulated to support the idea that the presence of divalent cations in the external environment is required for animal cells to be able to adhere to one another. It was found that many (but not all) other tissues could also be dissociated upon removal of divalent cations from the environment (for reviews see Steinberg, '58; and Curtis, '62). These dissociated cells could often be caused to reaggregate if and only if divalent cations were restored (Allison and Lancaster, '64; Feldman, '55; Galtsoff, '25; Moscona, '62; Steinberg, '62).

One hypothesis which has been put forth to account for this dependence of cell adhesion upon divalent cations has had rather wide acceptance (Gray, '31; Hoeber, '45). The ability of animal cells to adhere to one another is supposed to require an intercellular glue whose stability is dependent upon the presence of divalent cations in the medium. This is similar to the calcium pectinate which is thought to "glue" plant cells together (True, '22). If divalent cations are removed, the intercellular glue is supposed to dissolve and the cells are now no longer able to adhere to one another. A variant of this hypothesis was proposed by Steinberg ('58),

in which the divalent cation itself is the glue, forming salt bridges between opposed anionic sites on adhering cells.

An alternative hypothesis, and the one which has been examined in the present study, has proposed that the major role of divalent cations lies in their ability to reduce the negative charge at the surfaces of cells. The fact that different cations have differing abilities to promote reaggregation of dissociated tissue cells, *all at the same degree of reduction of surface charge*, suggests that divalent cations do indeed have adhesion-related actions other than their ability to lower surface charge. The fact that reaggregation in the presence of strontium or barium ions is minimal would seem to indicate that these other "adhesion-related actions" are of considerable importance.

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## ADDENDUM

An interesting abstract published by Lipson, Dodelson, and Hays ('65) *Clin. Res.*, 13: 237 came to the author's attention only after this study had been submitted for publication. These investigators compared the abilities of various inorganic cations to reduce the net negative surface charge of toad bladder epithelium cells (measured by electrophoresis of dissociated cells) with the abilities of these ions to promote re-establishment of cell adhesions (measured by the re-establishment of electrical resistance between luminal and serosal sides of the epithelium in the absence of other multivalent cations). Their findings are quite in accord with those presented in this paper; although all ions tested in electrophoresis experiments ( $Mg^{++}$ ,  $Ca^{++}$ ,  $Ba^{++}$ ,  $Cd^{++}$ , and  $Co^{++}$ ) could lower net negative charge, only  $Ca^{++}$  among the above ions was capable of restoring trans-membrane resistance.  $Sr^{++}$ ,  $Mn^{++}$ , and  $Al^{+3}$  also had the latter capacity.

The first of these was the... the second... the third... the fourth... the fifth... the sixth... the seventh... the eighth... the ninth... the tenth... the eleventh... the twelfth... the thirteenth... the fourteenth... the fifteenth... the sixteenth... the seventeenth... the eighteenth... the nineteenth... the twentieth... the twenty-first... the twenty-second... the twenty-third... the twenty-fourth... the twenty-fifth... the twenty-sixth... the twenty-seventh... the twenty-eighth... the twenty-ninth... the thirtieth... the thirty-first... the thirty-second... the thirty-third... the thirty-fourth... the thirty-fifth... the thirty-sixth... the thirty-seventh... the thirty-eighth... the thirty-ninth... the fortieth... the forty-first... the forty-second... the forty-third... the forty-fourth... the forty-fifth... the forty-sixth... the forty-seventh... the forty-eighth... the forty-ninth... the fiftieth... the fifty-first... the fifty-second... the fifty-third... the fifty-fourth... the fifty-fifth... the fifty-sixth... the fifty-seventh... the fifty-eighth... the fifty-ninth... the sixtieth... the sixty-first... the sixty-second... the sixty-third... the sixty-fourth... the sixty-fifth... the sixty-sixth... the sixty-seventh... the sixty-eighth... the sixty-ninth... the seventieth... the seventy-first... the seventy-second... the seventy-third... the seventy-fourth... the seventy-fifth... the seventy-sixth... the seventy-seventh... the seventy-eighth... the seventy-ninth... the eightieth... the eighty-first... the eighty-second... the eighty-third... the eighty-fourth... the eighty-fifth... the eighty-sixth... the eighty-seventh... the eighty-eighth... the eighty-ninth... the ninetieth... the ninety-first... the ninety-second... the ninety-third... the ninety-fourth... the ninety-fifth... the ninety-sixth... the ninety-seventh... the ninety-eighth... the ninety-ninth... the hundredth.

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