STUDIES OF CELL DEFORMABILITY

IV. A Possible Role of Calcium in Cell Contact Phenomena

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ABSTRACT

Cells grown in suspension culture were incubated with EDTA-disodium salt and shown to have more easily deformable surfaces and raised electrophoretic mobility than controls, following this treatment. The reversibility of these observations by the addition of calcium ions, and other parallel experiments, support the conclusion that, in these cells, calcium is bound to anionic sites at the cell periphery, some of which are located at the cellular electrokinetic surface. These cells should, therefore, exhibit demonstrable calcium-sensitive aggregation, if current theories on the role of calcium in the physiological situation are correct. The fact that no calcium-sensitive aggregation was observed suggests that calcium does not form "bridges" between the adjacent anionic sites on different cells, and does not act directly by its effects on the diffuse electrical double-layer in this situation. An alternative hypothesis is advanced for the role played by calcium in cell adhesion and separation processes.

In the previous paper of this series, some effects of incubating murine ascites sarcoma 37 cells with EDTA were described, and a model for calcium-binding within their peripheral zones, in the presence of physiological quantities of calcium ions, was tentatively suggested (1). In the present communication, further experiments on EDTA-treatment of other cells are described which enable another model to be advanced which relates to calcium in the cell periphery, to cell adhesion, and cell separation. The two models are complementary rather than mutually exclusive, and will be discussed in general biological context.

MATERIAL AND METHODS

Throughout the experiments, use was made of RPMI No. 41 cells which were derived from a human osteogenic sarcoma (2), and which have been maintained in suspension culture at 37°C in synthetic medium RPMI 906 (3) plus 5% calf serum. The withdrawn samples contained between 2.5×10^5 and 5.0×10^5 cells per ml, of which at least 94% were viable as judged by their ability to exclude trypan blue.

Cells removed from the culture vessels were washed in either Hanks' balanced salt solution (HBSS) which contains 0.0013 M $CaCl_2$ and 0.002 M $MgCl_2$ in addition to univalent ions, or calcium-magnesium-free HBSS (CMF), and then incubated in either 0.5% (0.013 M) disodium EDTA (EDTA) dissolved in CMF and having a final pH of 5.7, or control solutions, at 37°C for 30 min. Following incubation, the cells were washed twice in either CMF or HBSS at pH 7.0–7.3.

For the purpose of testing the specificity of EDTA for calcium and magnesium in its effect on cell deformability, cells were also incubated for 30 min at 37°C in 0.5% magnesium disodium ethylenediamine tetraacetate ($MgNa_2EDTA$) in CMF, and in a 0.5% solution of the calcium disodium salt of EDTA ($CaNa_2EDTA$) in CMF. In some experiments, an attempt was made to restore the original properties to EDTA-treated cells by reincubating them for 30 min at 37°C in HBSS, CMF + Mg^{++} containing 0.002

M. $MgCl_2$, or CMF + Ca^{++} containing 0.0013 M. $CaCl_2$.

Viability tests were made on cells incubated for 30 min at 37°C in 0.5% EDTA in CMF and CMF alone. After incubation, the cells were washed twice in CMF, resuspended in conditioned medium, and incubated in test tubes on a roller drum. 3 days later, the total cell count in each tube was made, including both free cells and cells detached from the culture tubes by incubation for 40 min at 37°C in 0.2% trypsin (Difco 1:250) in CMF. Viability was also assessed by trypan blue uptake.

Cellular deformability was assessed as previously described (1, 4), by measuring the negative pressure in centimeters of Brodie's fluid (relative density 1.007), applied at an accurately maintained rate of −0.2 cm per sec, required to suck a hemispherical bulge from cells into fluid-filled, smooth square-ended pipettes of 5–6 μ internal diameter.

Cellular electrophoretic mobility was measured in a cylindrical cell apparatus of the type described by Bangham et al. (5). Cells were timed to travel 25 μ in both directions alternately, at 37°C, with a potential difference of 50 volts applied over 16.5 cm.

In aggregation experiments, cells were washed once in CMF, and next incubated at 37°C for 15 and 30 min, in either CMF or 0.5% EDTA in CMF; they were then washed and resuspended in CMF. Measured quantities of cell suspension were added to 20 times their own volume of various solutions described below to give a final concentration of approximately 20,000 cells per ml, gently centrifuged at approximately 10 *g* for about 1 min, and incubated at 37°C for 15 min and 2 hr. Specimens of each deposit were gently withdrawn with Pasteur pipettes and placed into flat-bottomed cavity slides. By the use of a graticule eyepiece, the percentage of cells present as singlets, doublets, triplets, or in groups of four, was determined. The numbers of free single cells remaining were expressed both as a percentage of singlets plus doublets plus triplets plus quadruplets (i.e. per cent of cell groups) and as the percentage of the total numbers of cells present. Of the many solutions tested, only one group will be described:

Cells incubated in CMF were washed, suspended, centrifuged, and incubated in CMF or HBSS; cells incubated in 0.5% EDTA in CMF were washed, suspended, centrifuged, and incubated in CMF or HBSS; a mixture of equal volumes of cells incubated in CMF and 0.5% EDTA in CMF were washed, suspended, centrifuged, and incubated in CMF; cells incubated in HBSS, were washed, suspended, centrifuged, and incubated in HBSS.

As a test of the sensitivity of the agglutination technique for quantitating cell adhesion, cells washed in HBSS were suspended in doubling dilutions of antiserum, anti-S, prepared in rabbits against another strain of cultured human cells. The antiserum was previously heated at 56°C for 30 min to inactivate complement. Agglutination was just perceptible when anti-S was mixed with RPMI No. 41 on a microscope slide. In the "tube" agglutination experiments, the cell concentrations and other procedures were similar to those used in the previous experiments.

RESULTS

Viability Tests

The initial inoculum of cells per culture tube was 154×10^3. After 3 days in culture, there was a mean of 1407 ± 107 (SE) $\times 10^3$ cells in each of 10 cultures containing cells previously incubated with 0.5% EDTA in CMF, compared with $1484 \pm 136 \times 10^3$ cells in cultures containing cells incubated with CMF alone. This difference is not significant ($t = 0.482$ with 18 df; $0.7 > p > 0.6$).

Trypan blue exclusion tests showed no differences in vaiability between cells incubated for 15 min at 37°C in HBSS, CMF, and 0.5% EDTA, when compared with cells freshly removed from suspension culture (6% dead). After 2 hr of incubation, 10% were dead (i.e. stained) in HBSS, 9% in CMF, and 25% in 0.5% EDTA.

Cell Deformability

The results shown in Table I indicate that, compared with controls incubated in HBSS, cells incubated in CMF show no change in this mechanical parameter ($t = 1.65$ with 158 df; $p \doteq 0.1$). Following incubation in 0.5% EDTA in CMF, the cells are more easily deformable than those incubated either in HBSS ($t = 2.90$ with 88 df; $0.025 > p > 0.020$) or in CMF ($t = 2.02$ with 188 df; $0.05 > p > 0.025$). Incubation of cells in $CaNa_2EDTA$ in CMF produced no detectable change in deformability compared with CMF-treated cells ($t = 0.04$ with 138 df; $p > 0.9$). On the other hand, incubation of cells in $MgNa_2$ EDTA produced a significant increase in deformability, compared with CMF-treated controls ($t = 4.22$ with 148 df; $p < 0.001$).

Double incubation experiments showed that, after EDTA-treatment, reincubation in CMF + Ca^{++} resulted in cells deformable by 5.02 cm of negative pressure. Although this pressure is not significantly higher than the 4.97 cm required to deform cells treated with EDTA alone ($t = 0.344$ with 68 df; $0.8 > p > 0.7$), it is not significantly lower than the corresponding pressure (5.34 cm) required for cells incubated in CMF alone ($t =$

TABLE I

Negative Pressures Required to Produce Standard Hemispherical Deformations of RPMI No. 41 Cells into 5-μ Pipettes after the Indicated Treatments

Incubation media	Deformation pressures in cm Brodie's fluid ± SE (No. of observations)
Single incubations:	
HBSS	5.72 ± 0.19 (30)
CMF	5.34 ± 0.10 (130)
EDTA in CMF	4.97 ± 0.15 (60)
$MgNa_2EDTA$ in CMF	4.12 ± 0.33 (20)
$CaNa_2EDTA$ in CMF	5.51 ± 0.59 (10)
Double incubations:	
EDTA then CMF	5.03 ± 0.24 (20)
EDTA then CMF + Ca^{++}	5.02 ± 0.18 (35)
EDTA then CMF + Mg^{++}	4.78 ± 0.27 (20)

1.476 with 163 df; $0.2 > p > 0.1$). In contrast, when EDTA-treated cells were reincubated in CMF + Mg^{++}, the mean deformation pressure of 4.78 cm is not higher than the pressure required for EDTA-treated cells, but is significantly lower than the mean pressure required to deform cells incubated in CMF only ($t = 1.99$ with 148 df; $0.05 > p > 0.02$). When EDTA-treated cells were reincubated in CMF alone, the deformation pressure of 5.03 cm was not significantly different from either cells incubated in CMF alone ($t = 1.11$ with 148 df; $0.3 > p > 0.2$) or in EDTA ($t = 0.197$ with 78 df; $0.9 > p > 0.8$).

Electrophoretic Mobilities

Cells were treated as in the deformability and aggregation experiments, and their electrophoretic mobilities were measured, in an attempt to define their electrokinetic status during these experiments. The results are shown in Table II. *t* Tests reveal that incubation of No. 41 cells in CMF alone results in their having a significantly higher mobility than those treated in HBSS alone ($t = 3.4$ with 125 degrees of freedom; $0.01 > p > 0.001$). After treatment of cells with EDTA, their mobilities measured in CMF were significantly higher than those of cells incubated in CMF alone ($t = 3.2$ with 150 degrees of freedom; $p \doteq 0.001$).

The experiments in which after EDTA-treatment the cells were incubated for various lengths of time in CMF or HBSS indicate that the mobilities of the cells in CMF show an initial slight reduction, but after 30 min remain steady for 2 hr. On the other hand, the mobilities of the cells in HBSS fall, within 30 min, to a level not significantly different from that of cells treated only with HBSS, and then remain steady, within the limits of experimental error, for 2 hr.

Cells measured in media of constant ionic strength, but containing various amounts of calcium chloride, show a progressive decrease in electrophoretic mobility over the range of 0–0.05 M $CaCl_2$. This loss is shown decrementally ($\Delta\mu$/molarity $CaCl_2$) in Fig. 1.

Cell Aggregation

The results shown in Table III reveal a progressive increase in the percentage of single cells as the concentration of antiserum is decreased. A *t* test shows that there are significantly fewer free, single cells in 100% antiserum than in 12.5% antiserum ($t = 3.60$ with 14 degrees of freedom; $0.01 > p > 0.001$).

The results summarized in Table IV show the percentages of cells remaining as singlets, after preincubation in HBSS, CMF, or 0.5% EDTA in CMF, followed by centrifugation and reincubation in serum-free CMF, 0.5% EDTA in CMF, or HBSS for 15 min and 2 hr. The data do not reveal detectable changes in cell aggregation after preincubation in HBSS, CMF, or EDTA followed by washing and incubation in HBSS for 15 min or 2 hr. After preincubation in EDTA solution, followed by washing and incubation in HBSS, CMF, or EDTA, there are no detectable changes in cell aggregation after 15 min, but after 2 hr there are more free cells in EDTA than in either CMF or HBSS. However, dye-exclusion viability tests showed that at least 25% of the cells were dead, after incubation in EDTA for 2 hr. Preincubation in CMF followed by washing and reincubation in this medium for 15 min resulted in 33.3% of the total cells being present as singlets; this percentage is not significantly different from the 37.4% following preincubation in CMF and reincubation in HBSS ($t = 1.28$ with 17 degrees of freedom; $0.3 > p > 0.2$) or from the 39.4% single cells present after preincubation in HBSS and reincubation in this medium for 15 min ($t = 0.90$ with 17 degrees of freedom; $0.4 > p > 0.3$). After 2 hr of reincubation, even these differences in means are reduced. Thus, when comparable treatment groups are examined, no

TABLE II

The Electrophoretic Mobilities of RPMI No. 41 Cells, Expressed in μsec^{-1} $volt^{-1}$ cm, after the Treatments Indicated

Initial incubation	2nd incubation	Measured in	Mobilities ± SE (No. observation)
CMF	CMF	CMF	−1.534 ± 0.03 (64)
HBSS	HBSS	HBSS	−1.402 ± 0.03 (63)
EDTA	CMF	CMF	−1.684 ± 0.03 (76)
EDTA	CMF—30 min	CMF	−1.555 ± 0.04 (66)
EDTA	HBSS—30 min	HBSS	−1.418 ± 0.04 (60)
EDTA	CMF—1 hr	CMF	−1.530 ± 0.04 (61)
EDTA	HBSS—1 hr	HBSS	−1.395 ± 0.04 (52)
EDTA	CMF—2 hr	CMF	−1.519 ± 0.05 (52)
EDTA	HBSS—2 hr	HBSS	−1.363 ± 0.04 (52)

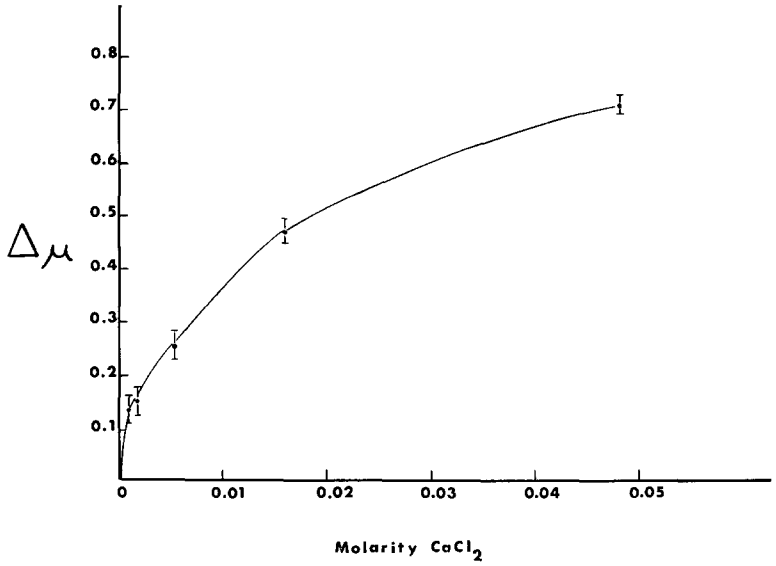

FIGURE 1 The decrease in electrophoretic mobility ($\Delta\mu$) of RPMI No. 41 cells as a function of $CaCl_2$ concentration in their suspending fluids. Each point shows the mean and standard error of 40–50 observations. All suspending fluids were of equal ionic strength containing varying ratios of NaCl and $CaCl_2$, and were made isotonic with sucrose. The bulk-phase pH value was 7.0–7.4 throughout. Further experimental details are given by Weiss and Mayhew (34).

effects of calcium on cell adhesion is detectable by this technique.

DISCUSSION

The viability tests show that incubation of RPMI No. 41 cells with 0.5% EDTA for up to 30 min does not produce detectable lethal changes in them. These results are consistent with the morphological studies made by Dornfeld and Owczarzak (6) on cultured chick fibroblasts. The 15-min experiments are, therefore, concerned with living cells; the experiments in which cells were incubated for 2 hr in EDTA solutions contain too many dead cells to permit interpretation.

As noted earlier, after EDTA was dissolved in CMF the pH of the mixture was 5.70. The pK

values of the carboxyls of EDTA are as follows: pK_1 = 1.996, pK_2 = 2.672, pK_3 = 6.161, and pK_4 = 10.26 (7), and this is reflected in the pH-sensitive dissociation of metal complexes with EDTA. Thus at pH 5.7, approximately 75% of the EDTA molecules will be doubly ionized and 25% triply ionized, whereas at pH 7.0 approximately 75% will be triply ionized and 25% doubly ionized. As calcium has its main affinity for triply ionized EDTA, it is expected that at the lower pH the EDTA will chelate less calcium. Some idea of the magnitude of this effect is given by Chaberek and Martell's data (7) which show that approximately 1.0 and 1.75 moles of EDTA are required to complex 1 mole of calcium at pH values of 7.0 and 5.7, respectively. Thus, although EDTA at pH 5.7 is not chelating calcium as efficiently as it would at pH 7.0, it is nonetheless chelating it.

Evidence that EDTA-treatment produces changes in the peripheries of these cells is provided by data from the deformability and electrophoresis experiments, and these will now be considered separately.

Single incubations of cells in CMF alone enabled them to be deformed by negative pressures of 5.34 cm of Brodie's fluid, compared with 5.72 cm in HBSS which contains both calcium and magnesium ions. This difference is not statistically significant, and subsequent comparisons of deformation pressures following other treatments were made against cells incubated in CMF, since this solution was used as a vehicle. In contrast to the ascitic form of sarcoma 37 cells described in the previous paper in this series (1), in which a 42% reduction in pressure was required to produce a standard deformation after incubating them in 0.2% EDTA, the RPMI No. 41 cells showed only a 13% mean reduction after incubation in 0.5% EDTA; pilot experiments which are not reported showed no detectable deformability changes in

TABLE III

The Percentage (± SE) of the Total Cells Present Remaining in the Single State, after Incubation in Doubling Dilutions of Weak Antiserum

Each percentage is the mean of eight experiments.

	Antiserum concentration			
	100	50	25	12.5
	%	%	%	%
Percentage total cells free ± SE: Anti-S	25.7 ± 3.4	32.2 ± 3.5	40.4 ± 2.8	49.8 ± 5.8

TABLE IV

The Percentage (± SE) of Single Cells in the Media Shown, after 15 Min and 2 Hr of Incubation at 37°C

In each experiment, 200 cells or aggregates were counted.

Initial incubation in:	Final incubation in:	15 min of final incubation Per cent singlets ± SE (No. expts.) in		2 hr of final incubation Per cent singlets ± SE (No. exp.) in	
		Total cell	Cell groups	Total cells	Cell group
HBSS	HBSS	39.4 ± 3.7 (10)	63.0 ± 3.2 (10)	28.5 ± 2.4 (10)	53.2 ± 2.6 (10)
CMF	CMF	33.3 ± 2.9 (9)	58.7 ± 4.9 (9)	31.6 ± 6.9 (7)	52.5 ± 7.0 (7)
CMF	HBSS	37.4 ± 3.5 (10)	60.6 ± 3.5 (10)	32.7 ± 2.0 (10)	58.5 ± 2.1 (10)
EDTA	HBSS	43.7 ± 3.0. (10)	66.6 ± 2.5 (10)	32.1 ± 3.4 (10)	58.0 ± 3.4 (10)
EDTA	CMF	41.9 ± 2.4 (19)	66.8 ± 2.6 (19)	33.6 ± 4.0 (17)	56.6 ± 4.0 (17)
EDTA	EDTA	41.5 ± 2.7 (9)	69.3 ± 3.6 (9)	51.6 ± 4.5 (7)	72.8 ± 3.8 (7)
CMF* / EDTA	CMF	37.7 ± 1.7 (9)	66.6 ± 3.8 (9)	28.4 ± 3.5 (7)	52.0 ± 4.0 (7)

* 50% of cells were incubated initially in CMF and 50% in EDTA, before mixing and incubating in CMF.

lower concentrations of EDTA. This smaller reduction has made the demonstration that the effect of EDTA is mediated through chelation of calcium ions more difficult. However, the fact that the calcium disodium salt of EDTA is without demonstrable effect on cell deformability, whereas the magnesium and disodium salts produce significant reduction in this parameter, is in accord with the progressive relative affinities of these salts for calcium ions (7).

Other suggestive evidence that EDTA acts through calcium chelation comes from the experiments in which EDTA-treated cells have their mean deformabilities restored to a pressure not significantly different from the controls, by reincubation with CMF + Ca^{++}, but not by reincubation in CMF + Mg^{++}. The fact that following EDTA-treatment reincubation in CMF also apparently restores the deformability to a level not significantly different from the control is in accord with a similar observation made on ascites tumor cells (1) and explained in terms of a slow leak of calcium from an inner "compartment" into the cell peripheral zone.

Although EDTA may also produce intracellular effects, Dornfeld and Owczarzak (6) were unable to demonstrate these, and Wolpert (8) suggests that the deformability measurements are insensitive to intracellular changes of the type compatible with viability. Within the limits of sensitivity of the deformability experiments, it is concluded that EDTA makes the RPMI No. 41 cells more easily deformable by removing calcium from their peripheries.

The apparently calcium-dependent surface changes produced by incubation of RPMI No. 41 cells with EDTA are consistent with the work of others. Thus, Borei and Björklund (9) showed by centrifugation experiments that the surfaces of EDTA-treated, unfertilized sea urchin eggs were less rigid than in controls. On the other hand, Mitchison (10) was unable to demonstrate changes in the deformability of EDTA-treated, unfertilized sea urchin eggs, using a microelastimeter of the type on which the present apparatus was based. Mitchison's experiments were made with 0.001 M EDTA, compared with 0.013 M used in the present experiments, and changes of less than 20%, based on five measurements, were disregarded; it is, therefore, impossible to compare Mitchison's findings with my own. Dornfeld and Owczarzak (6) showed that the surface changes produced by them in mouse fibroblasts were not produced by calcium, magnesium, or ferrous chelates of EDTA, and that the EDTA effects themselves were rapidly reversed by its removal. Robbins and Micali (11) also noted changes in the "cortex" of HeLa cells following EDTA treatment which were reversible by addition of calcium ions. My own previous work on the reversal of increased deformability of EDTA-treated murine ascites tumor cells by reincubating them in calcium-containing media (1) also supports the suggestion that deformability experiments can be used to indicate that calcium is bound in the cell periphery, that it affects the mechanical strength of this region, and that it may be removed by EDTA.

Measurements of cellular electrophoretic mobility showed that cells incubated in EDTA and observed in CMF had higher mean mobilities than cells incubated and measured in HBSS. EDTA-treated cells measured in CMF had significantly higher mobilities than EDTA-treated cells measured in HBSS.

Examination of the Helmholtz-Smoluchowski equation shows that electrophoretic mobility, μ, may be related to surface charge density, σ, by

$$\mu = \frac{\sigma}{K\eta}$$

where $1/K$ is the effective electrical double-layer thickness, and η the dynamic viscosity at the cellular hydrodynamic slip plane. Thus, regardless of changes in electrophoretic mobility brought about by binding of calcium ions to the cell surface, changes can also arise from the diminution of $1/K$ brought about by the presence of calcium ions in the cellular microenvironment.

Surface charge density may be related to ionic concentration, n, and valency, z, within the double-layer by a form of the Gouy-Chapman equation quoted by Haydon (12):

$$\sigma = \sqrt{\frac{DkT}{2\pi}} \cdot \sqrt{\sum n_i \left(e^{-\frac{z_i \epsilon \psi_0}{kT}} - 1\right) + \sum n_j \left(e^{-\frac{z_j \epsilon \psi_0}{kT}} - 1\right)}$$

where, ψ_0 = surface potential; ϵ = electronic charge; Z_i and Z_j = valency of species "i" and "j"; n_i and n_j = number of ions per ml of species "i" and "j"; k = boltzmann constant, and D = dielectric constant in the double-layer. The effect

on the double-layer of the additional calcium and magnesium ions present in the HBSS, compared to the CMF, will be to decrease the electrophoretic mobility by less than 2%. Therefore, the differences of 9–16% observed between the mobilities of cells suspended in CMF and HBSS following EDTA-treatment cannot be attributed to a first-order effect of calcium and magnesium ions on the electrical double-layer surrounding the cells, but strongly suggest that incubation with EDTA removes bound calcium ions from the cellular electrokinetic surface, which are replaced to some extent from HBSS.

In the previous study on sarcoma 37 cells, it was shown that the increased deformability on EDTA-treatment was not accompanied by detectable changes in cellular electrophoretic mobility; and it was suggested that at *physiological concentrations* calcium does not bind appreciably to anionic groups located closer than about 10 A from the hydrodynamic slip plane of these cells. The possibility was raised that sarcoma 37 cells may, in fact, exist in the free ascitic form because their calcium-binding anionic sites are located too deeply within their peripheries to form calcium "bridges" with adjacent cells. In contrast to the ascites tumor cells, the RPMI No. 41 cells show evidence (Table II and Fig. 1) of binding calcium to sites detectable electrophoretically, and hence located superficially. If calcium plays a part in promoting cell contact, and then forms bridges between peripheral anionic groups of adjacent contacted cells, it should be possible to show that agglutination of RPMI No. 41 cells is dependent on environmental concentration of calcium ions.

The technique of quantitating cell adhesion by agglutination experiments has been used by Armstrong (13) and Wilkins et al. (14, 15) among others; and experiments made with agglutinating antisera show it to be a sensitive test of cell adhesion as used in the present study. It should be noted that, in other studies on agglutination with No. 41 cells, as few as 30% of singlets have been observed in "normally" growing populations in spinner flasks, and up to 90% singlets in populations incubated for 45 min in EDTA. Thus, this particular system is capable of detecting agglutination changes beyond the ranges shown in Tables III and IV.

The experimental data shown in Table IV fail to reveal an effect of calcium on cell aggregation in that, in comparable treatment groups, the presence of calcium does not demonstrably promote adhesion, and its absence does not demonstrably prevent it. It is unlikely that the effects of calcium in the adhesive process are masked by its leakage from the cells into their environment, as Levinson has shown that the calcium content of both Ehrlich ascites cells (C. Levinson, 1967. Submitted for publication) and RPMI No. 41 cells (unpublished data) is independent of external calcium concentration within experimental limits, which are capable of detecting a 10% change. RPMI No. 41 cells contain 0.013 μM Ca per 10^6 cells, and were used in a concentration of approximately 2×10^4 cell per ml of suspending fluid in the agglutination experiments. Therefore, leakage of 10% of their calcium content into the suspending fluid would raise the environmental calcium concentration by only 0.000026 μM per ml. The possibility that many of the potential calcium-binding sites were already occupied in CMF-treated cells, and that this prevented them calcium-bridging to similar adjacent cells, was tested by mixing equal numbers of EDTA-treated and CMF-treated cells, and observing their aggregation patterns in CMF. As shown in Table IV, no significant increase in cell to cell adhesion was demonstrated in these "mixed" cultures.

It might also be argued on the basis of the deformability data that, after pretreatment with EDTA, incubation of the cells in either CMF or CMF + Ca^{++} results in physiological saturation of calcium-depleted binding sites, with calcium ions moving out from an inner compartment, or in from the environment, respectively. The fact that there is an increase in electrophoretic mobility of these cells following treatment with EDTA and subsequent incubation in CMF and HBSS suggests that calcium removed from the cellular electrokinetic surface is not entirely replaced from either the environment or from inner compartment(s) within the time limits of the present experiments. As the electrophoretic mobilities of EDTA-treated cells measured in HBSS were lower than those of EDTA-treated cells measured in CMF, it appears that replacement of calcium chelated from their electrokinetic surfaces occurs faster from the medium than from outward diffusion from internal regions. Thus, over the time taken for at least the 15-min agglutination experiments, cells pretreated in EDTA and reincubated in CMF will have less calcium bound to their electrokinetic surfaces than similarly treated cells reincubated in HBSS.

The increased aggregation of CMF-treated cells reincubated in CMF, compared with those first treated with EDTA and then incubated in CMF for 15 min (t = 2.0 with 26 degrees of freedom; $p \simeq 0.05$), provides, at first sight, limited solitary support to the theory that calcium, within the physiological range, plays some part in cell aggregation, since on both electrophoretic and deformability evidence incubation in CMF removes less calcium from these cells than incubation in EDTA solution. On the other hand, the data in Table IV show that this difference between the two treatment groups is not maintained after 2 hr of reincubation of the cells in CMF. In addition, when groups were subjected to similar pretreatment in EDTA, and then reincubated for 15 min in HBSS, CMF, or EDTA, no differences in agglutination were detectable. The diminished viability in the cells reincubated for 2 hr in EDTA prevents the use of these agglutination results in the present discussion.

It has been suggested at various times that calcium ions may promote cell contact by reduction of the potential enerby barriers keeping cells apart. In the case of cells which have made contact with each other, the divalent calcium ions may promote their mutual adhesion by "bridging" between adjacent anionic sites on the cells themselves or intervening macromolecules. Alternatively, calcium ions may affect cell contact interactions by other mechanisms. Each of these possibilities will be discussed in the light of the present experimental evidence.

The possible relevance of the theories of Derjaguin and Landau (16) and Verwey and Overbeek, (17), known as DLVO-theory, to the problems of cell contact has been discussed by Curtis (18), Pethica (19), Weiss and Woodbridge (20) and Weiss (21). In general terms, the electrostatic energies of repulsion, V_R, tend to keep cells apart, while the London-Van der Waals' attractive energies tend to bring cells together. Some of the pitfalls in the application of the DLVO theory to this biological situation have been discussed elsewhere (20, 22). The energy of repulsion between two hemispherically ended cell processes may be approximated to:

$$V_R = \tfrac{1}{2}\, Dr\, \psi_0^2 \log_e(1 + e^{-Kd})$$

where ψ_0 = surface potential; D = dielectric constant; r = radius of curvature of cell processes; $1/K$ is the Debye-Hŭckel parameter of effective thickness of the diffuse ionic double-layer; and d = distance separating the cell processes. Consideration of this equation for V_R reveals that calcium ions may promote cell contact by reduction of the Debye-Hŭckel parameter. At 25°C,

$$1/K = 3.05 \int I \text{ A (23), where}$$

$$I = \textstyle\sum_i c_i\, z_i^2 \text{ (24), where}$$

c_i = ionic concentration, and z_i = valency. Thus, addition of calcium ions will produce a reduction in V_R (through $1/K$). This aspect of DLVO thoery is applicable in general terms to the adsorption of virus particles (25) and agglutination of leukocytes (14, 15). The theory was also invoked to account for some observations (26) in which it was shown that the percentage of cells shaken off glass surfaces to which they were attached, was sensitive to the calcium concentrations in their culture media. However, this interpretation of the experimental data is probably incorrect since it could at most explain cell attachment, but not cell detachment. The general weakness in all of these types of experiments is that an effect of calcium in reducing $1/K$, and hence V_R, is expected to be demonstrable only in media of unphysiologically low ionic strength. In physiological media, which have high ionic strength in colloid terms, the effect of physiological concentrations of divalent cations on $1/K$ is expected to be slight, as shown in the previous consideration of the Gouy-Chapman equation. Also, if this mechanism plays a part in cell adhesion in the physiological environment, it should be demonstrable in the data shown in Table IV. The failure to demonstrate an equivocal effect of calcium on cell agglutination is in agreement with Armstrong's (13) experiments on cells from EDTA-dissociated embryonic chick limb buds, in which culture in similar ionic strengths of the divalent cations calcium, magnesium, strontium, and barium did not produce cell aggregation of the same magnitude, as would be expected if the primary effect of these ions was to reduce the effective thickness of the electrical double-layer.

In addition to diminishing the potential energy of repulsion between cells by reducing $1/K$, divalent ions could also reduce electrostatic repulsion by direct binding to anionic sites, thereby bringing about reduction in surface potential, ψ. The measurements of electrophoretic mobility shown in Fig. 1 may be interpreted as demonstrating

ion-binding of this nature; and the measurements on EDTA-treated cells in HBSS and CMF show that in "physiological" environments the amounts of divalent cations present can indeed affect surface potential. However, in spite of these calcium- and magnesium-induced reductions, cell agglutination was not demonstrably affected by incubation in HBSS compared with CMF.

It could be argued that the failure to demonstrate an effect of calcium on cell agglutination, under the conditions described here, in cells preincubated in either CMF or HBSS, and reincubated in these media, is due to saturation of calcium-binding surface sites after both preincubations, so that calcium-bridging is, in fact, occurring in both reincubation media. However, if the calcium-bridge hypothesis is tenable, it would be expected that if a mixture is made of CMF-treated cells in which, on electrophoretic evidence, much of their surface calcium remains bound, and EDTA-treated cells in which much of the surface-bound calcium has been removed, then agglutination through calcium-bridging should be favored. The experimental data do not, therefore, support the concept of calcium-bridging in cells in which it should be demonstrable.

Much of the confusion, in the literature, about the role of calcium in cell adhesion stems from the view that cell separation is simply the reverse of cell adhesion. Thus, the classical experiments of Herbst (27) in which sea urchin embryos were dissociated into single cells by removal of calcium ions, and many other dissociation experiments, have been interpreted as indicating that the divalent ions are involved in holding one cell to another. It has been suggested, as a result of experimental and theoretical considerations (28, 29), that, whereas cell adhesion occurs by means of interactions between the surfaces of two adjacent cells, separation need not occur in the same spatial location but is more likely to occur by means of nonlethal microruptures in the weakest parts of the whole three-dimensional cellular peripheral zone, and is thus dependent on the cohesive strength of the peripheries of individual cells.

It is known from work done on a variety of other cells that calcium plays some role in promoting cell aggregation (30–33). In aggregation experiments, aggregates result when the forces of adhesion or cohesion are greater than the forces of distraction. The distractive forces themselves may result from active cell movements, which may in themselves be calcium-dependent; shearing forces generated in various shakers or Brownian movements of filamentous processes. When these distractive forces exceed either the adhesive forces holding one cell to another cell, or the cohesive forces maintaining the structural integrity of parts of the peripheral zone of individual cells, then separation will be favored. Thus, calcium can act either by direct surface activity or alternatively by its action(s) within the cellular peripheral zone. The present experimental data do not reveal evidence of true direct surface activity, although it is possible that deeply bound calcium could orient or stabilize other groups involved in cell adhesion. On the other hand, the fact that calcium-removal makes RPMI No. 41 cells more easily deformably may indicate that their peripheral zones are weaker or less cohesive, and, therefore, that the presence of calcium in this region makes separation of adherent processes more difficult, whereas loss of calcium facilitates cell separation. It is possible that the various experiments purporting to show that calcium promotes cell adhesion do, in fact, show that calcium hinders separation, and that this explanation is more consistent with experimental data than explanations based on reduction of electrostatic energies of repulsion, or calcium "bridging."

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