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On the Role of Metal Cations in Cellular Adhesion: Cation Specificity

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ABSTRACT Of the four common alkaline earth cations, only Ca^{++} is able to protect *Rana pipiens* embryos from dissociation of the epidermis into isolated cells by EDTA. Presumably this is because only Ca^{++} is able to satisfy the divalent cation requirement for cellular adhesion in this tissue. Although early stage embryos are completely dissociable by EDTA, the axial musculature, brain and spinal cord become resistant to dissociation by stage 17. Tetraphenyl boron is also effective in disaggregating amphibian embryo tissues.

An important attribute of most cell types of multicellular organisms is their mutual adhesiveness. This permits the formation of tissues from collections of individual cells. Cell surface events are presumed to be the factors most likely involved in adhesion mechanisms since adhesions are established and maintained between cell surfaces. Almost all of the various hypotheses which have been concerned with mechanisms of adhesion have dealt principally with cell surface phenomena.

One phenomenon common to adhesion of almost every cell type which has been studied is a requirement for divalent alkaline earth cations. Removal of divalent cations promotes disaggregation of tissues into isolated cells. The cells of tissues dispersed in this manner can often be caused to reaggregate into tissue masses but only if divalent cations are added back to the medium (for references see Armstrong, '66). The data presented in this paper deal with the specificity of cation involvement.

MATERIALS AND METHODS

The eggs of *Hyla regilla* were collected in the Vaca Hills west of Davis. They were stored at 4°C until needed and then allowed to develop at 16°C in pond water. The eggs of *Rana pipiens* and *Xenopus laevis* were obtained by hormonally induced ovulation (Wright and Flathers, '61). The jelly layers of prehatched embryos of the first two species were removed with watchmakers forceps, and those of *Xenopus* were removed by incubation with cysteine (Gusseck and Hendrick, '67). The hyaline membrane could then be removed with forceps. Disaggregation was carried out at room temperature in the wells of three-depression spot plates (Pyrex cat. no. 7740) placed in 100 mm Petri dishes to retard evaporation. All solutions were made up in doubly distilled water, the second distillation being from a glass still. Stages 19–20 (newly hatched embryos – Shumway, '40) *Rana* embryos were used most extensively, but most of the results obtained with Rana were confirmed with *Xenopus* embryos (Nieuwkoop and Faber Stages 8–30, in New, '66). *Hyla* embryos were used for a few experiments. Calcium concentrations were determined using a Beckman atomic absorption spectrometer.

RESULTS

The metal cation chelating agent disodium ethylene diamine tetraacetate (Na_2-EDTA) was very effective in disaggregating several of the tissues of amphibian embryos into single cells. In later stage embryos (for example, stage 19 *Rana pipiens* embryos), EDTA disaggregated epidermis and the underlying mesenchyme and yolky endoderm but did not affect brain, spinal cord or somite tissues (fig. 1). In the concentrations employed (see table 1), the first signs of disaggregation were evident within a few minutes as one or a few foci of loosened and disaggregating epidermal cells. Disaggregation continued by the enlargement of these foci until the entire ectoderm was dissoci-

Fig. 1 Stage 18+ *Rana pipiens* embryo after having been treated for about 20 minutes with 10m*M* Na_2 EDTA. The epidermis and underlying mesenchyme have disaggregated completely and have been scraped away with a hair-loop. The yolky endoderm is dissociating along its posterior margin but is still being held in place elsewhere by a thin non-cellular membrane. The somites, brain and spinal cord are not dissociated by EDTA.

ated. EDTA treatment of *Rana* embryos at earlier stages of development (stages 14–16; neural fold to closed neural tube stages) resulted in complete dissociation of all tissues. The axial mesoderm of stages 17–18 which had not yet condensed into somites could be dissociated by EDTA, but that which had condensed could not. A similar decrease in ease of dissociation of amphibian embryos with increasing age has been noted by Curtis ('60) and Jones and Elsdale ('63).

Table 1 presents the abilities of the various common alkaline earth cations to suppress the disaggregating effects of EDTA. Although dissaggregation did not occur as rapidly as in the presence of EDTA alone, only calcium was fully effective in preventing disaggregation (fig. 2). That calcium ion did actually prevent disaggregation, as opposed to merely slowing the process down, is demonstrated by the ability of embryos to heal massive epidermal wounds completely (fig. 3). Strontium was partially effective in preventing disaggregation also. Wound healing in Sr-EDTA (20 mM Sr – 10 mM EDTA) was limited to a rounding off of the cut edges of the wound. Wounds were stabilized in this configuration; disaggregation at the borders did not occur (for 2–3 hours at least) but neither was there further closure of the wound. The borders of wounds made in embryos immersed in Mg EDTA or Ba EDTA showed signs of cell disaggregation within a few minutes.

The ability to heal large epidermal wounds was the most sensitive assay for disaggregation. Although intact embryos did not spontaneously disaggregate in solutions one millimolar in both EDTA and Mg^{++}, Ba^{++}, or Sr^{++}, they did not show wound healing in these solutions, either. Instead, the exposed margins of epidermal wounds became the site of epidermal disaggregation which spread over the entire embryo. Intact epidermis apparently presents a barrier which resists the effects of EDTA at these reduced concentrations. This idea is further suggested by the fact that embryos could heal very small epidermal wounds in 1 mM Mg EDTA, Sr EDTA and Ba EDTA as long as there was minimal disturbance of the wound borders. If the borders of small wounds were gently teased free from the underlying mesen-

TABLE 1

Dissociation of Rana pipiens embryos (stage 19) by EDTA

Solution [1] (concentration in millimole/liter)					Results	
Na_2 EDTA	Mg^{++}	Ca^{++}	Sr^{++}	Ba^{++}	Disaggregation [2]	Wound healing [3]
0	0	0	0	0		yes (3) [4]
0	10	0	0	0	no (3)	yes (3)
0	0	0	10	0	no (3)	yes (3)
0	0	0	0	10	no (3)	
10	0	0	0	0	yes (74)	no (4)
10	10	0	0	0	yes (157)	
10	0	10	0	0	no (4)	yes (13)
10	0	0	10	0	yes (3)	no (4)
10	0	0	0	10	yes (3)	
10	20	0	0	0	yes (9)	no (7)
10	0	0	20	0	no (5)	slight [5] (5)
10	0	0	40	0		slight (4)
10	0	0	0	20	yes (8)	no (4)
1	0	0	0	0	yes (56)	no (1)
1	2	0	0	0	no (6)	no (4)
1	0	1	0	0	no (1)	yes (10)
1	0	0	2	0	no (4)	no (6)
1	0	0	0	2	no (9)	no (12)
0.1	0	0	0	0	no (2)	yes (10)

[1] All solutions made up in Ca^{++} free Holtfreter's solution, pH 7.3 (Hamburger, '60).

[2] When disaggregation occurred, epidermis and underlying mesenchyme and endodermal tissues were all dissociated completely into single cells ("yes"). The first patches of disaggregating epidermis were evident within 3–30 minutes. In some solutions, small numbers of epidermal cells were lost from the embryo, but no patches of disaggregation occurred. These were included in the "no" category.

[3] Wounds were made by dissecting out a square of epidermis from the flank. In general, one of two effects was noted; either a closure of the epidermis over the wound area resulting in complete healing ("yes"), or a disaggregation of the epidermal cells at the wound border which spread over the embryo ("no").

[4] Numbers in parentheses represent number of cases.

[5] "Slight" means that the borders of the wound did not disaggregate but also did not close appreciably over the wound area.

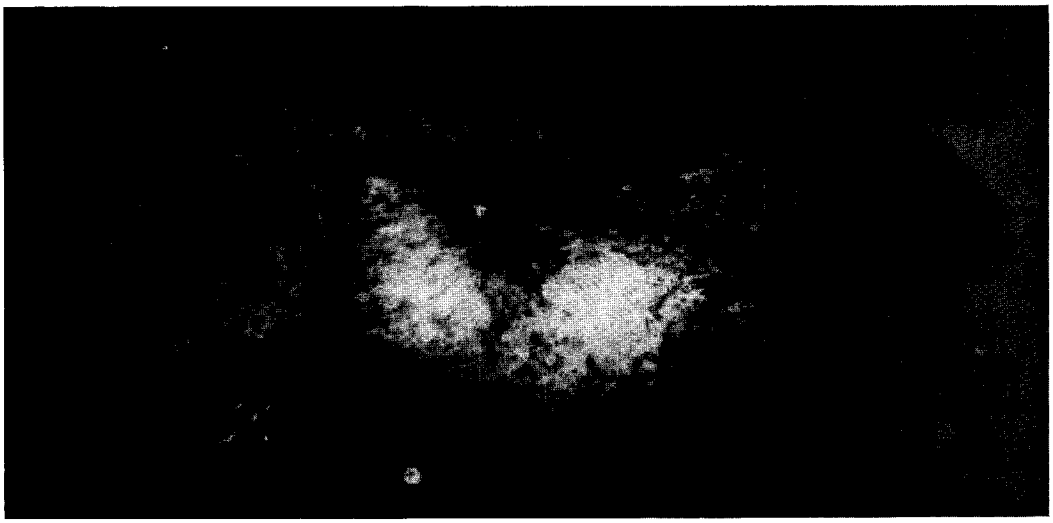

Fig. 2 Stage 18 *Rana pipiens* embryo is in the process of wound healing in 10 m*M* Ca-EDTA. The area of the wound is now less than one-fourth of the original wound.

chyme, disaggregation soon followed. It appears that access has to be provided to the under-surfaces of the epithelium for disaggregation to occur.

Sodium tetraphenyl boron (TPB) has been demonstrated to be effective in disaggregating mouse liver tissue (Rappaport, '66; Rappaport and Howze, '66a,b; Epstein, '67; Friedman and Epstein, '67). It is also effective in disaggregating amphibian embryo ectoderm, mesenchyme and endoderm (table 2). The mode of action of TPB must be different from that of EDTA since equimolar Ca^{++} has no effect on TPB action. Rappaport suggested that TPB acts by binding K^+ instead of Ca^{++} and supported this by showing that other K^+ binding agents, such as sodium picrate had a similar effect on mouse liver tissue. Since sodium picrate does not promote disaggregation of amphibian tissue (table 2), TPB may be acting in some fashion other than by binding K^+. Disaggregation by EDTA did not involve K^+ binding since even a twofold excess of K^+ did not effect the action of EDTA, and wound healing of epidermal wounds proceeded normally in the absence of K^+.

The viability of cells dissociated by the various EDTA-containing solutions remained unaffected for at least one to two hours following disaggregation as evidenced by the ability of dissociated cells to exclude nigrosin (Kaltenbach et al., '58). Ciliary activity of epidermal cells also was unimpaired for at least an hour following disaggregation. Thus it does not seem likely that EDTA-mediated dissociation is the result of cell injury. It should be noted, however, that after rather prolonged

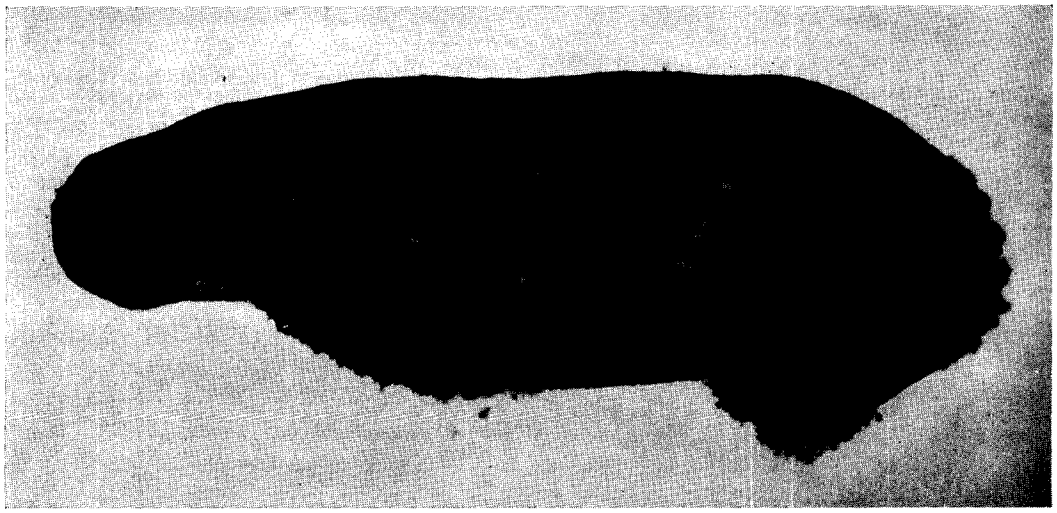

Fig. 3 Early stages of disaggregation of the epidermis mediated by 10 m*M* Mg-EDTA. The initial sign of dissociation is the appearance of patches of disaggregating epidermal cells. In this case, these have occurred on the sucker, over the posterior region of the belly and at the tip of the head. As disaggregation proceeds, the initial patches broaden in area until the entire epidermis has disaggregated.

TABLE 2

Dissociation of Rana pipiens embryo (Stage 19) ectoderm by Sodium tetraphenylboron (TPB)

Solution (concentrations in millimoles/liter)			Results	
TPB	Picrate	Ca++	Disaggregation	Wound healing
1.0	0	0	yes (5)	
1.0	0	1.0	yes (4)	
0	1.0	0	no (3)	yes (3)
0	5.0	0		yes (5)

treatment with 1m*M* 2,4-dinitrophenol (but not 1m*M* KCN) epidermis did dissociate.

The viability of cells was adversely affected by dissociation with TPB. Ciliary motion ceased soon after disaggregation and very few dissociated cells were able to exclude nigrosin. Similar results were obtained with mouse liver. In this case, the dissociated cells appeared to be fixed when examined by time lapse microcinematography as the cells were absolutely rigid with no observable motion whatsoever of their surfaces or cytoplasm. Since TPB disrupts mitochondrial structure (Harris and Leone, '66) and uncouples oxidative phosphorylation in isolated mitochondria (Utsumi and Packer, '67), it might be expected that it would be harmful to whole cells as well.

CONCLUSION

A variety of experiments have demonstrated that Ca^{++} is required for cellular adhesion. In the light of this fact, the usual interpretation of the mechanism of EDTA action as a tissue disaggregating agent is that it removes Ca^{++} ions from the intercellular space. The observation that Ca EDTA does not promote disaggregation of amphibian embryo epidermis supports the notion that EDTA acts by binding Ca^{++}. Also EGTA, a divalent cation chelating agent which shows a very strong preference for Ca^{++} over the other alkaline earths (Boyd et al., '65), is about equal to EDTA in effectiveness. Treatment of embryos with Mg-, Sr- or Ba-EDTA presumably results in removal of Ca^{++} from the embryo by formation of the Ca-EDTA complex and the concomitant liberation of free Mg^{++}, Sr^{++} or Ba^{++}. Ca^{++} was detected in various dissociation media (10m*M* EDTA and 10m*M* Mg-EDTA were examined) used to dissociate stage 19 embryos (about 1×10^{-8} moles was removed by the dissociation medium from an embryo during dissociation). Since Mg^{++}, Sr^{++} and Ba^{++} are ineffective (or only partially effective in the case of Sr^{++}) in suppressing the disaggregating effects of EDTA, it seems clear that these ions are unable to substitute for Ca^{++}.

Thus in amphibian embryo ectoderm, Ca^{++} is required for adhesion, and the requirement is specific, in the sense that Mg^{++}, Sr^{++} or Ba^{++} will not substitute for it.[1] Any attempt to account for the role of divalent cations in adhesion must also be able to account for this specificity. Cation specificity in cell-to-cell adhesion is apparently a very general occurrence as it has been demonstrated for a variety of tissues using several different techniques for assaying adhesiveness (tee table 3). It is interesting that different tissues show differing specificities. For example, Mg^{++} and Ca^{++} are effective with chick embryo limb bud tissue and Ca^{++} and Sr^{++} are effective with frog urinary bladder, but Mg^{++} is not effective with frog bladder and Sr^{++} is not effective with limb bud. Divalent cations are also required for adhesion of cells to protein-coated glass surfaces (Fischer et al., '58; Lieberman and Ove, '58; Weiss, '60; Garvin, '61). Cation specificity has also been demonstrated for this system (Fischer et al., '58; Garvin, '64, '65). Divalent cations are not required for adhesion to clean glass (Easty et al., '60; Taylor, '61, '62; Nordling et al., '65).

Alkaline earth cations are involved in a variety of processes occurring in cells. For example, the normal function of many enzymes (Williams, '60) and the selective permeability characteristics of biological membranes (Manery, '66) both require divalent cations. If the study of cation involvement in cellular adhesion is going to provide useful information regarding mechanisms of adhesion, then it is necessary that cations be involved in only a small number of processes required for adhesion. Probably the most important site of action of Ca^{++} in adhesion is at cell surfaces. Although critical evidence for this is lacking, Curtis ('57), working with dissociated *Xenopus* embryo cells, found that both the binding of the Ca^{++} lost during dissociation and the recovery of adhesiveness occurred almost instantaneously, after Ca^{++} is added back to the medium. Since transport of calcium into cells is slow (Rudenberg, '50; Hodgkin and Keynes, '57) probably only surface Ca^{++} was re-

[1] Some results obtained by Steinberg ('57) are somewhat at variance with these. Steinberg found that Mg^{++} and Sr^{++} (but not Ba^{++}) are able to support the initiation of reaggregation of dissociated newt embryonic cells. The differences may be the result of the use of different assays for cell adhesiveness or differences between urodele and anuran embryos.

TABLE 3

Spectra of divalent cation capabilities for promoting cell adhesion

Tissue	Ability to promote adhesion [1]											Reference
	Mg^{++}	Ca^{++}	Sr^{++}	Ba^{++}	Mn^{++}	Fe^{++}	Co^{++}	Ni^{++}	Cu^{++}	Cd^{++}	Al^{+3}	
Blastula (amphibian)	0	+	0	−			0	0				Steinberg ('57,'58)
Bladder epithelium (toad)	−	+	+	−	+		−	−		−	+	Lipson, Dodelson and Hays ('65)
Gastric mucosa (bullfrog)	−	+	−	−								Forte and Nauss ('63)
Limb bud (chick embryo)	+	+	−	−								Armstrong ('66)
Polymorphonuclear leukocyte (rabbit, human)	+	+			+	−		+			−	Allison and Lancaster ('64)
Polymorphonuclear leukocyte (sheep)	0	0		+					+			Wilkins, Ottewill and Bangham ('62)
Macrophage red cell (mouse, sheep)	+	+		−					−		−	Lee and Cooper ('66)
Corneal epithelium (cow)	0	+	+	0								Buschke and White ('49)

[1] Adhesion promotion: "+" means cation is effective; "0" means cation is weakly effective; "−" means cation is not demonstrably effective under the conditions employed.

moved during dissociation and is involved in adhesion. The observation that various alkaline earth cations besides Ca^{++} will support reaggregation of at least some cell types suggests that the permeability properties of the plasma membrane are not involved with adhesion, as Mg^{++}, Sr^{++}, and Ba^{++} are not able to replace Ca^{++} in maintaining membrane impermeability (Maizels, '60). Apparently leaky cells are perfectly able to adhere to, and aggregate with, one another.

One hypothesis which fails to account for a specificity of divalent cation requirement is the surface charge reduction hypothesis of Curtis ('62, '66). Curtis has suggested that divalent cations, by virtue of their positive electrical charge, act by reducing the magnitude of the net negative electrostatic charge known to be present at the surfaces of cells. Reduction of electrical charge would result in a reduction of repulsive electrostatic forces tending to drive cells apart. It is difficult to account for the observed cation specificity if surface charge reduction is the principal mode of action of divalent cations, as one would expect different cations to be more nearly equal in their abilities to promote adhesion (especially since, at least with dissociated chick embryo limb bud cells, binding of the different alkaline earth cations to cell surfaces shows little or no specificity (Armstrong, '66)).

The disaggregating ability of TPB was unexpected and its interpretation is not clear. In the amphibian embryo, TPB disaggregates exactly the same tissues as does EDTA. Somites, brain and spinal cord are unaffected. Rappaport has suggested that TPB acts by sequestering K^{+}. If this is true for amphibian ectoderm also, then removal of either Ca^{++} or K^{+} causes disaggregation. The fact that picrate did not cause disag-

gregation suggests that perhaps TPB is acting in some other manner than through potassium binding. In this regard, TPB binds substituted amino groups as well as K^+ (Flaschka and Barnard, '60). The binding of the positively charged divalent cations to cell surfaces is thought to occur by virtue of an association with ionized anionic groups which are part of cell surface macromolecules. However, if this binding also involves coordination with nearby substituted amino groups (as the substituted amino groups of EDTA act to stabilize the Ca^{++}-EDTA complex (Martell and Calvin, '52)), complexing of these by TPB might lead to destabilization of the surface Ca^{++} complexes, liberation of Ca^{++} and loss of cellular adhesion. If this is true, then it should be possible to demonstrate TPB bound to the surfaces of TPB-dissociated cells. TPB binding to mitochondrial proteins has been demonstrated by Utsumi and Packer ('67). That basic groups may be involved in Ca^{++} binding is suggested by the fact that Ca^{++}-binding to cell surfaces is reduced at high pH (Steinberg, '62; Collins, '66) and high pH solutions are able to effect disaggregation of amphibian embryo tissues (Holtfreter, '43) and bovine corneal epithelium (Buschke and White, '49).

An alternative to the charge reduction model of Curtis is the cation bridge model of Steinberg ('58) in which Ca^{++} is supposed to constitute the "glue" between cells by linking anionic groups of apposed cell surfaces with ionic bonds. Some measure of specific involvement of divalent cations in adhesion might be explainable on the basis of an extension of this model in which each Ca^{++} ion is bound to one or two ligands from each cell (as in chelates), in addition to its binding with an ionized anionic group from each cell.

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