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The Mechanisms of Glutaraldehyde-Fixed Sarcoma 180 Ascites Cell Aggregation

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Summary. Sediment height analysis was employed to investigate the mechanisms of cell aggregation by glutaraldehyde-fixed sarcoma 180 ascites cells. The aggregation of these cells proceeds by a polymer bridging mechanism in which the surface molecules of one cell associate directly with the surface molecules of adjacent cells by nonbonding interactions. The ability of adhesive surface macromolecules to serve as polymer bridges is regulated by hydrophobic and coulombic interactions. Hydrophobic interactions are not significantly involved in polymer bridging *per se,* but instead appear to operate either intramolecularly or between adjacent molecules of the same cell surface, and regulate the conformation and ability of such molecules to form stable intermolecular associations with the surface adhesive molecules of a nearby cell. A disruption of these intrasurface hydrophobic interactions generally promotes cell aggregation. Coulombic forces generated by the fixed charges of surface molecules inhibit aggregation; their diminution by charge neutralization promotes aggregation. It is likely that coulombic repulsive forces regulate intramolecular associations, interactions between adjacent molecules arising from the same cell surface, and interactions between macromolecules arising from different cell surfaces. The actual forces which serve to aggregate two fixed cells are not hydrophobic, but have characteristics commonly attributed to hydrogen bonding. Ion-pairing does not seem to play a role in the aggregation of fixed cells under physiological electrolyte conditions, nor does disulfide bridging.

The regulation of cell adhesiveness plays a prominent role in such diverse biological functions as cell motility, cell morphology, growth regulation, morphogenesis, wound healing, regeneration, and tumor invasiveness and metastasis (Weiss, 1967; Sanford *et al.,* 1970; Revel, Hoch & Ho, 1974). Despite its obvious importance, the mechanisms underlying cell adhesiveness are poorly understood. Both surface charge phenomena and surface-bound macromolecular adherents have been implicated in the process (Weiss, 1967; Muller & Zahn, 1973), but the nature and extent of their involvement is not clear. The present study employs sediment height analysis to investigate the first step in the adhesive process, that of initial association during cell aggregation. Because living cells

will not tolerate the range of conditions required for these experiments, glutaraldehyde-fixed sarcoma 180 (S180) ascites cells were used as a model system for determining the effects of salts and organic solvent denaturants upon cell aggregation. The results obtained favor the view that the outer portion of cell surfaces, presumably the external surface coat, interacts directly with the outer surface region of other cells during aggregation, that the ability of surface molecules to engage in aggregation is regulated by intrasurface hydrophobic and electrostatic interactions, and that aggregation itself involves nonhydrophobic intermolecular associations between cell surfaces.

Materials and Methods

Cells

Sarcoma 180 (S180) ascites cells were grown intraperitoneally in six-week-old Swiss white mice and harvested on the seventh day after inoculation. For maintaining the line, 0.2 ml of ascites fluid, diluted fivefold with Alsever's solution, was injected intraperitoneally into each mouse. Harvested cells were washed five times with 10–20 volumes of 0.145 M NaCl and pelleted at 4 °C by centrifugation at 300 rpm in an International refrigerated centrifuge. Such low speed centrifugation permitted separation of the large ascites cells from the smaller red blood cells and lymphocytes. Ascites cells comprised 98% or more of the final population.

Following washing, the S180 cells were fixed for 30 min at 4 °C in 2% glutaraldehyde containing 0.145 M NaCl and 5 mM Tris buffered to pH 7.5. Cells were then washed four times in 10–20 volumes of glass distilled water and stored in a solution of 0.23 M sucrose plus 0.03 M NaCl at 4 °C until use. Cells prepared in this manner retained qualitatively stable aggregative properties for at least one week, but suffered a gradual diminution in the amplitude of their response to salts and solvents over this period. All experiments were performed within three days of cell fixation.

Sediment Height Analysis

The final sediment height achieved by a cell suspension allowed to settle by gravity was used as an index of cell aggregation. Before use, cells were washed five times with 10–20 volumes of glass distilled water, then mixed with the appropriate test solutions and allowed to equilibrate for at least 2 hr at room temperature. Except for the studies of Fig. 10, all inorganic cations employed were chloride salts. Each suspension contained 7.5% cells by volume, corresponding to a number concentration of about 2×10^7 cells/ml. Suspensions were mixed by vigorous pipetting, yielding preparations in which more than 98% of the cells existed as singlets. A sample of 7.5 ml of each suspension was pipetted into a vertical clear plastic column with an internal diameter of 6 mm and a smooth flat rubber plug fitted onto the bottom. Cells were allowed to sediment by gravity to the bottoms of the columns at room temperature, and the height of each sediment was measured as a function of time until settling was completed.

Data is presented as the sediment height ratio, that is, the final sediment height attained by a test sample divided by the final sediment height of a reference. For inorganic ion

experiments the reference solution was distilled water; for all other experiments 0.145 M NaCl was used as a reference solution. Measurements of sediment height were made to the nearest half millimeter. Reference samples yielded final heights of 20–25 mm. Final sediment heights of identical columns differed by a maximum of 1 mm, and usually by about 0.5 mm. All illustrations represent an average of at least three separate experiments. Organic solvents were either of spectroscopic grade or the best commercially available quality; salts were reagent grade.

Sedimentation Velocity

The sedimentation velocity of cell aggregates was obtained by measuring sediment height as a function of time. During the early and late stages of cell settling, the accumulation of cell sediment was nonlinear with time. But during the middle stages the sedimentation rate was both linear and maximal. Velocity was determined as the change in sediment height per unit time over the linear interval.

To correct observed sedimentation velocities (V) for differences in solution viscosity (η) and density (ϱ_s), the simple form of Stoke's Law for spherical particles was assumed: $V=\{k(\varrho_c-\varrho_s)M^{2/3}\}/\eta$, where ϱ_c is cell density, M is the mass of an aggregate, and k is a system parameter. To correct for differences in solution density and viscosity, a corrected sedimentation velocity (V') was obtained by multiplying the observed velocity V by the ratio $(\varrho_c-\varrho_s)/\eta$ for the reference solution divided by $(\varrho_c-\varrho_s)/\eta$ for the test solution. Differences between two samples in their corrected sedimentation velocities reflect differences in mass and therefore in the number of cells within the respective aggregates.

Solution viscosity was measured with a Dudley viscometer, and solution density was determined by weighing a known volume of each test solution. Cell density was calculated from the relationship $\varrho_c=x_d\varrho_d+x_s\varrho_s=(0.30)\,(1.29)+0.70\,\varrho_s$ where x_d and x_s are the volume fractions within a cell occupied by dry matter and solvent, respectively, and ϱ_d and ϱ_s are the corresponding densities of these two intracellular compartments. Since the fixed cells were found by isotope uptake studies to be permeable to small molecules, ϱ_s was taken to be equal to the density of the suspension fluid. The dry matter density ϱ_d was determined by the cesium chloride buoyant density of the fixed cells. The volume fraction x_d was calculated from the tritiated water exclusion volume of a centrifuged cell pellet assuming a 26% interstitial space (spherical close packing); x_s is the remaining volume fraction $(1-x_d)$.

Results

The Sediment Height Method

For technical reasons, most studies of particle aggregation must rely upon surrogate measurements. The reliability of a particular technique is gauged by the extent to which it provides results consistent with those obtained with one or more independent methods. Sediment height analysis of polydisperse colloidal suspensions has been a commonly employed technique for several decades. Its utility arises from the fact that the final height of a sediment is proportional to the extent of particle aggrega-

tion independently determined by such methods as light transmission, sedimentation velocity, fluid filtration rate, viscosity, subsidence rate, and electrophoretic mobility (Slater & Kitchener, 1966; Harding, 1972, Dollimore & Horridge, 1972, 1973). The more extensively a suspension of particles aggregates as it settles out of solution, the greater will be its final sediment height in a column of specified geometry.

To determine whether the final sediment height of glutaraldehyde-fixed S180 cells reflected the tendency for such cells to aggregate under a variety of experimental conditions, an attempt was made to compare the results of sediment height studies with the results of three separate techniques.

The use of fluid filtration rate through a cell sediment (LaMer & Healy, 1963) proved impossible because the fluid flow caused a progressive packing of the cells which occluded further flow. An attempt to measure the disappearance of single cells as the result of pair and aggregate formation (Wilkens, Ottewill & Bangham, 1962*a*) was also unsuccessful. The shearing stress involved in collecting samples for counting in a hemocytometer resulted in nearly total disaggregation. Attempts to measure the disappearance of single cells by direct observation with a water-immersion lens were similarly unsuccessful because of the rapidity with which cells and their aggregates settled out of solution.

The measurement of sedimentation velocity proved straight forward, and a corrected value was calculated as described in Materials and Methods. The behavior of different settling columns was normalized by expressing the momentary sediment height as a fraction of the final sediment height. If the final sediment height is a valid reflection of the tendency for settling particles to aggregate, then it should be directly proportional to sedimentation velocity corrected for solution viscosity and density. Fig. 1 demonstrates that the sediment height ratio correlates well with the approximated sedimentation velocity, and is thus a reliable indicator of the tendency of S180 cells to aggregate with one another.

To correct observed sedimentation velocities for differences in solution viscosity and density, the simple form of Stoke's Law for spherical particles was employed. This form of Stoke's equation assumes that aggregates are all of the same size (monodisperse), have smooth surfaces, a solid and spherical geometry, a uniform density without spaces and channels, and a uniform packing of cells within an aggregate. Cell aggregates do not rigorously fulfill these assumptions, and as a result the corrections for solution viscosity and density effects are approximate rather than rigorous.

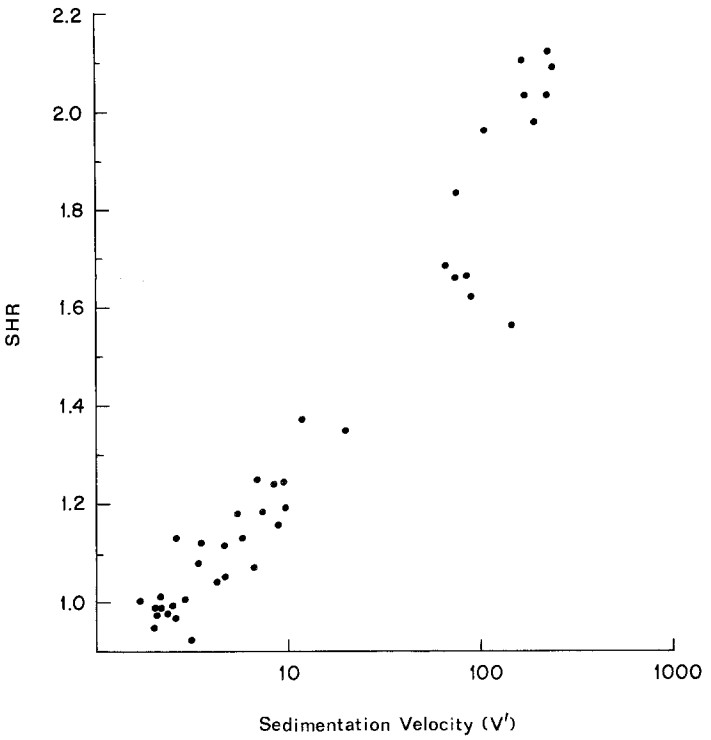

Fig. 1. The sediment height ratio (SHR) as a function of the corrected sedimentation velocity (V'). Cells were suspended in various electrolyte solutions and allowed to settle by gravity. To obtain the sediment height ratio, the final sediment height achieved by a test solution was divided by the final height attained by a reference solution (cells in distilled water). To obtain the sedimentation velocity, the percent (multiplied by 10) of the final sediment height accumulating per minute was calculated from the increase in sediment height with time, and corrected as described in Materials and Methods. The data displayed represent all of the data illustrated in Figs. 7–10

Organic Solvent Effects upon Sediment Height

The sediment height ratio (SHR) is the final sediment height of S180 cells in a test solution divided by the final height in a reference solution. For organic solvents, the reference solution was 0.145 M NaCl (Figs. 2–6), and all solvents were dissolved in 0.145 M NaCl. For inorganic electrolyte studies, the reference solution was distilled water, in which all salts were dissolved (Figs. 1, 7–10).

Fig. 2 illustrates the effects of low to moderate concentrations of straight-chain alcohols upon the sediment height ratio of fixed S180 cells. Methanol produces a modest increase in the SHR of about 10% over the 0–4 M range. Ethanol evokes a substantially greater SHR increase

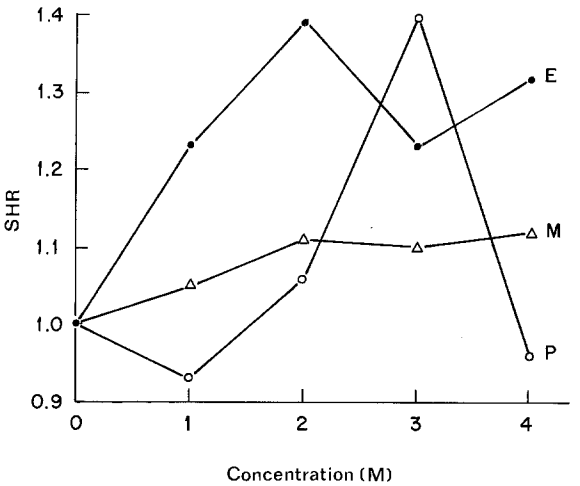

Fig. 2. The sediment height ratio (SHR) as a function of alcohol concentration for methanol (M), ethanol (E), and propanol (P)

from 0–2 M; there is a decline in SHR from 2–3 M ethanol, followed by a resumed SHR promotion above 3 M. Propanol causes a slight inhibition of the SHR at 1 M, a pronounced increase between 1 and 3 M, and a second decline from 3 to 4 M. In the higher alcohol concentration range of 4–12 M (data not shown), both methanol and ethanol evoke a continued rise in the SHR to values at 12 M of 1.97 and 2.15, respectively. Propanol does not have this effect.

With the butanol series of alcohols (Fig. 3), *n*-butanol is the most effective at increasing the SHR and produces more than a doubling of the SHR at 0.5 M. A similar stimulation of the SHR was obtained with isobutanol, but at a slightly higher concentration. Although sec-butanol produces a comparable SHR elevation, a much higher concentration is required. Finally, tert-butanol produces only a modest 20% increase in the SHR.

Fig. 4 demonstrates the influence of ketones upon the SHR. 2-Propanone has no effect at concentrations of 2 M or less, but considerably raises the SHR at 3 and 4 M. 2-Butanone reduces the SHR at low concentration, but elevates it considerably at concentrations above 1.5 M. At low concentrations 3-pentanone reduces the SHR; the low solubility of this ketone precluded the use of higher concentrations.

Urea and formamide reduce the SHR by 30–40% at 8 M concentration (Fig. 5). The effects of methylurea are essentially identical to those of

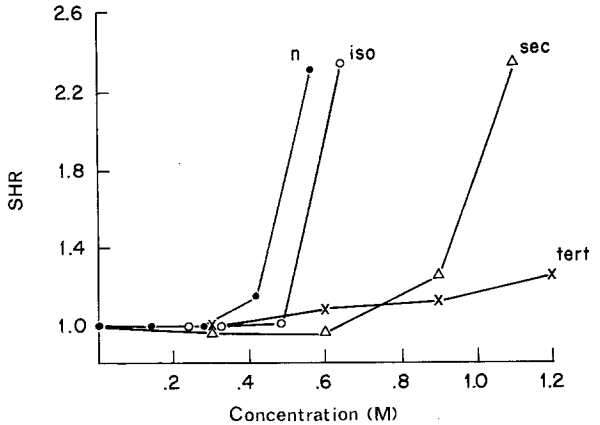

Fig. 3. The sediment height ratio (SHR) as a function of butanol concentration for *n*-, iso-, sec-, and tert-butanol

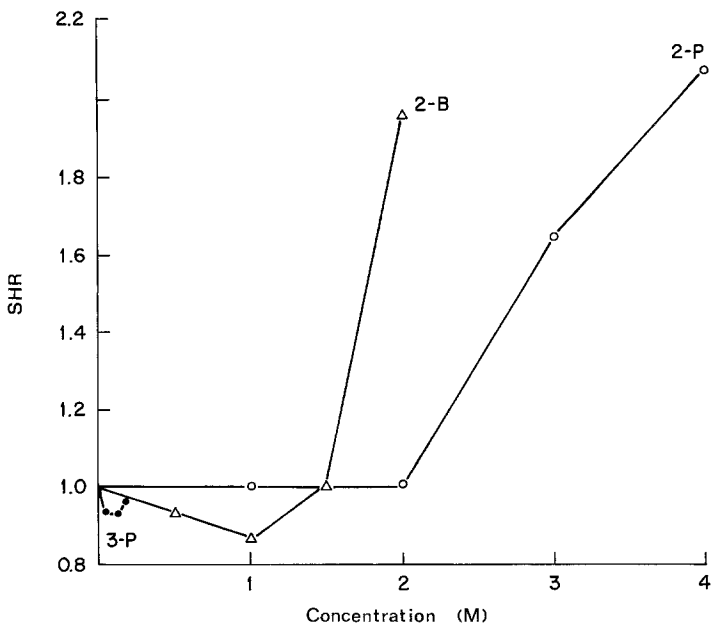

Fig. 4. The sediment height ratio (SHR) as a function of concentration of 2-propanone (2-P), 2-butanone (2-B), and 3-Pentanone (3-P)

urea. N,N-dimethylformamide, however, considerably elevates the SHR at concentrations above 2 M.

Fig. 6 illustrates the effects of polyhydric alcohols upon the SHR. Low concentrations of 1,2-propanediol slightly elevate the SHR, but higher

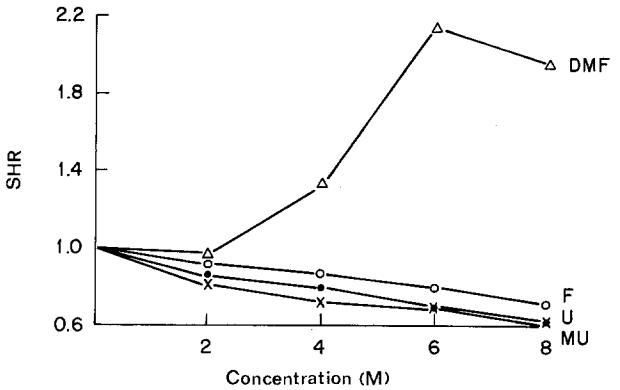

Fig. 5. The sediment height ratio (SHR) as a function of concentration for urea (U), methylurea (MU), formamide (F), and dimethylformamide (DMF)

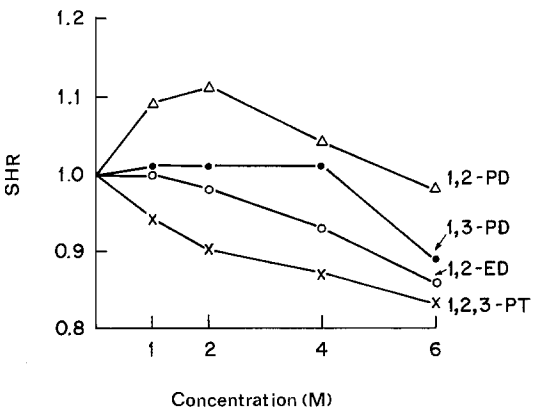

Fig. 6. The sediment height ratio (SHR) as a function of polyhydric alcohol concentration for 1,2-propanediol (1,2-PD), 1,3-propanediol (1,3-PD), 1,2-ethanediol (1,2-ED), and 1,2,3,-propanetriol (1,2,3-PT)

concentrations produce a progressive reduction. 1,3-Propanediol has no significant effect at concentrations of 4 M or less, but is slightly inhibitory at 6 M. 1,2-Ethanediol modestly reduces the SHR at concentrations above 1 M, while 1,2,3-propanetriol causes a somewhat more pronounced reduction at all concentrations employed.

Ion Effects upon Sediment Height

Fig. 7 shows the influence of four monovalent cations upon the SHR. Over the concentration range of 1–100 mM, the curves for K, Cs, and

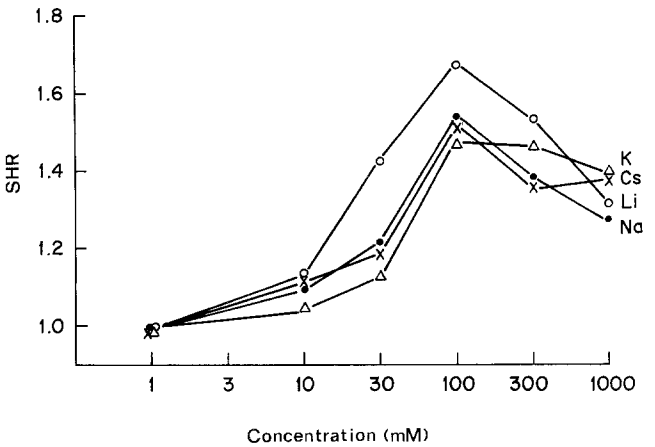

Fig. 7. The effect of low to moderate concentrations of monovalent cations upon the sediment height ratio (SHR). All cations were chloride salts. Cation effects are typical of a charge neutralization-charge reversal process

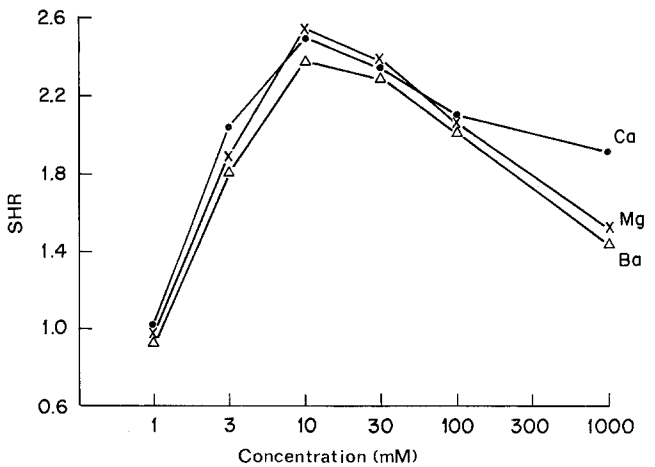

Fig. 8. The effects of low to moderate concentrations of divalent cations upon the sediment height ratio (SHR). All cations were chloride salts

Na are very similar. The curve for Li is displaced somewhat above the others, but not greatly so. For each cation the SHR rises until a maximum value is achieved at a concentration of 100 mM. At higher concentrations the SHR declines. While monovalent cations have a substantial effect upon sediment height, the SHR does not distinguish greatly between the species of monovalent cations employed.

A similar situation is found with divalent cations over the same concentration range (Fig. 8). Except at 1000 mM, the SHR curves for Ca, Mg,

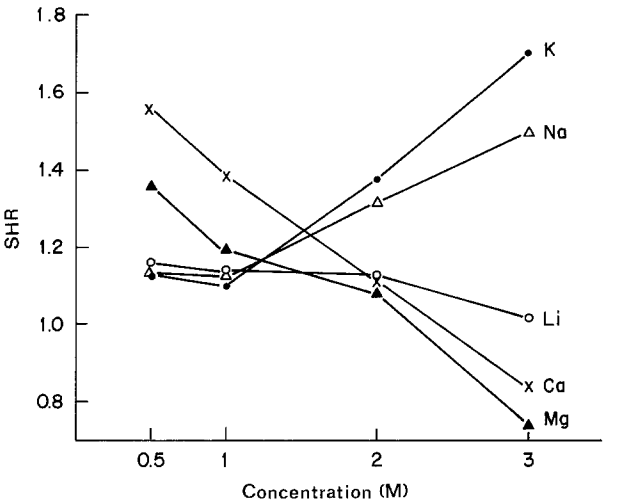

Fig. 9. The effect of high cation concentrations upon the sediment height ratio (SHR). All cations were chloride salts. Cation effects display a characteristic lyotropic salting in-salting out pattern

and Ba are essentially identical. Again, the species of divalent cation plays little role in determining the influence of a cation upon SHR behavior. A comparison of Figs. 7 and 8 reveals that divalent cations produce a maximum SHR at a concentration about one order of magnitude less than is required with monovalent cations; that is, 10 mM versus 100 mM. Moreover, the maximal SHR obtained with divalent cations (2.4–2.6) is substantially greater than that produced by monovalent cations (1.5–1.7). Clearly it is the valence of a cation rather than the species which determines the SHR of S180 cells at concentrations of 1 M or less.

Fig. 9 investigates SHR behavior at higher salt concentrations. As salt concentration is raised from 0.5 to 3 M, the SHR values for Ca and Mg decline. Li also produces a decline, though a very small one. With Na and K, however, the SHR reaches a minimum and then begins to rise again. The dependency of SHR upon valence rather than the species of an ion breaks down at high concentration. At an electrolyte concentration of 3 M, the SHR values for Ca and Mg are both about 0.8, for Li about 1.0, for Na about 1.5, and for K about 1.7. At high concentrations, then, SHR value is clearly more lyotropic (ion-species specific) than valence dependent.

All cells investigated electrophoretically appear to possess an excess of negative fixed surface charge (Weiss, 1967). If a particle possesses fixed surface charges of only one electrical sign, then one bulk phase

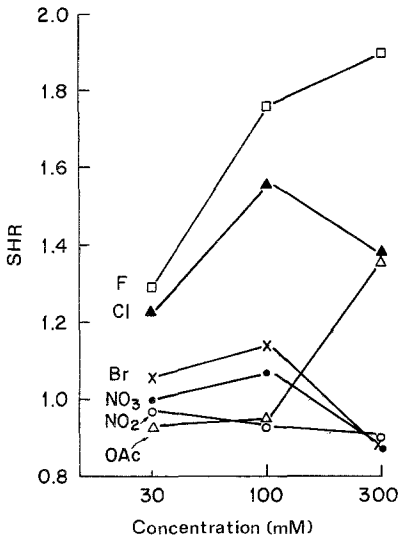

Fig. 10. Anion effects upon the sediment height ratio (SHR). All anions were sodium salts. The pattern of anion effects suggests that S180 cells possess fixed charges of both electrical signs in their surface region

co-ion can be substituted for another with little effect upon particle behavior (Verwey & Overbeek, 1948). However, if the particle possesses fixed surface charges of both electrical signs, as is the case with some cells (Ward & Ambrose, 1969), then its colloidal behavior will be sensitive to the type of anion in the bulk phase (Verwey & Overbeek, 1948). It can be seen from Fig. 10 that the SHR of fixed S180 cells is quite sensitive to the type of bulk phase anion employed. This result indicates that S180 cells possess fixed surface charges of both electrical signs.

Discussion

It is generally accepted that covalent bonds are not involved in cell aggregation and adhesion (Curtis, 1967; Weiss, 1967). Nonbonding interactions between macromolecules may involve London-Van der Waals forces, hydrophobic interactions, ion-pairing, hydrogen bonding, and electrostatic effects (Ramachandran & Sasisekharan, 1970). By determining the effects of organic denaturing solvents and electrolytes upon intermolecular associations, it is possible to determine at least in part the types of nonbonding interactions which are responsible for intermolecular associations within a particular polymer system.

In the present investigation, organic solvents and electrolytes are employed to assess the role of various nonbonding interactions in the aggregation of glutaraldehyde-fixed S180 cells. Since, when a suspension of particles is allowed to settle by gravity the final sediment height is proportional to the tendency of the particles to aggregate while settling (Slater & Kitchener, 1966; Harding, 1972; Dollimore & Horridge, 1972, 1973), sediment height analysis was used to investigate the aggregation of glutaraldehyde-fixed S180 cells.

Solvent Effects

Moderate concentrations of alcohols and ketones competitively disrupt macromolecular hydrophobic interactions. Their efficacy improves with increasing effective hydrocarbon chain length, decreases with chain branching, and does not coincide with their ability to lower the dielectric constant of an aqueous solution (Herskovits, 1962, Schrier & Scheraga, 1962; Robinson & Jencks, 1965; Emerson & Holtzer, 1967; Harrap, 1969; Kaminsky & Davison, 1969; Herskovits, Gadegbeku & Taillet, 1970*a*). The order of effectiveness in disrupting hydrophobic interactions is *n*-propanol > ethanol > methanol for unbranched alcohols, *n*- > iso- > sec- > tert- for the butanol series, and 2-butanone > 2-propanone for the ketones (Emerson & Holtzer, 1967; Harrap, 1969; Herskovits *et al.*, 1970*a*).

Figs. 2–4 show that moderate concentrations (1–3 M) of alcohols and ketones promote glutaraldehyde-fixed S180 cell aggregation, and that the order of effectiveness is as expected for a hydrophibic mechanism of solvent action. Since the solvents promote rather than inhibit aggregation, hydrophobic interactions between surface molecules of different cells cannot substantially contribute to aggregation, for if they did aggregation would be inhibited. The most likely interpretation of these data is that moderate concentrations of alcohols and ketones disrupt hydrophobic interactions within or between nearby macromolecules of the same cell surface, and that in the process cause a folding-out of buried regions thereby rendering a greater portion of individual surface adhesive molecules available for interaction by a nonhydrophobic mechanism with surface molecules of nearby cells.

Low concentrations of alcohols and ketones can strengthen hydrophobic interactions by creating the equivalent of a mixed micelle (Emerson & Holtzer, 1967). The ability of a solvent to promote this stabilizing effect at low concentration increases with solvent hydrophobicity up to some point, but then declines with further hydrophobicity (Emerson & Holtzer, 1967; Herskovits *et al.*, 1970*a*; Kaminsky, Wright & Davison,

1971). Figs. 2 and 3 show that low concentrations of *n*-propanol but not of methanol, ethanol, or *n*-butanol inhibit aggregation. Similarly, Fig. 4 shows that low concentrations of 2-propanone fail to inhibit aggregation, 2-butanone does inhibit, while 3-pentanone inhibits, but less so than 2-butanone. This is the pattern of effect which one would expect for a mixed-micelle type of stabilization in which low solvent concentrations caused a slight condensing of macromolecular conformation that modestly reduced the proportion of individual surface adhesive molecules available for interaction with their counterparts arising from nearby cells.

At high concentration, alcohols and ketones can cause a compacting of macromolecular architecture by promoting a folding-in of polar residues (Davidson & Fasman, 1967; Kaminsky & Davison, 1969; Herskovits *et al.*, 1970*a*; Kaminsky *et al.*, 1971). The inhibition of aggregation by higher concentrations of *n*-propanone (3–4 M, Fig. 2) probably reflects such a mechanism. Because of limited solubility, high ketone and butanol concentrations could not be used.

Solvents such as the ureas, amides, and polyhydric alcohols can disrupt hydrophobic interactions (Robinson & Jencks, 1965; Herskovits, Taillet & De Sena, 1970*b*; Herskovits, Taillet & Gadegbeku, 1970*c*), but they can also disrupt hydrogen bonds (Gordon & Jencks, 1963; Robinson & Jencks, 1965; Harrap, 1969; Russell & Cooper, 1969). For a competitive disruption of hydrogen bonds, solvent efficacy is proportional to hydrogen bond donor strength (Robinson & Jencks, 1965; Umberger, 1967), but declines with increasing solvent hydrophobicity (Robinson & Jencks, 1965). If hydrogen bonds contributed substantially to the aggregation of fixed S180 cells, one would expect urea, methylurea, and formamide to inhibit aggregation, while the more hydrophobic dimethylformamide should probably promote the process. Similarly, 1,2,3-propanetriol should considerably inhibit aggregation, while the more nonpolar propane-1,2-diol should have little inhibitory effect and might perhaps cause a slight promotion; ethane-1,2-diol and propane-1,3-diol should be intermediate in their effects. Figs. 5 and 6 show just this pattern of solvent influence. This indicates that hydrogen bonding is an important contributor to the aggregation of fixed S180 cells.

Table 1 examines the effects of several structurally related solvents. Those solvents with a highly polar component such as a carbonyl group are effective inhibitors of aggregation. When additional polar moieties such as amino groups are added, the inhibitory effectiveness is increased. Adding a small nonpolar group to a central carbonyl has little effect. Surrounding the polar region with methyl groups, as with acetone and

Table 1. Effect of 4 M solvents on SHR[a]

Solvent	SHR
0.145 M NaCl	1.00
Formamide	0.85
Acetamide	0.89
N,N-Dimethylformamide	1.31
Urea	0.73
Acetaldehyde	0.85
Acetone	2.25
Dimethylsulfoxide	1.64

[a] All solvents 4 M dissolved in 0.145 M NaCl.

N,N-dimethylformamide, reverses the inhibition and leads to a substantial promotion of aggregation. Blocking one polar group but leaving another exposed, as with methylurea, has little effect.

Thus as a general conclusion, organic solvents which possess at least one polar residue which is neither surrounded nor blocked by nonpolar groups are effective inhibitors of aggregation and behave in accordance with the expectations for a solvent disruption of hydrogen bonds between cell surfaces. When the polar moieties of such solvents are blocked or surrounded by nonpolar groups, the solvents promote aggregation by disrupting hydrophobic interactions which do not contribute to aggregation *per se,* but can indirectly influence the process by regulating conformation and the accessibility of potential macromolecular adhesive moieties.

Disulfide Bridges and Ion-Pairs

The SHR of fixed S180 cells was determined in the presence of high concentrations of three different disulfide bond-breaking agents. Cysteine (100 mM) reduced the SHR slightly to 0.92. Mercaptoethanol (100 mM) had a similar effect, lowering the SHR to 0.87. Dithiothreitol (20 mM) elevated the SHR to 1.09. The lack of a consistent pattern of effect by these agents, together with their modest influence upon the SHR, suggests that disulfide bridges are of marginal importance in regulating the ability of fixed S180 cells to aggregate.

Ion-pairs are disrupted by salt concentrations in the range of a few tenths to a few tens mM of NaCl (Balazs, Davies, Phillips & Scheufele, 1968; Phillips, Power, Robinson & Davies, 1970). All organic solvents used in this investigation were 145 mM NaCl. It is unlikely that ion-pairs would contribute to aggregation under these circumstances.

Ionic Effects

If charge interactions play a role in fixed-cell aggregation, one would expect to observe charge neutralization and charge reversal effects. The addition of small amounts of salt to a suspension of fixed cells should promote aggregation by partially neutralizing excess fixed charges in the cell surface region, thereby reducing coulombic repulsive forces. At higher electrolyte concentrations charge reversal should occur, an excess of mobile counter-ions should accumulate in the surface region, and the coulombic repulsive forces should again increase thereby inhibiting aggregation. At low to moderate concentrations, the ability of cations to cause these effects should be primarily a function of valence rather than of ionic species (Verwey & Overbeek, 1948; Wilkens *et al.*, 1962*a, b*; Weiss, 1967).

If fixed S180 cell aggregation is coagulatory, that is, if the cell surfaces do not make actual contact with one another, but are instead held in close proximity by London-Van der Waals forces propagated through intervening solvent, then very high cation concentrations should further inhibit aggregation by intensifying charge reversal. However, if cell aggregation involves polymer bridging by adhesive macromolecules of one cell which interact directly with those of another cell, then one would expect to observe the salting-out or salting-in of cells at high cation concentrations.

As Figs. 7 and 8 illustrate, low to moderate salt concentrations of both mono- and divalent cations first promote and then inhibit aggregation as would be expected for a charge neutralization/charge reversal phenomenon. This indicates that the net charge in the cell surface region regulates the ability of fixed S180 cells to aggregate. In the 1–1000 mM range, salt effects depend primarily upon cation valence rather than species.

At high electrolyte concentration, however, an entirely different pattern of effects upon the sediment height ratio is observed (Fig. 9). Na and K progressively increase the SHR at high concentration, while Li has little effect and Ca and Mg reduce the SHR. Moreover, the SHR no longer displays a marked valence dependence, but instead depends upon the type of cation employed. These are typical characteristics of polymer bridging, but are not expected for coagulation (Verwey & Overbeek, 1948; LaMer & Healy, 1963).

It is interesting to note that for salt effects upon protein conformation, Ca and Mg commonly cause salting-in while Na and K tend to promote salting-out; Li is usually intermediate (Von Hippel & Schleich, 1969).

Table 2. Summary of solvent effect

Solvent	Concentration	Postulated mechanism of aggregation	Expected effect	Is effect observed?
Alcohols, Ketones	low	hydrophobic interactions indirectly retard aggregation	slight decrease in SHR as solvent hydrophobicity increases up to some point, then loss of effect thereafter	Yes
	moderate	cells aggregate by hydrophobic interactions	reduction of SHR by alcohols, ketones	No
	moderate	hydrophobic interactions indirectly retard aggregation	increase in SHR by alcohols, ketones	Yes
	high	aggregation inhibited by folding-in of polar residues	SHR decreases as solvent hydrophobicity increases	Yes
Ureas, Amides	moderate	cells aggregate by hydrophobic interactions	reduction of SHR by urea, methylurea, formamide, dimethylformamide	No
	moderate	cells aggregate by hydrogen bonding	SHR reduction by urea, methylurea, formamide but increase by dimethylformamide	Yes
Polyhydric alcohols	moderate	cells aggregate by hydrophobic interactions	SHR reduction	No
	moderate	cells aggregate by hydrogen bonding	SHR reduction by 1,2,3-propanetriol, 1,2-ethanediol, and 1,3-propanediol but SHR increase by 1,2-propanediol	Yes

This same pattern is observed with the sediment height ratio at high electrolyte concentration.

These findings indicate that surface macromolecules of fixed S180 cells interact directly with similar molecules arising from other cells and serve as macromolecular bridges to adhere two cells to one another. The ability of such molecules to form intermolecular associations is regulated by their net charge, which in turn is a function of local ion concentrations.

Electrolytes probably regulate the ability of surface macromolecules to form intermolecular associations both with the macromolecules of other cell surfaces and with adjacent macromolecules of the same surface. Both effects presumably play a role in aggregation. That the extent of aggregation depends upon anion species (Fig. 10) indicates that fixed S180 cells bear surface charges of both electrical signs (Table 2).

Fixation Artifacts

Because living cells will not tolerate the range of conditions employed in the present study, glutaraldehyde-fixed S180 cells were used as a model system for examining the mechanisms of cell aggregation. Generalization of the present findings from the model system to living cells is limited by the potential artifacts which might arise from fixation.

Glutaraldehyde can be expected to react with and mask positive surface charges such as the amino-, imino-, and guanidinium-groups of proteins, glycoproteins, lipids, and lipoproteins (Weiss, Bello & Cudney, 1968; Weiss & Subjeck, 1974), thereby increasing surface electronegativity (Ward & Ambrose, 1969). While increasing surface electronegativity, fixation should not qualitatively alter surface electrokinetic properties providing all surface cations are not masked. The finding that fixed S180 cells bear positive fixed charges is almost certainly valid for living S180 cells, and demonstrates that with fixed cells not all surface cations are masked by the fixative. The cation concentrations (Figs. 7 and 8) which elicit maximum aggregation of fixed cells agree reasonably well with those reported for maximum aggregation and minimum electrophoretic mobility of other types of living cells (Wilkens *et al.*, 1962*a, b*).

The reaction with and masking of positive surface charges by glutaraldehyde, and the solvent's potential ability to react with aldehyde residues of surface carbohydrates (Robertson & Schultz, 1970) should reduce the ability of adhesive macromolecules of nearby cells to hydrogen bond with one another. This would lead to an underestimation of the involvement of hydrogen bonds in aggregation. Counteracting this tendency is the potential ability of free fixative aldehyde residues to themselves participate in hydrogen bonding. Which effect predominates cannot be assessed at the present time.

The ability of glutaraldehyde to cross-link macromolecules (Quiocho & Richards, 1964; Quiocho, Bishop & Richards, 1967) probably increases the rigidity of fixed-cell surface molecules and thereby leads to an underestimate of their ability to undergo conformational changes in response

to denaturing solvents. Cross-linking probably also reduces the ability of macromolecules to interdigitate, thus weakening the adhesive interactions of fixed cells. However, such cross-linking may well be advantageous in that it tends to preserve normal surface architecture. Glutaraldehyde is known to be a particularly gentle fixative that allows the preservation of many enzyme activities (Sabatini, Bensch & Barrnett, 1963) and causes less conformational denaturation than other commonly employed fixatives (Lenard & Singer, 1968).

That fixed S180 cells are particularly susceptible to dissociation by gentle shearing indicates that free fixative aldehyde residues arising from one cell surface do not covalently bond to the surface of another cell by aldol condensation.

Glutaraldehyde can polymerize with itself by aldol condensation (Robertson & Schultz, 1970). It is possible that such polymers might be responsible for the macromolecular bridging indicated by Fig. 9. This is unlikely for two reasons. First, such bridging is mediated by polymers of high molecular weight (LaMer & Healy, 1963). Self-polymerization of glutaraldehyde produces primarily dimers; trimers are but marginally detectable, while higher order polymers are quite rare (Robertson & Schultz, 1970). Such fixative polymers are probably much too small to serve as macromolecular bridges. Second, surface coat macromolecules are typically several hundred angstroms in length (Ito, 1965; Rambourg & Leblond, 1967; Price, 1970) and do possess the attributes required for macromolecular bridging. Since cross-linking of macromolecules within a surface by glutaraldehyde would be expected to restrict their efficacy as bridging agents by reducing their flexibility (LaMer & Healy, 1963), it is likely that macromolecular bridging makes an important contribution to the aggregation of living S180 cells.

References

Balazs, E.A., Davies, J.V., Phillips, G.O., Scheufele, D.S. 1968. Polyanions and their complexes. Part IV. A pulse radiolysis study of the interaction between methylene blue and heparin in aqueous solution. *J. Chem. Soc. (C)*, 1424

Curtis, A.Z.G. 1967. The Cell Surface: Its Molecular Role in Morphogenesis. Logos Press, London

Davidson, B., Fasman, G.D. 1967. The conformational transitions of uncharged poly-L-lysine. α-helix-random coil-β structure. *Biochemistry* **6**:1616

Dollimore, D., Horridge, T.A. 1972. The optimum flocculant concentration for effective flocculation of China clay in aqueous suspension. *Water Res.* **6**:703

Dollimore, D., Horridge, T.A. 1973. The dependence of the flocculation behavior of China clay-polyacrylamide suspensions on the suspension pH. *J. Colloid Interface Sci.* **42**:581

Emerson, M.G., Holtzer, A. 1967. The hydrophobic bond in micellar systems. Effects of various additives on the stability of micelles of sodium dodecyl sulfate and of *n*-dodecyltrimethylammonium bromide. *J. Phys. Chem.* **71**:3320

Gordon, J.A., Jencks, W.P. 1963. The relationship of structure to the effectiveness of denaturing agents for proteins. *Biochemistry* **2**:47

Harding, R.D. 1972. Heterocoagulation in mixed dispersions—Effects of particle size, size ratio, relative concentration, and surface potential of colloidal components. *J. Colloid Interface Sci.* **40**:164

Harrap, B.S. 1969. The effect of aliphatic alcohols on the thermal stability of tropocollagen under acidic conditions. *Int. J. Protein Res.* **1**:245

Herskovits, T.T. 1962. Nonaqueous solutions of DNA: Factors determining the stability of the helical configuration in solution. *Arch. Biochem. Biophys.* **97**:474

Herskovits, T.T., Gadegbeku, B., Jaillet, H. 1970*a*. On the structural stability and solvent denaturation of proteins. I. Denaturation by the alcohols and glycols. *J. Biol. Chem.* **245**:2588

Herskovits, T.T., Jaillet, H., DeSena, A.T. 1970*b*. On the structural stability and solvent denaturation of proteins. III. Denaturation by the amides. *J. Biol. Chem.* **245**:6511

Herskovits, T.T., Jaillet, H., Gadegbeku, B. 1970*c*. On the structural stability and solvent denaturation of proteins. II. Denaturation by the ureas. *J. Biol. Chem.* **245**:4544

Ito, S. 1965. The enteric surface coat on cat intestinal microvilli. *J. Cell Biol.* **27**:475

Kaminsky, L.S., Davison, A.J. 1969. Effects of organic solvents on the spectrum of cytochrome *c*. *Biochemistry* **8**:4631

Kaminsky, L.S., Wright, R.L., Davison, A.J. 1971. Effect of alcohols on the rate of autoxidation of ferrocytochrome *c*. *Biochemistry* **10**:458

LaMer, V.K., Healy, T.W. 1963. Adsorption-flocculation reactions of macromolecules at the solid-liquid interface. *Rev. Pure Appl. Chem.* **13**:112

Lenard, J., Singer, S.J. 1968. Alteration of the conformation of proteins in red blood cell membranes and in solution by fixatives used in electron microscopy. *J. Cell Biol.* **37**:117

Muller, W.E.G., Zahn, R.K. 1973. Purification and characterization of a species-specific aggregation factor in sponges. *Exp. Cell Res.* **80**:95

Phillips, G.O., Power, D.M., Robinson, P.C., Davies, J.V. 1970. Ion binding of penicillins to proteins. *Biochim. Biophys. Acta* **215**:491

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

Quiocho, F.A., Bishop, W.H., Richards, F.M. 1967. Effect of changes in some solvent parameters on carboxypeptidase-A in solution and in cross linked crystals. *Proc. Nat. Acad. Sci. (USA)* **57**:525

Quiocho, F.A., Richards, F.M. 1964. Intermolecular cross linking of a protein in the crystalline state: Carboxypeptidase-A. *Proc. Nat. Acad. Sci. (USA)* **52**:833

Ramachandran, G.N., Sasisekharan, V. 1970. Conformation of polypeptides and proteins. *Advanc. Protein Chem.* **23**:283

Rambourg, A., Leblond, C.P. 1967. Electron microscope observations on the carbohydrate-rich cell coat present at the surface of cells in the rat. *J. Cell Biol.* **32**:27

Revel, J.P., Hoch, P., Ho, D. 1974. Adhesion of culture cells to their substratum. *Exp. Cell Res.* **84**:207

Robertson, E.A., Schultz, R.L. 1970. The impurities in commercial glutaraldehyde and their effects on the fixation of the brain. *J. Ultrastruct. Res.* **30**:275

Robinson, D.R., Jencks, W.P. 1965. The effects of compounds of the urea-guanidinium class on the activity coefficient of acetyltetraglycine ethyl ester and related compounds. *J. Amer. Chem. Soc.* **87**:2462

Russell, A.E., Cooper, D.R. 1969. Structural and functional factors in the hydrogen bonding of polar organic solvents to acid-soluble collagen. Effect on renaturation kinetics and thermal stability. *Biochemistry* **8**:3980

Sabatini, D.D., Bensch, K., Barrnett, R.J. 1963. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17**:19

Sanford, K.K., Barker, B.E., Parshad, R., Westfall, B.B., Woods, M.W., Jackson, J.L., King, D.R., Peppers, E.V. 1970. Neoplastic conversion *in vitro* of mouse cells: Cytologic, chromosomal, enzymatic, glycolytic, and growth properties. *J. Nat. Cancer Inst.* **45**:1071

Schrier, E.E., Scheraga, H.A. 1962. The effect of aqueous alcohol solutions on the thermal transition of ribonuclease. *Biochim. Biophys. Acta* **63**:406

Slater, R.W., Kitchener, J.A. 1966. Characteristics of flocculation of mineral suspensions by polymers. *Disc. Faraday Soc.* **42**:167

Umberger, J.Q. 1967. Solvent proticity via erythrosin internal conversion. *J. Phys. Chem.* **71**:2054

Verwey, E.J.W., Overbeek, J.T.G. 1948. Theory of the Stability of Lyophobic Colloids. Elsevier, Amsterdam

Von Hippel, P.H., Schleich, P.H. 1969. The effect of neutral salts on the structure and conformational stability of macromolecules in solution. In: Structure and Stability of Biological Macromolecules. S.N. Timasheff and G.D. Fasman, editors. Vol. 2, Chapt. 6. Marcel Dekker, New York

Ward, P.D., Ambrose, E.J. 1969. Electrophoretic and chemical characterization of the charged groups at the surface of murine CL3 ascites leukemia cells. *J. Cell Sci.* **4**:289

Weiss, L. 1967. The Cell Periphery, Metastasis, and Other Contact Phenomena. North Holland Publishing Co., Amsterdam

Weiss, L., Bello., J., Cudney, T.L. 1968. Positively charged groups at cell surfaces. *Int. J. Cancer* **3**:795

Weiss, L., Subjeck, J.R. 1974. The densities of colloidal iron hydroxide particles bound to microvilli and the spaces between them: Studies on glutaraldehyde-fixed Ehrlich ascites tumor cells. *J. Cell Sci.* **14**:215

Wilkens, D.J., Ottewill, R.H., Bangham, S.D. 1962*a*. On the flocculation of sheep leucocytes. I. Electrophoretic studies. *J. Theoret. Biol.* **2**:165

Wilkens, D.J., Ottewill, R.H., Bangham, A.D. 1962*b*. On the flocculation of sheep leucocytes. II. Stability studies. *J. Theoret. Biol.* **2**:176