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Influence of polymer concentration and molecular weight and of enzymic glycocalyx modification on erythrocyte interaction in dextran solutions

A. J. Baker [1, 2], **W. T. Coakley** [1], **D. Gallez** [3]

[1] School of Pure and Applied Biology, University of Wales College of Cardiff, Cardiff CF1 3TL, UK
[2] Division of Biotechnology, PHLS CAMR, Porton Down, Salisbury SP4 0JG, UK
[3] Service de Chimie Physique, Universite Libre de Bruxelles, CP 231, Campus Plaine, B-1050 Brussels, Belgium

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Abstract. Erythrocytes adhere to each other when suspended in supra-threshold concentrations of dextran of molecular mass of 40 kD or greater. The plasma membranes are parallel to each other over the entire length of the contact seam at the lower effective polymer concentrations. When cells are pretreated with the proteolytic enzyme pronase or the sialidase neuraminidase the membranes are not parallel but make contact at spatially periodic locations along the membrane surface. Pronase induced reduction of cell electrophoretic mobility rapidly reaches a limiting value. Nevertheless, prolonged pre-exposure to enzyme leads to a continuing reduction in contact separations. This result taken with the observation that, for equal loss of electrophoretic mobility, a shorter contact separation results from pronase rather than neuraminidase pre-treatment implies that a non-electrostatic consequence of pronase pre-treatment dominates membrane interaction in the experimental regimes examined here. The average lateral contact separation for different enzyme regimes lay in the range 3.3 μm to a limiting lower value of about 0.7 μm. There was a good correlation between the logarithm of a contact separation index (the approach of separation distance to its limiting value) against the logarithm of a derived index related to net attractive interaction for a wide range of experimental conditions. Treatments which increased attraction or decreased repulsion (e.g. increased dextrans concentration or enzyme pre-treatment) lead to shorter lateral contact separation. This result is qualitatively consistent with the predicted behaviour for the dominant wavelength arising from interfacial instability of a thin aqueous film between adjacent membranes.

Key words: Cell adhesion – Dextran – Erythrocyte adhesion – Membrane fusion – Interfacial instability – Fluid film instability

Correspondence to: W. T. Coakley

Introduction

Maintenance of the organisation of eukaryotic cells normally requires that the cells' intracellular membranes remain separate and stable as they transiently, through thermal movements, approach each other. In other cases, formation of local contact is a feature of close approach of one membrane to another or to a solid substratum e.g. during the acrosome reaction (Russell et al. 1979), during cortical granule exocytosis (Schuel 1985), in cell-cell contact (Andre and Bongrand 1990) or in cell locomotion (Vasiliev 1987). Fusion of membranes in vitro (e.g. cells in polyethylene glycol (Pontecorvo 1975) and plant protoplasts in dextran solution (Kameya et al. 1981) requires modification of the repulsive net cell-cell interaction which normally maintains suspended cells as a monodispersion. There is thus a general interest in understanding how membranes avoid coming into close contact with each other and how repulsive interactions can be overcome so that membranes may come into close contact. It is also important to establish whether close contact, when it occurs, consists of a continuous seam of parallel membranes or is localised.

The adhesion of erythrocytes suspended in solutions of either polycations (Katchalsky et al. 1959, Tilley et al. 1987, Coakley et al. 1991), polysaccharides (Coakley et al. 1991, Darmani and Coakley 1990, Skalak and Zhu 1990) or lectins (Coakley et al. 1991, Darmani et al. 1990, Gokhale and Medha 1987) has been studied as a model membrane-interaction system where attractive macromolecule-associated interactions can effectively counter the normally repulsive net membrane interaction. The erythrocyte system is a useful model because it may be expected that a set of basic biophysical responses to changes in membrane interactions will be easier to detect in the relatively simple erythrocyte than in a eukaryotic cell containing a cytoskeleton where biochemical responses, which can further complicate interpretation, may be triggered.

Previous studies in which attention has been paid to the seam of erythrocyte adhesion show either than (in 3%

or 4% w/v 75 kD dextran (Jan and Chien 1973, Skalak et al. 1981) or in 2% w/v 450 kD dextran (Darmani and Coakley 1990) the plasma membranes of interacting cells are parallel to each other along the length of the contact seam or (in polycation (Coakley et al. 1985, Hewison et al. 1988) or lectin solutions (Darmani et al. 1990, Darmani and Coakley 1991) regions of close membrane-membrane approach were separated by regions of larger separation so that the seam was characterised by spatially periodic contact regions. The parallel membrane adhesion of erythrocytes in dextran was attributed (Jan 1979) to location of the membranes at a minimum in the net interaction energy profile arising from macromolecular cross-linking, van der Waals attraction and electrostatic repulsion. An attractive intermembrane effect due to surface depletion of polymer (demonstrated by Evans and Needham (1988) in studies of liposome interactions) may also be relevant to erythrocyte adhesion by dextran. More comprehensive analyses of membrane interaction (adapted from treatments of droplet and bubble behaviour) also include the dynamics of the thinning fluid film between membranes as they are drawn together under the influence of a net attractive force (Dimitrov 1982, Radoev et al. 1983). These analyses predict that a film thinning under the influence of a net attractive pressure may reach a "transition thickness" at which fluctuations of the thin film grow spontaneously, leading either to rupture of the thin film at spacially periodic points or to formation of a film with smaller thickness. The condition for spontaneous rupture of a non-thinning inter-membrane aqueous layer is not very different from that for a thinning layer while analysis of the former provides good physical insight to the rupture process (Dimitrov 1982, Dimitrov and Jain 1984). The effects of charge density, hydration and macromolecular cross-linking have been included in stability analyses for a non-thinning water layer between membranes (Gallez et al. 1984, Gallez and Coakley 1986, Prevost and Gallez 1984). This thin film instability model shows that the onset of instability and the rupture of the thin film depend on the interplay of destabilising attractive and stabilising repulsive interactions.

A recent study (Darmani and Coakley 1990) of cells in 2% w/v of 450 kD dextran showed that the outcome (plane parallel contacts or spatially periodic contacts) of membrane contact could be controlled by the extent of cell pre-treatment with the proteolytic enzyme, pronase. The average lateral separation of contacts was dependent on the duration of pronase pre-treatment of the cells. The present paper extends the earlier study (Darmani and Coakley 1990) of the consequences of modifying the cell surface to include an examination of the effect of a range of dextran molecular weights and concentrations on the contact mode of normal cells and of cells which were pre-treated with a protease (pronase) or with the glycosidase (neuraminidase) which removes the sialic acids responsible for most of the erythrocyte surface charge. It shows that experimental procedures which reduce repulsion or increase attraction lead to shorter separation of spatially periodic contacts. This result is in agreement with the variation of the wavelength predicted by the thin film instability model.

Materials and methods

a Erythrocyte suspension

Human erythrocytes were obtained by finger puncture from a single donor into phosphate buffered saline (PBS) containing 145 mM NaCl and 5 mM phosphate. The pH was adjusted to 7.32 by dropwise addition of 1% NaOH solution. The cell suspension was centrifuged at 1 000 g for 1 min. The supernatant and the buffy coat were removed by aspiration. The erythrocytes were resuspended and washed in PBS at 1 000 g before finally resuspending, to a cell concentration of $3 \cdot 10^7$ cells per ml, in PBS containg 0.25 mg ml^{-1} bovin serum albumin (to avoid cell crenation; BDH Ltd). Experiments were completed within 2 h of finger puncture.

b Enzyme pre-treatment

Pronase (Boehringer Mannheim) was made up to the required concentration in PBS containing 0.25 mg/ml bovine serum albumin (BSA). Equal volumes of pronase solution and cell suspension were mixed. The pronase concentrations quoted in the results were the final concentrations in the cell/pronase mixture. The resulting suspension was held at 25 °C for a known time, with occasional shaking. The suspension was then diluted 10 fold with PBS and centrifuged at 1 000 g for 1 min. The supernatant was removed by aspiration and the cells were resuspended in PBS/BSA. Neuraminidase (Type VI, Sigma Ltd) was made up to 0.2 units/ml in PBS (neuraminidase treated erythrocytes cup slightly as do cells in 0.25 mg/ml BSA, therefore the anit-crenation agent BSA was not added to the PBS). The cells were held at 25 °C for a known time and treated as described above (for cells exposed to pronase) until final resuspension in PBS.

c Exposure to dextran

0.5 ml of dextran (450 kD, Pharmacia Ltd; 9.6, 20, 40 and 72 kD, Sigma Ltd) solution in PBS/BSA was added to 0.5 ml of an appropriate cells suspension (in a test tube) and held at room temperature for 30 min with occasional shaking. The cells were fixed at room temperature by addition of an equal volume of 6% w/v glutaraldehyde (50% purity, Sigma Ltd) in PBS for 5 min. The concentrations of dextran quoted in the results were the concentrations in the final suspensions.

d Light and election microscopy

Initial evaluation of cell clump morphology and contact seam characteristics was carried out on fixed cells by bright field microscopy using a Nachet 400 research microscope with a × 63 oil immersion objective. Fixing the cells avoided any problems related to the cell-crenating "glass effect" often observed when erythrocytes settle on microscope slides. Spatial periodicity of the contact seam

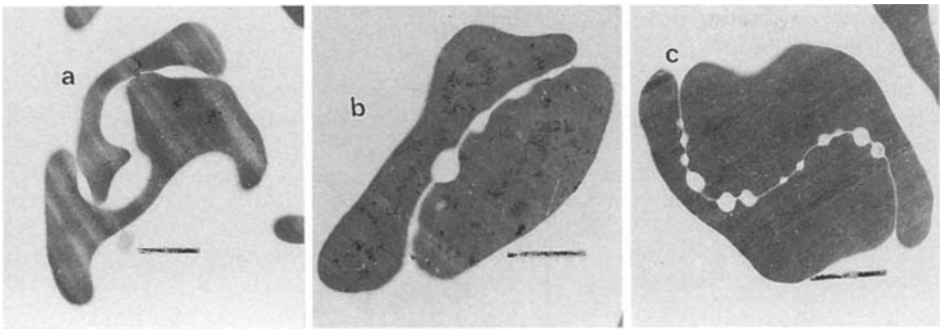

Fig. 1. Transmission electron micrographs showing an example of spatially periodic **a** long (2.3 μM) lateral separation (30 min pre-treatment with 0.06 μg/ml pronase and then exposure to 6% w/v 450 kD dextran); **b** medium (1.3 μM) lateral separation (60 min pre-treatment with 0.5 mg/ml pronase and then exposure to 5% w/v 20 kD dextran) and **c** short (0.66 μM) lateral separation (60 min pre-treatment with 0.5 mg/ml pronase and then exposure to 8% w/v 20 kD dextran)

was also more easily resolved in fixed cells. Light micrographs of dextran-induced cell clumps showing examples of spatial periodicity of the contact seam in fixed and in unfixed erythrocytes have previously been published (Darmani and Coakley 1990).

In preparation for electron microscopy the fixed cells were centrifuged (1 000 g for 1 min), resuspended in 3% glutaraldehyde (25% EM Grade, AGAR Scientific) in 0.1 M phosphate buffer, pH 7.4 and incubated for 1–3 h at 4 °C. The cells were resuspended and held overnight in 0.1 M phospate buffer. The cells were washed again and post-fixed in Millionig's buffered osmium tetroxide for 1 h at 4 °C. The cells were then dehydrated by serial washing and resuspension in increasing concentrations of ethanol (50%, 70%, 90%) for 15 min each at 4 °C, 30 min in 100% ethanol at 4 °C and 30 min in 100% ethanol at room temperature. The cells were transferred to suspension in 100% Spurr resin, through a series of Spurr/ethanol steps. The cells were in resin overnight. The resin was polymerised in an oven at 60 °C for 10 h. 60 nM section were then cut.

e Measurements of contact spacing

Measurements were made either from electron micrographs or by video analysis. In the case of electron microscopy the lateral separation of contact points along the seam of agglutination was measured for a run of from 3 to 10 contact points along the seam. An average spacing was calculated for that seam. The process was repeated, typically for 8–12 cell contact seams, and an overall average spacing was calculated for the experimental conditions under which the cells had been exposed to dextran. In the case of the video analysis the fixed cells were examined by light microscopy using a Zeiss microscope with ×100 objective. Output from a camera (Panasonic, W-V 1900) was processed by a Seescan (Seescan Ltd., Cambridge, UK) image processor. The separation of individual contact points was measured and recorded. A total of at least 35 measurements were made from cells from each sample and a mean separation was calculated for the sample.

f Electrophoretic velocity measurements

Electrophoretic velocity measurements were initially carried out in low ionic strength buffer (14.5 mM NaCl, 280 mM erythritol, 5 mM phosphate buffer, pH 7.32) so that the low currents required to achieve cell movement would minimise the production of cell-convecting thermal gradients. Measurements were later carried out in PBS to allow comparison with previously published data for enzyme induced reduction of electrophoretic velocity. The conditions of measurement are referred to, when required, in the Results section. 0.5 ml of cell suspension was diluted ×10 with PBS, centrifuged at 1 000 g for 1 min and resuspended in 10 ml of either PBS or of low ionic strength buffer. The resulting suspension was loaded into a 40 mm long rectangular cross-section (4 mm × 1 mm) glass electrophoresis cell (for PBS samples) or in a 37 mm long, rectangular cross section (10 mm × 1 mm) cell (for low ionic strength samples). The low ionic strength samples were exposed to a 2 mA current in a standard Mark II cytophorimeter (Rank Brothers, Bottisham, Cambridge, UK) while the cells in PBS were exposed to 5 mA in the modified Mark II system described by Sutherland and Pritchard (1979). The electrophoretic velocity in the stationary layer was measured for enzyme pre-treated cells and expressed as a percentage of the velocity of control erythrocytes.

Results

(i) *Overview of contact formation for different pronase pre-treatments, dextran concentrations and sizes*

The contact seams of cell aggregates formed on exposure to dextrans of different molecular masses (20 kD–450 kD) and concentrations (2% w/v–8% w/v) following pronase pre-treatments of different durations (5 min–60 min) were evaluated by light microscopy. The contact categories established for scoring were, parallel cell membranes (P) and local contacts estimated to have long ($L > 1.8$ μM), medium (1.2 μM $< M < 1.8$ μM) or short ($S < 1.2$ μM) lateral separations. The transmission electron micrographs of Fig. 1 show an example of an L, M, and S category. The dominant categories of contact are shown for different experimental conditions in Table 1.

The main initial conclusions from Table 1 are that: (a) Spatially periodic contacts are a feature of almost all cases where pronase pre-treated cells adhere on exposure to dextran: (b) The lateral separation of contact regions shortens with increasing dextran concentration: (c) The lateral separation shortens with increasing pronase pre-

Table 1. Light microscopy evaluation of lateral contact separation for cells pre-treated with 0.5 mg/ml pronase for times ranging from 0 (controls) to 60 min and then exposed to 2, 4, 6 or 8% w/v dextran. *P* indicates parallel membranes; lateral separation categories, (L > 1.8 μM), (1.2 < M < 1.8 μM), (S < 1.2 μM). N represents "no adhesion". The superscripts give the number of times a category was examined. 2 to 4 individual results were obtained from blood from each finger puncture from a single donor

Treatment Time (min)	Dextran – 450 kDa Concentration			
	2%	4%	6%	8%
0	^{4}P	^{6}P	^{2}L	^{2}P
5	^{2}L	^{2}M	^{2}S	^{1}S
15	^{2}L, M	^{2}S	^{4}S	^{2}S
30	^{1}M	^{1}S	^{1}S	^{1}S
60	^{3}S	^{1}S	^{3}S	^{2}S
	Dextran – 72 kD			
0	^{2}P	^{1}P	^{2}L	^{2}P
5	^{1}M	^{1}S	^{1}S	^{1}S
15	^{1}S	^{1}S	^{3}S	^{1}S
30	^{1}S	^{1}S	^{1}S	^{2}S
60	^{2}S	^{1}S	^{2}S	^{3}S
	Dextran – 20 kD			
0	^{1}N	^{1}N	^{1}N	^{2}N
5	^{1}N	^{1}N	^{1}N	^{1}S
15	^{1}N	^{1}P	^{3}S	^{1}S
30	^{1}N	^{1}S	^{1}S	^{3}S
60	^{1}N	^{2}S	^{1}S	^{3}S

treatment times: (d) When adhesion occurs shorter wavelengths are a more common feature of the results with lower molecular weight dextrans.

No adhesion occurred in 10 kD dextran at the concentrations (2%–8% w/v) and the pronase pre-treatment times (up to 1 h) examined.

Table 1 therefore shows variation of lateral contact separation distance with dextran molecular weight, dextran concentration and pronase pre-treatment time. Experimental conditions of interest (in providing insight to mechanism) particularly include those where wavelength varies significantly and gradually with change in experimental variable. For the situations reported in Table 1 the greatest variety of wavelength categories (and therefore gradual significant change) was seen for cells pre-treated for different times with pronase and then exposed to 2% w/v 450 kD dextran. In constrast only category S spacings were seen in pronase pre-treated cells exposed to 6% w/v of 450 kD dextran and in 9 of the 10 regimes where cells were exposed to 20 kD dextran. For these latter situations experiments were carried out to establish whether refinement of the increment in variable size would identify conditions where spacings other than S would be observed.

(ii) *Contact separation for different dextran concentrations*

Figure 2 includes measurements (obtained using the video analysis system) of average lateral separation for cells

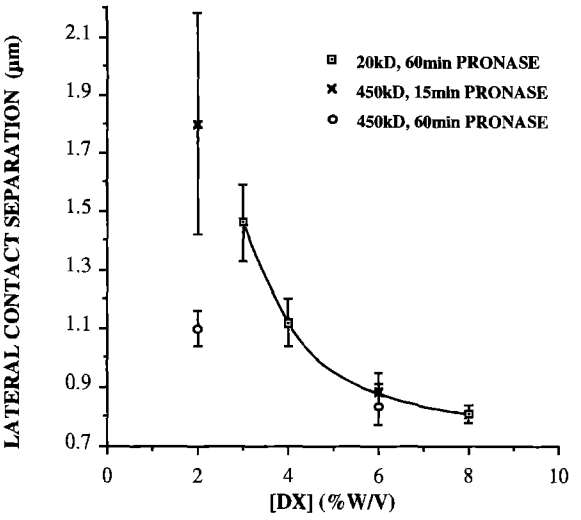

Fig. 2. Average lateral separation of contact points (with 95% conf. limits) obtained from video measurements for cells in different dextran concentrations: –□– cells pre-treated with pronase for 60 min and then exposed to 20 kD dextran: cells pronase pre-treated for 15 min –×– or –○– 60 min and then exposed to 450 kD dextran. The curve represents a trend for 20 kD data only. The cells for each data point came from separate finger punctures from the same donor

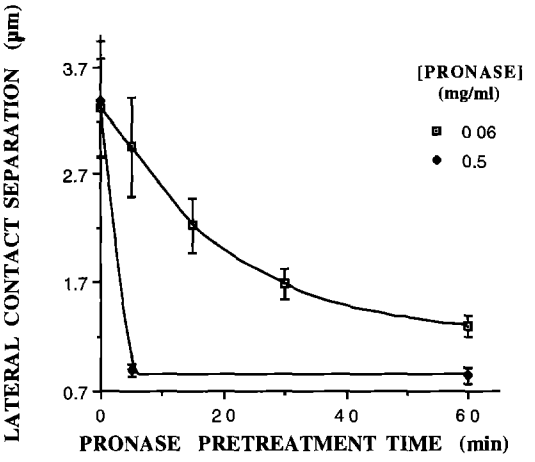

Fig. 3. Average lateral contact separation (with 95% conf. limits) obtained from video measurements for cells exposed to 6% w/v 450 kD dextran following pre-treatment for different times with pronase concentrations of 0.5 mg/ml –♦– or 0.06 mg/ml –□–. The cells for each set of data were obtained from different finger punctures from the same donor

which had been pre-treated with pronase for 60 min and then exposed to concentrations of 20 kD dextran in the range 3% w/v to 8% w/v. The average wavelength ranged from 1.46 μM (±0.13 (95% conf. limits)) to 0.82 μM (±0.03 (95% conf. limits)). The results showed that the appearance of the dominant S state for cells in 4% w/v 20 kD dextran in Table 1 is preceded at a lower dextran concentration (3%) by M sized lateral spacing. Figure 2 includes measurements of spacings for cells pretreated with pronase for 15 min or 60 min and then exposed to 450 kD dextran.

(iii) *Contact separation for mild pronase pre-treatment and exposure to 6% 450 kD dextran*

Measurements shown in Fig. 3 quantify the sharp transition from the L state of control cells in 6% 450 kD dextran (Table 1) to the S state in cells pre-treated with 0.5 mg/ml pronase for 5 min. The figure shows that there was little decrease in contact spacing for an increase in pre-treatment time from 5 to 60 min. We wished to avoid the difficulties of working reproducibly with enzyme pre-exposure times of less than 5 min. Consequently further experiments were carried out for 5 min or longer but at reduced enzyme concentrations (less than 0.5 mg/ml). Figure 3 also shows the dependence of contact spacing on time of preincubation with 0.06 mg/ml pronase. A gradual transition can be seen from 3.3 μM for control cells to 1.3 μM for cells pronase pretreated for 60 min. The lateral spacing for cells pre-treated with 0.25 mg/ml pronase for 5 min before exposure to 6% 450 kD polymer was 1.4 μM.

(iv) *Lateral separation distance for different dextran molecular weights*

Table 1 shows that, because of the absence of adhesion in many of the samples exposed to 20 kD dextran, the number of concentrations and pre-treatment times at which spacing could be compared for the three molecular masses was limited. Where a quantitative comparison could be made for all three masses (15 min pre-treatment with pronase, followed by exposure to 6% w/v of 450 kD, 72 kD or 20 kD dextran) there was little difference in the average measured spacings from two experiments. These measurements (0.89 μM, 0.84 μM and 0.95 μM in descending order of molecular mass) were close to the lower limit observed for such measurements in this work (see Sec. v below) and it may be that spacings were not strongly sensitive to molecular size on that account. When more limited comparisons were carried out at conditions closer to adhesion thresholds than were those of the above three-mass comparison the implications for dependence of wavelength on molecular weight were ambiguous, e.g. Table 1 shows that for some conditions (e.g. pre-treatment with pronase for 5 min and exposure to 2% or 4% dextran) the lateral spacing is longer for cells in 450 kD than in 72 kD dextran while Fig. 2 shows that a 1.1 μM wavelength is achieved at lower polymer concentrations (2%) for cells pre-treated with pronase for 60 min and exposed to 450 kD dextran than was the case (4%) for cells in 20 kD dextran.

(v) *Detection of lower limit of intercontact spacing*

The possibility was recognized that video-assessed light microscopy measurements leading to average wavelengths less than 1.0 μM might be biased against small spacings, because of resolution limits of the optical system. Video microscopy inspection showed that contact separation for the experiments of Table 1 was among the shortest in cells treated with pronase for 30 and 60 min

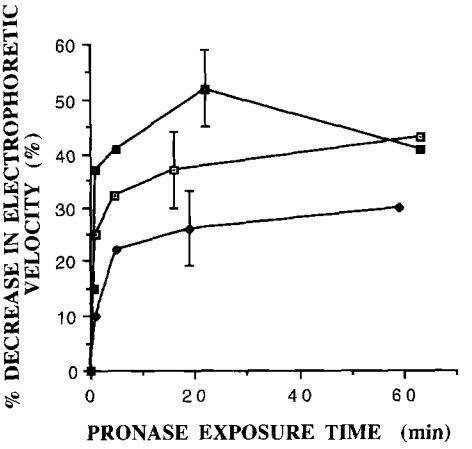

Fig. 4. Decrease in electrophoretic mobility (in PBS) for cell exposed to different concentrations (–□– 0.5, –■– 0.12 –◆– 0.03 mg/ml) of pronase for different times. The (typical) error bars reflect 95% confidence limits for the mean values. The cells were drawn from one donor. Data for each pronase concentration was based on blood from a single corresponding finger puncture

prior to exposure to 6% w/v 72 kD dextran. Measurements with the video system for these conditions gave average lateral separations of 0.8 μM with some individual readings down to 0.5 μM while transmission electron micrographs gave average spacings of 0.6 μM for cells exposed to the same experimental regime. Transmission electron microscopy measurements of average wavelengths for a number of other experimental regimes confirmed that 0.6 μM was the shortest average wavelength observed over the experimental range shown in Table 1. Measurement with either method showed that the lateral spacing tended towards a lower limit as the controlling variable, whether dextran concentration, or pronase pre-treatment time was increased to give short wavelengths.

(vi) *Electrophoretic mobility of enzyme treated cells*

The reduction in cell electrophoretic mobility following enzyme treatment was taken as an index of loss of sialic acid residues (through glycophorin degradation in the case of pronase (Gokhale and Medha 1987)) close to the outer surface of the glycocalyx. Figure 4 shows that the initial decrease in electrophoretic mobility (for cells in PBS) with time of exposure to pronase was sharp, reaching a limiting value within a few minutes for pronase concentrations of 0.5 mg/ml and 0.12 mg/ml. When mobility measurements were carried out in low ionic strength medium we consistently found a lower apparent charge removal (about 25%) in cells which had been exposed to 0.5 mg/ml pronase for 60 min. The difference in electrophoretic mobility change measured in the different ionic strengths may be due to the greater influence of charge deep within the glycocalyx on measured electrophoretic mobility as a result of the reduced charge shielding in the lower ionic strength medium (Donath and Pastushenko 1980). Exposure of erythrocytes to neuraminidase resulted in a much greater decrease in electrophoretic mobility (even in low ionic strength medium)

Table 2. Light microscopy evaluation of contact spacing in cells treated with neuraminidase (0.2 units/ml at 25 °C for times ranging from 0.5 min to 10 min) prior to exposure to dextran of different molecular masses and concentrations. The superscripts give the number of times a category was examined. 1 to 3 individual results were obtained from blood from each finger puncture from a single donor. Electrophoretic velocity measurements were made on the enzyme-treated cells in low ionic strength buffer. The reduction in electrophoretic velocity was expressed as a percentage of the velocity of control cells. Ranges of electrophoretic velocity for which results have been grouped are also shown

% Mobility Decrease	Dextran – 450 kDa Concentration			
	2%	4%	6%	8%
0	^{2}P	^{2}L	^{2}L	^{2}P
35	^{1}L			
55–58		^{2}L, M		
75–82	^{1}L	^{1}M	^{2}S	^{1}S
	Dextran – 72 kD			
0	^{2}P	^{2}P	^{2}P	^{2}P
20		Pa	^{1}P	
48–50	^{1}Pa	^{2}L, M	^{1}L, M	^{1}M
77–79	^{1}Pa	^{1}L	^{1}L, M	^{1}L, M
	Dextran – 20 kD			
0	^{1}N	^{1}N	^{1}N	^{1}N
70				^{1}N
79		^{1}Pa		

that was the case when cells were exposed to pronase. The 45% decrease of electrophoretic mobility of erythrocytes shown for the higher pronase concentrations in Fig. 4 and the 80% value observed for neuraminidase treated cells (Table 2) compare with decrease of 65% and 97% respectively reported earlier for the same enyzmes by Schnebli et al. (1976).

(vii) *The influence of pronase pre-treatment time on electrophoretic mobility and on contact separation*

Table 1 shows that the lateral spacing of contacts in 2% w/v 450 kD dextran decreased as the pronase pre-exposure time was increased to 60 min yet Fig. 4 shows that most of the electrophoretic mobility change for cells in 0.5 mg/ml pronase occurred within the first 5 min (confirming previous work (Darmani and Coakley 1990)). In order to establish that enyzme activity was required to bring about the changes in contact pattern following the increase of pronase pre-exposure from 5 and 60 min an experiment was carried out with heat denatured enzyme. Cells were exposed to 0.5 mg/ml pronase for 5 min and the suspension was split into three aliquots which were centrifuged. One aliquot was resuspended in the initial enzyme solution for a further 55 min, a second was resuspended in PBS buffer while the third aliquot was resuspended with heat denatured enzyme. Figure 5 shows an L/M category response for cells exposed to pronase for

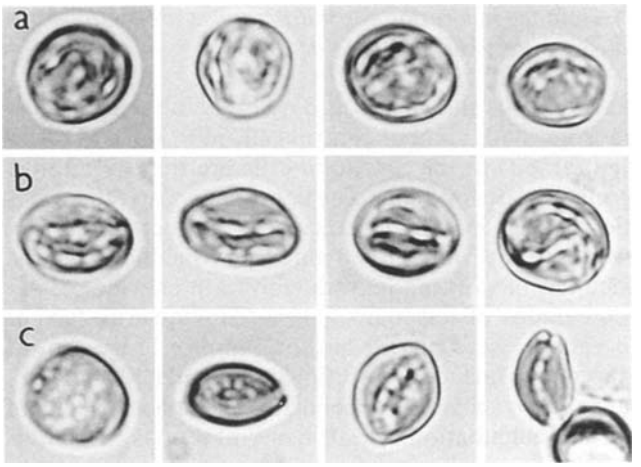

Fig. 5. Quadruplicate examples of erythrocytes exposed to 2% 450 kD dextran following 5 min pre-treatment with pronase and then suspension in **a** PBS, **b** heat denatured enzyme and **c** pronase, for 55 min. The lateral contact separation category is in the L-M range in **a** and **b** and is S in **c**

only 5 min and for cells exposed to heat denatured enzyme for the 55 min period. Cells exposed to pronase for the full 60 min showed an S category. The electrophoretic velocity (in PBS) was reduced by 36% after the initial 5 min enzyme treatment. The reductions were 36%, 38% and 32% respectively for the three samples above at the end of the total 60 min "pre-treatment" period. The experiment showed a continuing requirement for active enzyme to modify the cell glycocalyx despite the failure to detect further charge loss following the additional 55 min pre-treatment.

(viii) *Effect of neuraminidase pre-treatment on cell contact formation*

The light microscopy evaluations of contact seams of cells in dextran following neuraminidase pre-treatment are shown in Table 2. No adhesion was seen in neuraminidase pre-treated cells which had lost 70% of their mobility before exposure to 20 kD dextran in the concentration range 4%–8% w/v (Table 2) while pronase pre-treated cells in the same dextran solutions showed S category spacing for 40% loss of electrophoretic mobility (Table 1, Fig. 3). Spatially periodic contacts were observed in all neuraminidase pre-treated cases (35%–80% charge removal) for cells in 450 kD and 72 kD dextran (Table 2). The observed contact spacings were equal to or (usually) longer than those for cells in the same dextran solutions following 40% charge removal by pronase (Table 1, Fig. 4). Only a small number of neuraminidase pre-treatment experiments gave S category spacings (Table 2) compared to the situation for pronase pre-treatment (Table 1). Where adhesion at cell rims was observed in 4% w/v 20 kD dextran following 80% charge removal by neuraminidase (Table 2) the membranes made parallel contact, i.e. there was no evidence of localised point contact formation.

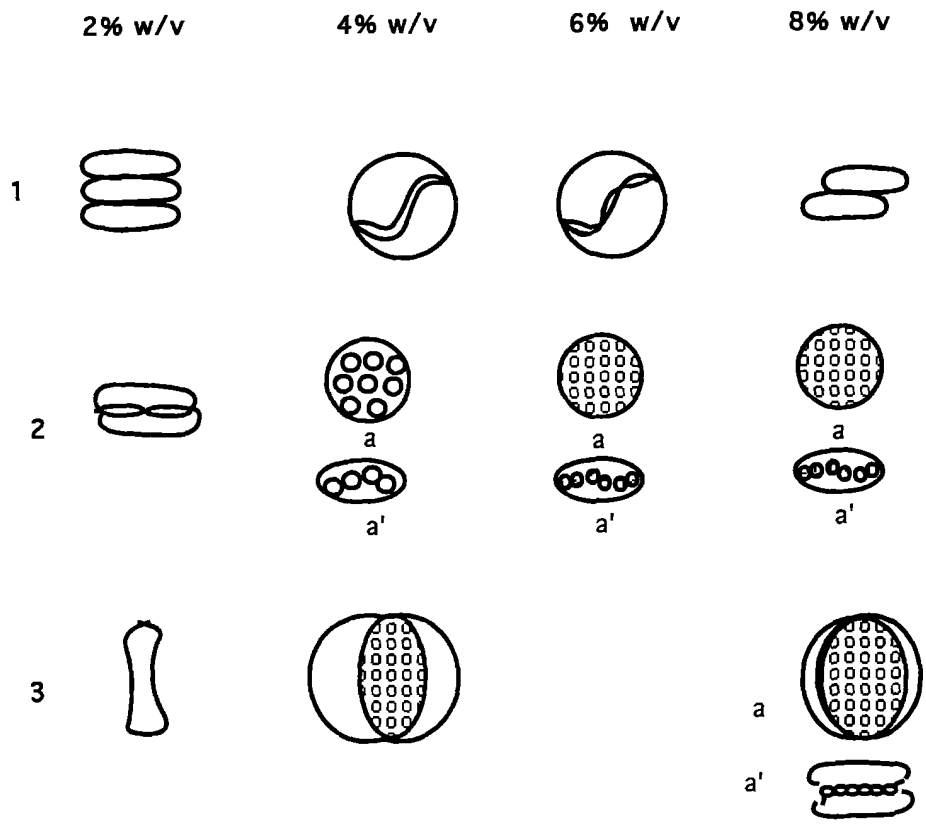

Fig. 6. Illustrations of the principal categories of cell plump morphology for a range of experimental situations. *Row* 1, normal erythrocytes in 450 kD dextran; *Row* 2, cells treated with pronase for 5 min before exposure to 450 kD dextran and *Row* 3, cells treated with pronase for 30 min before exposure to 20 kD dextran. a and a^1 represent plane and elevation views of the clumps respectively

(ix) *Morphology of dextran induced cell clumps*

The principal cell morphologies observed are illustrated in Fig. 6. Row 1 shows, for cells in 450 kD dextran, clumps of cells in parallel contact in 2% and 8% w/v dextran, spherical doublets with parallel membranes at 4% w/v and long lateral separation in 6% w/v. The Row 2 cells, pronase pre-treated for five minutes, show long wavelengths in rouleaux type aggregates formed on exposure to 2% w/v 450 kD dextran, medium wavelengths at 4% w/v and short wavelengths at 6% and 8% w/v. The cells in Row 3 were pronase pre-treated for 30 min and then exposed to 20 kD dextran. The cells in 4% and 8% w/v showed short wavelengths. The contact seam in the elevation sections of Row 3 show that the seam of contact consists of a straight line of discrete contacts with the individual cells retaining much of their normal biconcave shape. This outcome contrasts with the elevation sections of Row 2 where the doublet shape of cells in 6% and 8% w/v 450 kDa dextran is ellipsoidal and the short inter-contact spacings are distributed along a wavy line rather that the straight line of the cells in Row 3. The results in Fig. 6, and the larger pool of results of which they are representative, show that the lateral inter-contact distance is not closely related to the clump morphology.

Discussion

The measurements and observations above imply that the following generalisations hold within the range of variables examined: (*i*) Plane parallel contacts occurred when control cells were exposed to 2% w/v and 4% w/v dextran of molecular mass 450 kD and 70 kD. (*ii*) Spatially periodic contacts were a feature of almost all cases where pronase pre-treated cells were exposed to dextran. They were also seen in normal cells exposed to high (6%) concentrations of 450 kD dextran. (*iii*) The lateral contact separation decreased for increased pronase pre-exposure and for increased dextran concentration. (*iv*) For a given dextran concentration and molecular weight wavelengths were shorter for pronase pre-treated cells than for neuraminidase pre-treated cells even though the latter cells lost more charge.

Let us first review different points of view on the mechanism involved in adhesion of erythrocytes. It was shown previously that adhesion of normal erythrocytes occurs in solutions of dextran with a molecular weight of 40 kD or higher (Jan and Chien 1973) and that neuraminidase pretreatment of cells increased the extent of adhesion. A reduction of ionic strength reduced the adhesion of normal cells but had no effect of the adhesion of neuraminidase treated cells. Jan (1979) concluded that macromolecular bridging played a significant role in overcoming the electrostatic repulsion between cells. Evans and Needham (1988) drew attention to the osmotic effect of surface depletion of dextran in inducing mutual adhesion of liposomes. Donath et al. (1989) recognised the importance of surface depletion in dextran adhesion of erythrocytes but concluded that crosslinking was also contributing to interaction.

The observation in the present work (Table 2) that 80% reduction in electrophoretic mobility (which reflects a loss of glycophorin sialic acid residues (Schnebli et al.

1976), and therefore surface charge, of about 80%) by neuraminidase pre-treatment of cells allowed adhesion at 4% w/v 20 kD dextran is consistent with results of Jan and Chien (1973). Table 2 also shows that cell pre-treated with neuraminidase so that 70% of surface charge was lost remained monodisperse in 8% w/v 20 kD dextran while (for the same dextran condition) Table 1 shows adhesion of pronase pre-treated cells when the decrease in electrophoretic mobility (Fig. 4) was less than 70%.

The pronase and neuraminidase results of Tables 1 and 2 together with Fig. 5 suggest that a charge independent property, which normally contributes to the prevention of cell adhesion, is modified to a greater extent by pronase than by neuraminidase treatment. Schnebli et al. (1976) found that erythrocyte agglutination occured at a lower concentration of the lectin concanavalin A in cells pretreated with pronase rather than neuraminidase even though the latter enzyme reduced electrophoretic mobility more and released more sialic acid than did pronase. They concluded from a number of results of their own and of others in different systems that the effect of the enzymes was not due to loss specific lectin binding sites but was a non-specific effect probably due to removal of peptide and glycopeptide cell surface material which normally sterically hinders the agglutination reaction. (Pronase treatment may also lead to changes in the membrane interactions by changing the short-range dipole interactions of surface proteins). Later, Bell et al. (1984) considered that the repulsive barrier between cells arises mostly from a combination of electrostatic repulsion and of a glycocalyx steric stabilisation effect which arises as the macromolecular layers of approaching cells overlap and water of hydration is squeezed out of the intercellular gap. In their model the repulsive force results from a combination of the osmotic tendency of solvent to return into the intercellular layer and from the steric compression of the glycocalyx macromolecules. More recently available (Patel and Tirrel 1989) force balance measurements of stereorepulsion by adsorbed copolymers (a system with similar properties to a glycocalyx) suggest that the characteristic length for the exponential decay of stereorepulsion is shorter than the glycocalyx thickness.

Despite the emphasis placed on glycocalyx stereo-repulsion effects in explaining the above results of studies involving pronase the observation of Jan and Chien (1973) that normal erythrocytes remain mono-disperse in dextran solutions if the ionic strength is reduced to 50 mM shows that electrostatic effects can be significant in some cases. It is also necessary to recall that pronase treatment, and to a lesser extent neuraminidase treatment, of erythrocytes makes the cell surface more hydrophobic (van Oss et al. 1975; Gascoigne and Fisher 1984) and that hydrophobic interaction increase cell adhesion (Doyle and Rosenberg 1990).

When considering the problem of achieving very close pre-fusion approach of membranes Leiken et al. (1987) proposed an interesting model in which membranes lying adjacent to each other in an interaction energy minimum would be expected, because of random thermal fluctuations, to eventually penetrate a repulsive barrier at a point.

However the only model we know of which accomodates both outcomes in the present work (i.e. either plane parallel membranes or spatially periodic point contacts) is the view that as membranes approach each other the thinning water layer will drain so that the membranes remain parallel and reach an equilibrium separation or the thin water layer becomes unstable to give point contacts.

The instability criterion for a thin fluid film sandwiched between two continuous fluid phases is given (Gallez and Coakley 1986) by

$$k^2\,\sigma_t + k^4\,B - \mathrm{d}P_t/\mathrm{d}h < 0 \qquad (1)$$

where h is the film thickness, B is the bending modulus of the membrane (Evans 1983), $k = 2\pi/\lambda$ (λ is the wavelength of the disturbance which will rupture the thin film); the total surface tension $\sigma_t = 2\sigma_s + \sigma_r + \sigma_a$ where σ_s is the pure surface tension of the film, σ_r and σ_a are the surface tension contributions from sources of repulsive and attractive interactions respectively. Contributions to σ_r include hydration effects, stereorepulsion of the glycocalyx (Gallez and Coakley 1986, Bell et al. 1984) and electrostatic interactions (Prevost and Gallez 1984, Gallez and Coakley 1986). Contributions to σ_a include terms related to the long range van der Waals interaction, attractive macromolecular crosslinking (Gallez and Coakley 1986) and hydrophobic effects (see Doyle and Rosenberg 1990). P_t in (1) is the sum of the attractive and repulsive normal interaction pressures and h is the film thickness. Measurements show that a number of the normal pressures $P(h)$ contributing to P_t in a thin film, including electrostatic $P_E(h)$ (Luckham and Ansarifar 1990), hydration $P_{Ha}(h)$ (usually but not always (Rand et al. 1988)), hydrophobic $P_{Ho}(h)$ (Israelachvili and McGuiggan 1988, Israelachvili et al. 1989), adsorbed copolymer stereorepulsion $P_R(h)$ and macromolecular cross-linking $P_A(h)$ (Patel and Tirrel 1989) can be described by an equation of the form

$$P(h) = \pm P_0 . \exp(-h/L) \qquad (2)$$

where P_0 is the pressure when $h = 0$ and L is a characteristic length for a particular interaction. P_0 is positive when the interaction is attractive and negative when the interaction is repulsive. The magnitude of P_0 can depend on experimental conditions such as the cell surface charge density or adsorbed polymer concentration while the magnitude of L depends, *inter alia*, on ionic strength for electrostatic interactions and configuration for polymer crosslinking (Coakley et al. 1991). The wavelength of the fastest growing disturbance on a non-thinning film, where the bending term is ignored, is (Gallez and Coakley 1986)

$$\lambda = 2\pi(\sigma_t/(\mathrm{d}P_t/\mathrm{d}h))^{0.5} \qquad (3)$$

The results in the present work show that when wavelength changes under conditions where only one variable (e.g. dextran concentration, pronase exposure time or concentration) is being changed the wavelength first decreases gradually (Figs. 2, 3) and tends towards a limiting value of about 0.7 μM (Results, Sec. *v*). When, as for the conditions relevant to the experiments described here, the wavelength of the surface instability is much longer than

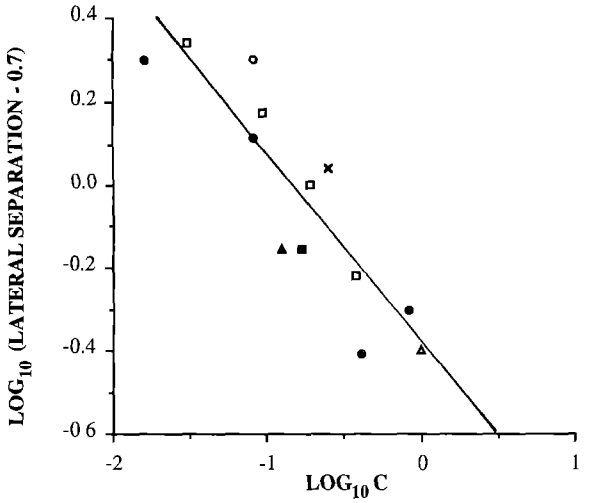

Fig. 7. A plot of the logarithm of $(\lambda - 0.7)$ against the logarithm of constraint "c" (normalised as described in the text) for cell suspended in 450 kD dextran following treatment at a pronase concentration of; –□– 0.06 mg/ml for different times (as in Fig. 3)2 0.25 mg/ml, 5 min, 6% w/v –▲– and 4% w/v –○– dextran. Cells pre-treated with 0.5 mg/ml for different times and exposed to 2% w/v polymer –●– (Darmani and Coakley 1990). Cells pre-treated with 0.5 mg/ml pronase for, –■– 5 min, 4% w/v; –×– 15 min, and –△– 60 min, 2% w/v. Measurements of subsets (–□–) and (–●–) were made from electron micrographs. The remaining data were from the video analysis system. The slope of the regression line is −0.46

the characteristics lengths associated with the type of terms described by (2), a change in interaction pressure will, except in some cases for electrostatic interaction (Darmani and Coakley 1990), have its greatest influence on the instability condition through its effect on the first derivative term dP_t/dh of (1). If the experimental procedures modify P_0 in (2) rather than L (an outcome which is broadly the case for change in dextran concentration, pronase concentration and pronase pre-treatment time) then the wavelength would, for a fixed value of h, be expected to change with P_T^*, the dominant net attractive interaction (The * symbol means that the total pressure P_t is attractive in the situation considered). A parameter, c, has been derived to characterise P_T^* by assuming that it is proportional to dextran concentration, pronase concentration and pronase pre-treatment time and by normalising the different experimental conditions so that c has a value of 1.0 when dextran concentration is 4%, pronase concentration is 0.5 mg/ml and pronase pre-treatment time is 30 min. A log-log plot of the average lateral separation (lambda) against c for 13 data points obtained for 450 kD dextran had a slope of −0.26 and a correlation coefficient of 0.89. The range of variable was 3, 8 and 12 fold for dextran concentration, pronase concentration and pronase pre-treatment time respectively. The plot was confined to lateral separation values greater than 1.0 μM in order to avoid the region where the separation came close to the limiting value.

When (because of the observed approach of lateral separation to a limiting value of 0.7 μM (a mean of 0.8 μM and 0.6 μM; Results, Sec. *v*) $\log(\lambda - 0.7)$ was plotted against $\log c$ the slope was −0.46 (s.d. = 0.07) and the correlation coefficient was 0.88. The approach of separation distance to its limiting value was thus well described by a relationship of the form $(\lambda - 0.7) = \text{Constant}.c^{-0.46}$. The index of c, derived from a normalised set of data representing a wide range of experimental conditions, is similar to the index (−0.5) for the theoretical dependence of λ on the derivative of the net attractive interaction pressure (dP_T^*/dh) in (3), for non-thinning films. Work is in progress now to establish conditions, from a theory of thinning films, which would predict a limiting wavelength.

The present work shows that changes in physicochemical parameters which increase cell-cell attraction or decrease repulsion lead to spatially periodic contacts rather than a parallel membrane configuration. This result supports the thin film instability model for the development of spatially periodic contacts. The lateral separation of contacts also decreases in size as the interactions become more attractive. Because of the dearth of independent data on the relative importance of the different consequences (particularly change in stereorepulsion and change in hydrophobicity) of pronase pre-treatment on cell-cell repulsion no attempt was made to link wavelength uniquely to the different changes in independent variable reported here. Now that the general consequences, for mode of adhesion and contact separation, of changing attraction of repulsion between membranes have been established, it is hoped that future work in which interaction is modified by ionic strength change without recourse to change in enzyme pre-treatment will provide a quantitative test of the instability theory.

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