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INTERFACIAL INSTABILITY AND MEMBRANE INTERNALIZATION IN HUMAN ERYTHROCYTES HEATED IN THE PRESENCE OF SERUM ALBUMIN

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The dynamics of morphological change, when human erythrocytes are heated through the spectrin denaturation temperature in the presence of bovine serum albumin, has been studied using differential interference contrast optics and a television video analysis system. Most washed (control) cells developed a wavy disturbance, with an average of 6.6 ± 0.4 (2 S.E.) waves per cell rim, when heated. The average number of waves per cell rim decreased and the percentage of heated cells showing morphological changes in the dimple region increased with increasing serum albumin concentration, reaching 100% at 1.0 g/l. The change in the dimple region of cells heated in the presence of serum albumin involved the growth of a regular wavy disturbance around the cell dimple rim. The development of the wavy disturbance on the dimple, which resulted in the internalization of membrane, has been examined as an example of an interfacial instability on a biological membrane. Scanning and transmission electron micrographs confirm membrane internalization.

Introduction

Human erythrocytes undergo morphological changes when heated to 50°C [1–3], the thermal denaturation temperature of the structural protein spectrin [4]. Two principal types of morphological change are observed [2,3]. The nature of the dominant shape change depends on properties of the cell surface and its suspending phase [3]. In one type of morphological change (category 1) a wavy outline develops around the cell rim and vesicles pinch from the crest of the surface wave. The category 1 response has been treated [3] as an example of an interfacial instability [5–7], i.e. the growth of a surface wave on an unstable surface. The second type of morphological change observed in heated cells (category 2) involved changes in the dimple region [3]. Phase contrast cinemicrographs of cells during heating suggested that membrane internalization may have been occurring in the dimple region.

In the present work television and video recording techniques have been used to study the dynamic aspects of morphological changes involving membrane internalization at the dimple of cells heated in the presence of bovine serum albumin. The use of differential interference contrast optics and changes in experimental technique, which allow observation at high magnification, have improved the visualization process. Optical observations are supplemented by scanning and transmission electron microscopy.

Materials and Methods

Preparation of blood samples. Human erythrocytes were obtained by a finger puncture and

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Abbreviations: DIC, differential interference contrast; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

collected into 5 mM Hepes-buffered saline (145 mM NaCl) of pH 7.4. The collected cells (about $5 \cdot 10^7$ cells/ml) were centrifuged in a MSE Chilspin centrifuge ($3000 \times g$, 5 mins) and resuspended twice in Hepes-buffered saline. The washed cells were centrifuged and resuspended in buffer containing different concentrations of bovine serum albumin, described as 'essentially fatty-acid free' (Fraction V, Sigma Ltd.).

Cleaning of microcapillaries. The 5 cm long pyrex glass rectangular cross section microcapillaries presently supplied to us require more preparative cleaning than previously [3] in order to avoid echinocyte formation and the occasional cell lysis at room temperature. New microcapillaries were boiled for one hour in a 50% concentration of a non-ionic and anionic surfactant Decon 75 (Decon laboratories Ltd.). The microcapillaries were then rinsed by boiling for one hour in several changes of distilled water. Finally the microcapillaries were stored in distilled water and dried by aspiration just before use. Following use in a heating experiment the microcapillaries were boiled in 2% Decon 75, washed and rinsed as before.

Cells, electrically insulated in microcapillaries, heated by externally applied 20 kHz current. The heating of cells in 5 cm long rectangular cross section, 100 μm pathlength, glass microcapillaries has been described elsewhere [8], except that in the present work the cell suspension filled the microcapillaries and no flow stress was applied to the cells. The microcapillary was bathed in saline on a microscope slide and the external saline was heated by 20 kHz current. The cells in the microcapillary were not exposed to the heating current.

Light microscopy and video recording. Changes in the morphology of cells heated as above were observed by differential interference contrast (DIC) optics on a Nachet NS 400 universal microscope using a $\times 40$ objective lens. Video records of the cells during heating (about 30 cells per video field) were analysed using a Sony 'Videostrobe' analyser. Individual frames recorded at a rate of 50 still-frames per second, could be studied to follow the development of morphological changes. The number of waves per cell rim was scored by counting the initial number of protuberances that developed on the cell outline. The cells which fragmented shortly after developing a wavy outline were scored as category 1 responses. Cells which did not develop a wavy outline around the periphery but showed changes involving the dimple region were scored as category 2 responses.

Oil immersion microscopy of cells heated by current from electrodes inserted into microcapillaries. In studies with a short working distance $\times 100$ oil immersion objective the cell heating arrangement was modified as follows. A cell suspension was drawn into a 2 cm long rectangular cross section microcapillary. Two thin platinum electrodes, connected to a 20 kHz power supply were inserted into the loaded microcapillary. The electrode tips were separated by a distance of 4 mm. The arrangement precluded the determination of heating rate. The cells were heated from room temperature by gradually increasing the output from the power supply. Morphological changes occurred after about 2 min heating. The output from the power supply was then held constant and the gradual development of the morphological changes observed.

Light and scanning electron microscopy of cells heated by an electric field, on a coverslip. A thin (0.085–0.130 mm) circular (6 mm diameter) coverslip was placed on a glass microscope slide. Two platinum electrodes (thickness 0.025 mm) were placed on the coverslip with a gap of 2 mm between the electrodes. A rectangular microcapillary (pathlength 50 μm), connected to a micrometer syringe containing 6% (v/v) glutaraldehyde in buffer, was positioned close to the gap between the two electrodes. A 22×22 mm coverslip (thickness 0.12–0.16 mm) was placed over the arrangement and firmly held in position by adhesive tape. A suspension of cells was introduced onto the small coverslip. The cells were heated by a 20 kHz current from the electrodes. When morphological changes were observed (using $\times 100$ oil immersion optics) the power supply was switched off and the cells allowed to cool for 2 min before being fixed by a slow addition of glutaraldehyde from the micrometer syringe. The arrangement was left in the fixative for 30 min before dismantling. The small coverslip to which the cells were attached was washed first in buffer and then twice in distilled water. The washed sample was then air-dried, gold coated and examined in a Cambridge 600 Stereoscan scanning electron microscope.

Electron microscopy of cells heated in suspension in a test tube. Erythrocyte suspensions were heated in a water-jacketed (4 ml) test tube. The cell suspension (4 ml) was placed in the test tube and incubated for 2 min at 37°C. 2 ml of the suspension was removed by pipette as a 37°C control sample and fixed in 2 ml of 6% (v/v) glutaraldehyde in buffer. The 2 ml remaining in the test tube was heated by circulating water from a waterbath set at 56°C. The temperature of the sample, monitored with a thermistor, rose from 37°C to 52°C in 45 s. The heating rate was 0.2 K/s at 52°C. When the temperature in the test tube reached 52°C the flow from the 56°C waterbath was stopped and replaced by flow from the 37°C waterbath. The sample temperature fell from 52°C to 37°C in 40 s. The cells were left standing at 37°C for 5 min and then gently poured into buffer containing glutaraldehyde. After a 1 h fixation in glutaraldehyde the cells were washed first in buffer and then twice in distilled water. A sample was removed, air-dried on a small coverslip, coated with gold and examined in a scanning electron microscope. The remaining fixed cells were prepared for transmission electron microscopy. Silver sections of the cells embedded in Araldite CY212 (Agar Aids Ltd.) were cut on a Porter-blum ultramicrotome and stained with 2% (w/v) uranyl acetate for 1 h. The sections were washed and restained with 2% lead citrate (in 10 mM NaOH, pH 12) for 10 min. Sections were examined in a Phillips EM 200 transmission electron microscope.

Results

The influence of serum albumin on the fragmentation of erythrocytes was determined by heating cells at 0.5 K/s on a glass microcapillary placed between electrodes on a microscope slide, as described. The data in Table I represents the pooled results obtained over a 2.5 h period from heated samples prepared from a single blood preparation. The order in which samples containing different concentrations of serum albumin were heated was varied so as to reduce any distortion of the concentration dependence data arising from time dependent changes in the red cell membrane. Table I shows that the average number of waves per cell decreased as the serum albumin concentration was increased to 0.075 g/l. Practically every cell showed a category 2 response (internalization) in samples containing more than 0.1 g/l bovine serum albumin. No wavy cells were observed at serum albumin concentrations of 1.0 g/l

TABLE I

THE INFLUENCE OF BOVINE SERUM ALBUMIN (BSA) CONCENTRATION ON THE BEHAVIOUR OF ERYTHROCYTES DURING HEATING THROUGH THE SPECTRIN INACTIVATION TEMPERATURE

The 'wavy cell' class represents those cells which developed a wavy outline on heating. Category 1 represents those wavy cells in which the disturbance around the rim grew to the point of vesicle formation. Category 2 response represents those cells showing no wavy outline but morphological changes involving the dimple region as the cell is heated through the spectrin inactivation temperature

BSA (g/l)	Number of of cells scored	Cell with wavy outlines (%)	Cells which fragmented after wave development (%)	Cells which internalized (Category 2, %)	Average number of wave/cell (±2 S.E.)	Range of wave/cell
0	158	92	72	6	6.6±0.4	5–9
0.001	110	93	70	5	6.5±0.3	5–9
0.01	95	96	82	3	6.4±0.4	5–9
0.05	99	63	25	37	5.5±0.4	4–9
0.075	118	35	19	65	5.2±0.5	4–7
0.1	60	7	7	93	na [a]	na
0.5	36	6	3	94	na	na
1	31	0	0	100	na	na

[a] na, an average number of waves per cell was not calculated because very few of the heated cells developed a wavy outline on the cell rim.

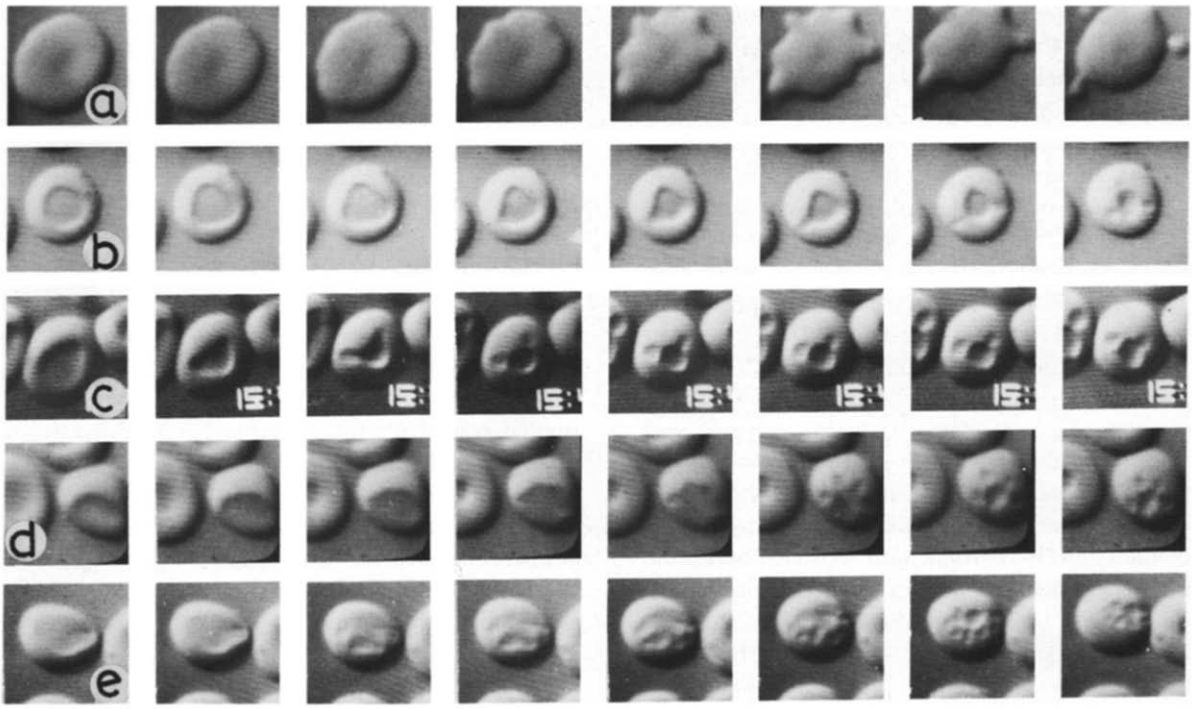

Fig. 1. Selected frames taken from differential interference contrast video records of cells heated through the spectrin denaturation temperature. The time interval in each sequence is 2 s. (a) Sequence showing a cell heated in buffer; an '8 wave' pattern develops around the cell rim. (b–e) Sequences of individual cells heated in buffer containing 1.5 g/l bovine serum albumin. These sequences were selected to illustrate the development of a wavy disturbance, leading to membrane internalization, around the dimple region of the cell.

or higher. Results very similar to those shown in Table I were obtained on three separate occasions.

In Table I the percentage of cells which develop a wave around the cell rim and the percentage of cells which develop a wave around the cell rim and produce vesicles from the wave crests (category 1) are differentiated. In some cases the wave growth stops before vesicles are produced. On continued heating of the latter cells at 0.5 K/s the wave subsides. About 10 s after the start of the wave growth, membrane internalization occurs at the centre of the cell dimple. However, Table I considers only those changes which occur at the spectrin denaturation temperature, and does not refer to any subsequent behaviour of the cells on continued heating. Occasionally a cell showed no sign of a morphological change, neither wave growth nor internalization during the heating process.

For high magnification studies, designed to examine the internalization process, erythrocytes were suspended in buffer containing 1.5 g/l bovine serum albumin. This concentration of serum albumin ensured that a category 2 response was obtained (Table I). Figs. 1 and 2 show human erythrocytes during heating through the spectrin inactivation temperature. The first frame of each sequence in Figs. 1 and 2 was taken at a point just before a morphological change became apparent. The subsequent frames were selected to show characteristics of the morphological changes. The total time for each sequence was between 1 and 2 s.

The heat-induced fragmentation of a control cell in buffer is shown in Fig. 1(a). A wavy outline developed around the cell rim as the cell was heated. The fifth frame of the sequence shows that 8 protuberances or wavecrests appeared around the cell rim. In subsequent frames the wavecrests grew to a point where breakup, forming vesicles at three of the wavecrests, occurred. The first frame of each of the sequences in Fig. 1(b–e) shows that

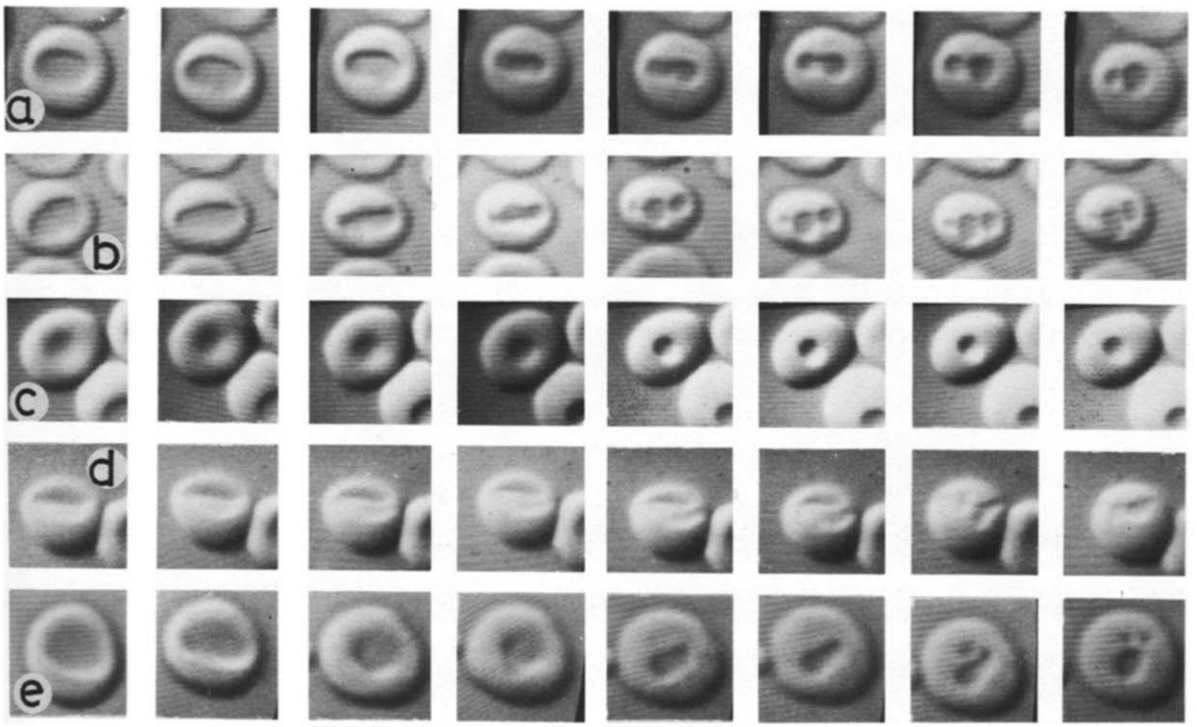

Fig. 2. Examples of morphological changes that occurred in the dimple region of erythrocytes heated in buffer containing bovine serum albumin. With the exception of (c) all of the sequences show the breakup of the dimple region of the cell.

the curvature of the cell dimple in cells in 1.5 g/l bovine serum albumin is more pronounced than is the case for the washed cell in Fig. 1(a). As the morphological changes proceeded the cell diameter decreased, the surface of the cell rose slightly and membrane internalization occurred. The sequence in Fig. 1(b) shows the dimple took up a triangular shape as it passed through the spectrin denaturation temperature. Satellite beads formed at the two lower corners of the triangle and the remaining dimple assumed a circular outline. A further bead appeared shortly afterwards in the position of the third corner of the triangle. The last frame of Fig. 1(b) shows a small circular dimple with three satellite beads. A shadow can also be seen in the central dimple-region which may represent a bead that formed below the dimple. Fig. 1(c) shows another cell in which the dimple took up a triangular shape when heated. Two large satellite beads formed from the corners of the triangle in subsequent frames. The sequence in Fig. 1(d) shows the development of a '6-wave pattern' around the rim of the dimple. In subsequent frames four satellite beads can be seen around the central dimple. Fig. 1(e) shows in the third frame the formation of a wavy outline on the cell dimple region. Subsequent frames show that changes in the dimple region led to the formation of a number of small beads surrounding the larger central form.

Fig. 2 shows some further examples of the morphological changes that occurred when erythrocytes were heated in buffer containing bovine serum albumin. In some instances the dimple took up a slit-like appearance and rapidly transformed to a cell with two or three circular (dimple) regions (Fig. 2(a,b)). These dimple regions remained closely associated with each other. In Fig. 2(c) the only observable morphological change was a decrease in the size of the dimple region as the cell was heated through the spectrin inactivation temperature. The first frame in Fig. 2(d) shows a cell with a small ridge across the dimple region. When the cell was heated this ridge formed a division across the dimple region producing two large beads. Fig.

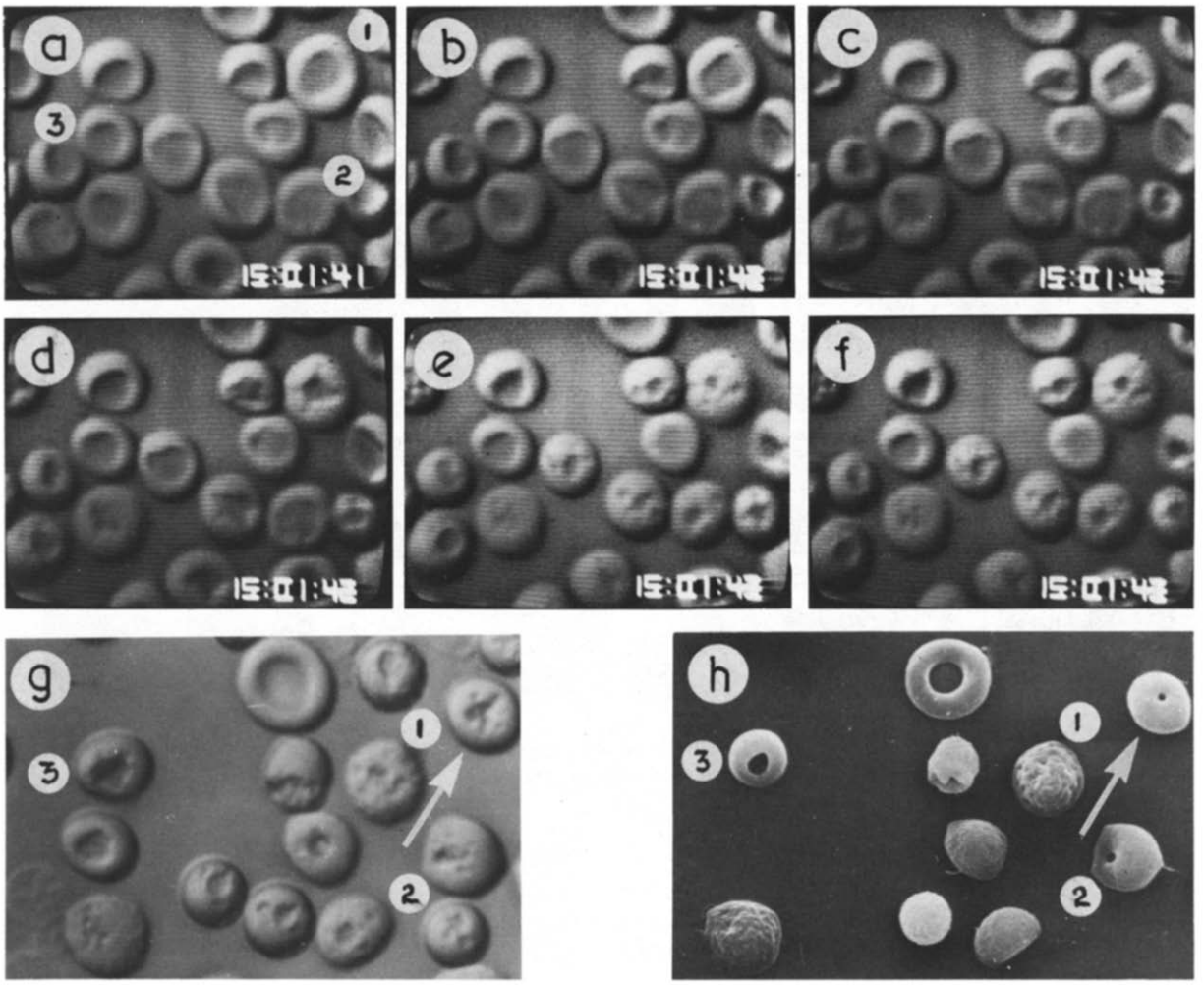

Fig. 3. (a–f) Selected frames taken from differential interference contrast video records of cells during heating through the spectrin inactivation temperature. The progress of the morphological changes can be followed in individual cells. The time display on the right-hand side of the TV screen shows the interval between frames (a) and (f) to be 2s. (g) DIC micrograph of the same field as that observed in the TV monitor, following fixation with glutaraldehyde. (h) Scanning electron micrograph of the same field of view as that seen in the optical micrograph. The DIC image of individual cells can be compared with the surface topography shown in the electron micrograph. Comparison of the arrowed cell, and cells 2 and 3 shows that the satellite beads in (g) are absent in (h) implying that the beads are intracellular.

2(e) shows a change in the cell dimple form as the cell passed through the spectrin denaturation temperature. In the fifth frame the dimple has become smaller and moved across the cell. The final frame of Fig. 2(e) shows that two satellite beads have formed around a larger central form. The bead on the right-hand side of the cell arose from the top right-hand corner of the dimple. The bead on its left probably arose from a bead formed below the dimple. In the fourth frame the shadow at the top left-hand corder of the dimple may represent a bead forming in the dimple.

In order to examine whether the DIC microscope image was showing invaginations of the cell surface or beads below the surface, erythrocytes were viewed during heating and fixation with glutaraldehyde using a $\times 100$ oil immersion objective. The same cells were then examined in a

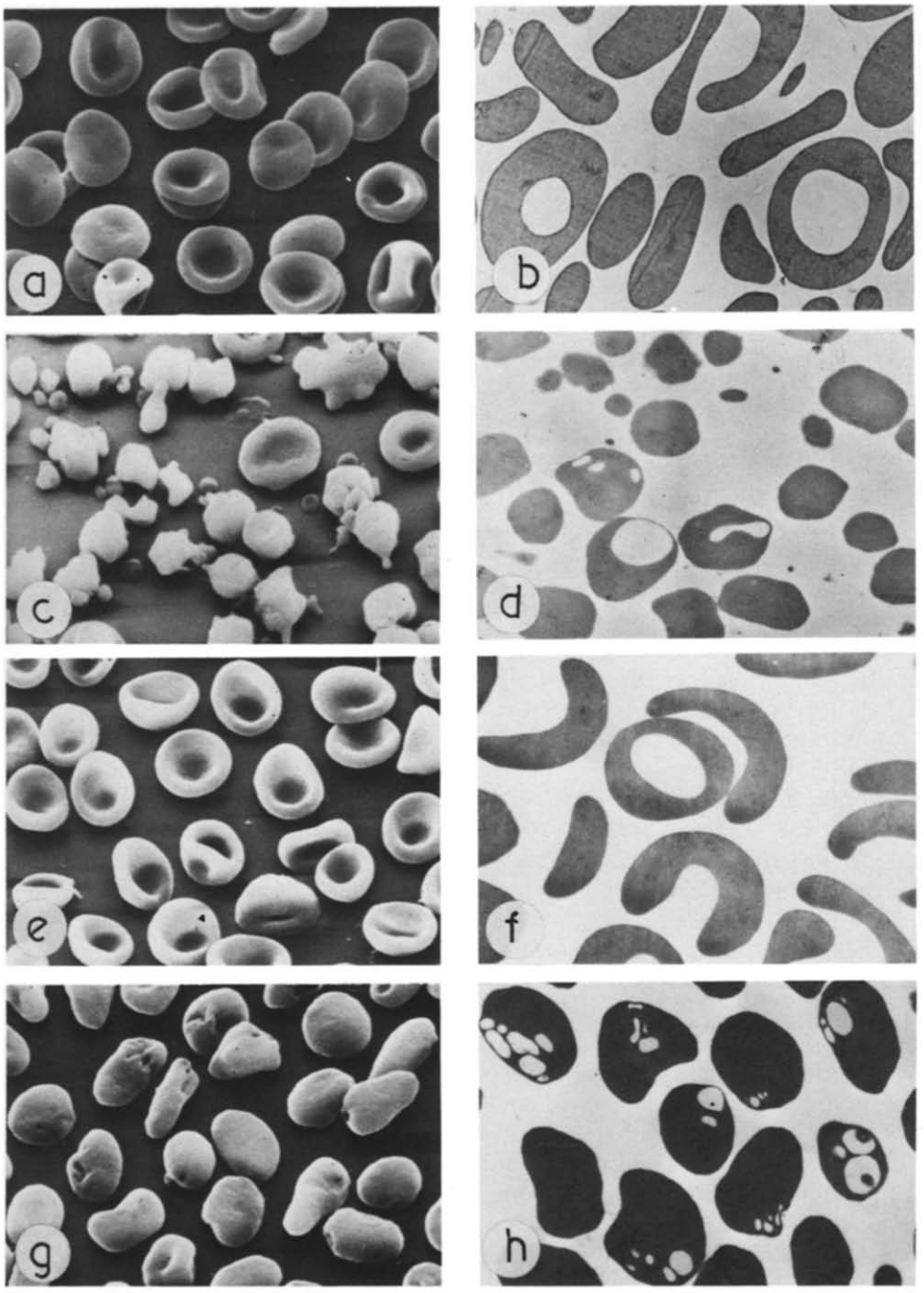

Fig. 4. Scanning electron micrographs (SEM) (a, c, e, and g) and transmission electron micrographs (b, d, f and h) of human erythrocytes incubated in a small test tube: (a) and (b) control cells, washed and resuspended in buffer at 37°C, (c) and (d) cells heated at a rate of 0.2 K/s to 52°C, (e) and (f) cells, washed and resuspended in buffer containing 1.5 g/l bovine serum albumin at 37°C, (g) and (h) cells in bovine serum albumin heated at a rate of 0.2 K/s to 52°C, note absence of cell fragmentation.

scanning electron microscope. Fig. 3(a–f) shows the morphological changes that occurred in 14 cells suspended in 1.5 g/l bovine serum albumin as they passed through the spectrin denaturation temperature. Fig. 3(g) is a DIC micrograph of the same cells as those shown in the video records 10 min after the cells were fixed with glutaraldehyde. Fig. 3(h) is a scanning electron micrograph of the field shown in Fig. 3(g).

The development of morphological change in individual cells can be observed in the sequence of Fig. 3(a–f); for example, the cell labelled (l) took up a square dimple form from which four beads developed. After the morphological changes had occurred the cells were allowed to cool and were observed on the television video system as glutaraldehyde was added. The flow of buffer caused some damage to the cells during the fixation procedure. Some cells lysed as the buffer started to flow over them, other cells in the field developed wrinkled surfaces as a result of the fixation procedure. A comparison of Figs. 3(g) and 3(h) shows that some cells were lost during the preparation for scanning electron microscopy. The electron micrograph in Fig. 3(h) was taken at a 10° tilt to visualize the cell surface topography. In some cases (cells 2 and 3) the surface topography is smooth, showing no sign of the beads seen in the DIC image 10 min after the addition of glutaraldehyde (Fig. 3(g)). The cell arrowed in Fig. 3(g) shows a dimple surrounded by five satellite beads. The SEM image shows that the arrowed cell has a small central hole. The latter cell together with cells '2' and '3' suggests that some beads are intracellular.

The scanning and transmission electron micrograph in Fig. 4 compares the effect of heating, in bulk suspension, washed erythrocytes in buffer containing 1.5 g/l bovine serum albumin with control cells. Fig. 4 (a) and (b) show scanning and transmission electron micrographs, respectively, of control cells, washed and resuspended in buffer at 37°C. Fig. 4(c) shows a scanning electron micrograph of control cells heated to 52°C. Many of the cells have fragmented into small spheres. The transmission electron micrograph (Fig. 4(d)) shows that electron transmitting spaces, or vacuoles rarely occur within the sections of heated washed cells. The electron micrographs of cells in 1.5 g/l bovine serum albumin at 37°C are shown in Figs. 4 (e) and (f). Following heating to 52°C most of the cells have a rounded appearance and there is little evidence of fragmentation (Fig. 4(g)). The transmission electron micrograph in Fig. 4(h) shows that most heated cells contain a number of various sized vacuoles. Fig. 4(h) is consistent with a view that heating erythrocytes in buffer containing bovine serum albumin causes membrane internalization and the formation of internal vesicles. The electron micrographs of cells heated in buffer containing bovine serum albumin (Figs. 4 (g) and (h)) contrast sharply with those heated in buffer alone (Figs. 4 (c) and (d)).

Discussion

The heated erythrocyte has been used as a model system in which to examine the shape changes produced by membrane stresses when the contribution of the cell cytoskeleton to the membrane elastic properties is reduced. Table I showed that the presence of serum albumin in the suspending buffer altered the shape change observed in heated cells. Increasing the bovine serum albumin concentration resulted in a decrease in the average number of waves per cell rim and an increase in the percentage of cells showing a category 2 response, i.e. morphological change at the cell dimple. Table I also showed that a number of cells which developed a surface wave on the cell rim did not produce vesicles from the wave crests. These 'late internalization' cells probably represent a state where the destabilizing stresses in the membrane were low.

Jay [9] has examined the influence of human and bovine serum albumin on the geometry of human erythrocytes at room temperature. From digitized profiles of erythrocytes hanging on edge from coverslips he calculated that the mean minimum thickness of cells at their dimple decreased by 9% and the mean maximum thickness at the rim, increased by 6% in 1.0 g/l bovine serum albumin, a concentration where the effect of the protein on cell shape reached saturation. Part of the 4% decrease in average cell diameter was attributed to the fact that some cells had assumed a cup or stomatocyte shape [9].

Many of the cells suspended in 1.5 g/l bovine

serum albumin in the present study had pronounced dimple curvature before the heat induced morphological change occurred (Figs. 1–4). However, Table I shows a significant category 2 response in heated cells at 0.05 g/l bovine serum albumin, a concentration much lower than the 1.0 g/l found by Jay to produce maximum shape changes at room temperature.

In plasma, serum albumin influences the concentration of lysophosphatides and free fatty-acids in the erythrocyte membrane [10]. Mohandas et al. [11] used albumin to remove previously added lysophosphatidylcholine from the outer leaflet of erythrocyte membranes, thus reversing the discocyte-echinocyte shape change observed when the lysophosphatidylcholine was added to a red cell suspension. The removal by serum albumin, of lysophosphatidylcholine and free fatty-acids from the outer monolayer of the cell membranes in the present work may be inducing a bending bilayer couple, of the type proposed by Sheetz and Singer [12], in the erythrocyte membrane.

When cells were heated in the presence of 1.5 g/l bovine serum albumin Fig. 1 and Fig. 3 (a–f) show that in some cases a regular wave form developed around the dimple region and satellite beads were produced at points around the wavy disturbance. Differential interference contrast optics enabled the category 2 response to be examined with greater clarity than could be obtained with the phase contrast optics employed in previous studies of heat induced morphological change [3]. Allen et al. [13] have pointed out that it is possible to be misled when interpreting differential interference contrast images into thinking that one is looking at a three dimensional image of surface topography. Consequently, in the present work, it was difficult to decide whether a detail of an image represented a surface depression or an intracellular object close to the cell surface but within the shallow depth of focus. A comparison of the appearance of cells in the optical microscope during heating and after fixation (Fig. 3(a–g)) with the same cells in the scanning electron microscope showed that, at least in some instances, the beads were beneath the cell surface. The transmission electron micrographs of cells heated in the presence of bovine serum albumin while in suspension support the conclusion that membrane internalization has occurred (Fig. 4h).

In our previous study [3] and here in Table I, erythrocytes were heated in a microcapillary placed between electrodes on a microscope slide. Under these conditions the microcapillary wall insulated the cells from electric field effects. However, to obtain the high magnification sequences in Figs. 1–3 an alternative heating arrangement was used. For this unique situation (because of the short working distance of the ×100 oil immersion lens) it was necessary to insert platinum electrodes into a microcapillary containing a suspension of cells. In this latter case the electric field strength between the inserted electrodes during heating was of the order of 25 V/mm. This field strength is lower than that of 210 V/mm required for the electrical breakdown of the human erythrocyte membrane [14]. It is also lower than the field strength of 50 V/mm at 2 MHz [15], required to form reversible contact between human erythrocytes. But it is within the range where orientation of cells can occur. If heating significantly affected membrane permeability the membranes of leaky haemolysing cells would have reduced electrical resistance and would allow intracellular currents

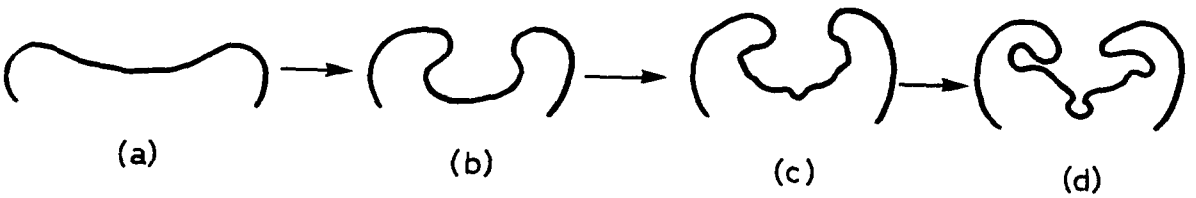

Fig. 5. Diagrammatic representation of the internalization of membrane; (a) The cell profile at a temperature just below the spectrin denaturation temperature. (b) The cell diameter decreases at the spectrin denaturation temperature, the cell dimple deepens and the membrane at the dimple develops a fold; (c) a regular disturbance grows on the edge of the membrane fold and (d) vesicles pinch from the crests of the growing disturbance.

to flow which might influence the fragmentation pattern in the heated cells. The haemolysis temperature of cells heated in microcapillaries over a wide range of heating rates is more than 14°C higher than the spectrin denaturation temperature [16]. There was no suggestion in the present work that serum albumin lowered the haemolysis temperature. It is unlikely therefore that undesirable intracellular currents occurred. In our high magnification study the electric field did not appear to significantly affect the morphological changes observed. Similar changes can be seen under a ×40 magnification, albeit with lower resolution, when cells are heated in a microcapillary (insulating the cells from electric field effects) placed between electrodes on a microscope slide [3,17]. The membrane internalization shown in Fig.'4 was also achieved in a system free from electric field effects.

Examination of the video records of cells during heating suggests that the main cell body tends towards a spherical shape and the cell diameter decreases as the spectrin is denatured. As the cell became more rounded the membrane of the dimple develops a fold (Fig. 5). It appears that the surface wave grows on the highly curved outline of that fold and that vesicles pinch from the crests of the wave as it moves into the fold. Besides the surface wave growth on the cell dimple the pinching of vesicles from the wavecrests (Fig. 1) and the morphological changes in Figs. 2 (a) and (b) are further examples of interfacial instabilities. Membrane internalization can occur without any apparent breakup of the internalized vesicle (Fig. 2c).

Features of the process of internalization of membrane when cells are heated in high serum albumin concentrations (Fig. 1(b–e)) are similar to those involved in the externalization of membrane in heated washed cells (Fig. 1a). Specifically vesicle formation in both cases follows the growth of a regular surface disturbance on the cell membrane. Also, the shape of the central dimple surrounded by satellite beads in internalized cells (Fig. 1(b–e)) is similar to the appearance of the main cell body surrounded by vesicles when membrane is externalized (Fig. 1a). The terms membrane internalization and externalization have been used here to describe the shape change leading to the production of small rounded membrane-bound bodies, often attached by a narrow neck to a main cell body. The final step of complete externalization or internalization, i.e. fusion of membrane at the narrow neck is not addressed. The observation that in heated cells significant internalization occurs at serum albumin concentrations which are low compared with those required to produce significant stomatocyte formation at room temperature suggests that the heated erythrocyte system would be a sensitive means of monitoring the influence of different agents on membrane bilayer bending.

Acknowledgements

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