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THE INFLUENCE OF TEMPERATURE AND INCUBATION TIME ON DEFORMABILITY OF HUMAN ERYTHROCYTES

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Summary

Human erythrocytes have been heated and stressed in a novel and controlled manner using rectangular microcapillaries. Heated cells attached to the capillary wall were stressed by liquid flow. Under particular conditions of stress, temperature and incubation time the body of the cell could be pulled in the flow, retaining a connection with the glass by means of a narrow process or tether. The tethers appear as: regularly beaded, irregularly beaded or without beads depending upon the incubation conditions. We have outlined the incubation regimes necessary to achieve these different responses in the temperature range 48—55°C. The cells become less deformable as the incubation is continued beyond an optimum time. The behaviour of the tether is compared with that of a viscoelastic liquid. Circular dichroism studies of ghost membranes show that the denaturation of membrane proteins is partially reversible when incubation times are similar to those required to bring about a loss of deformability.

Introduction

Human erythrocytes fragment when heated to temperatures in the region of 50°C [1]. It has been suggested that the fragmentation takes place during a transition of the red cell from a body with elasticity through a plastic stage to a cell which behaves in some respects like a highly viscous fluid [2]. Despite the fluid-like behaviour implied by the fragmentation process Teitel [3] found that cells incubated at temperatures in excess of 47°C for 20 min were less deform-

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able than control cells. He showed that the enhanced stiffness of the cells resulted primarily from a change in the biophysical behaviour of the membrane rather then from the morphological changes that resulted from heating. Williamson et al. [4] stressed cells attached to glass at various temperatures up to 50° C and found that the deformability of the cells increased markedly at the higher temperatures. At temperatures of 37° C and stresses above 0.2 N/m^2 a process or tether was frequently drawn out from the cell at its point of attachment to the glass cover-slip. At 48—50° C the entire cell was irregularly deformed and markedly elongated at stresses as low as 0.1 N/m^2. Cells heated to 49° C for 1 min before stressing at 37° C were less deformable than cells stressed without preheating. Williamson et al. [4] suggest that if cells are subjected to shear stress while a critical structural protein or other constituent was undergoing conformational changes which loosen its intramolecular associations with other membrane constitutents, cell membrane material will flow more easily. When the cell is cooled the conformational changes and altered intramolecular associations of membrane constituents might not be reversed, rendering the membrane less deformable than normal.

The four structural transitions of the erythrocyte membrane have been examined by Brandts et al. using scanning calorimetry and circular dichroism measurements [5]. They were able to demonstrate the involvement of spectrin in the 'A transition' which occurred at 49.5° C. They showed by scanning calorimetry at a heating rate of 20°/h that the 'A transition' was irreversible.

In this study we have incubated cells for various times at temperatures up to 55° C before exposing them to a stress pulse. We show that there is an optimal temperature and time regime that produces a highly deformable cell state. On continued incubation the cell becomes less deformable even at elevated temperatures. We suggest that the incidence and type of bead formed on the cell tether reflects the deformability state of the membrane. This study is the first occasion when environmental conditions have been so controlled that incubation regimes can be prescribed to bring about different stages of cell deformability. The information obtained may help in the identification of structures and enzyme activities important in controlling erythrocyte deformability. Circular dichroism measurements on ghost membrane preparations show that the change in the α-helix content of the membrane has a reversible and irreversible component following incubation.

Methods and Materials

Human erythrocytes were collected by finger pricks, diluted into phosphate buffered saline (150 mM NaCl in 5 mM sodium phosphate buffer, pH 7.4) to a concentration of approximately $5 \cdot 10^7$ cells/ml, held at room temperature and used within 1 h. The cells were stressed in 10 cm long glass rectangular precision microcapillaries with 0.1 mm wall thickness, 0.1 mm pathlength and 1.2 mm width (Microslides, Camlab Ltd.). A drop of cell suspension was placed on a clean surface. One end of the microcapillary was brought into contact with the drop. The cell suspension flowed along the microcapillary at a rate of the order of 1 cm/s. It was therefore, a simple matter to control the length of the column of cell suspension within the microcapillary. The end of the micro-

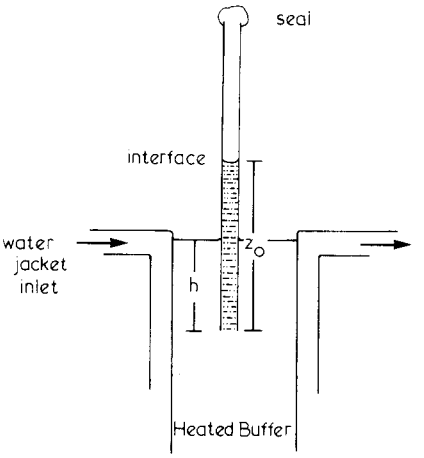

Fig. 1. Incubation conditions before stressing a column z_0 of cells in a vertical microcapillary immersed to a depth h.

capillary away from the cell suspension was sealed with a small amount of silicone grease. The sealed microcapillary was placed flat with its axis in the horizontal direction for 5 min to allow the red cells to sediment and attach to the glass. The microcapillary was then immersed vertically to a predetermined depth in buffer containing 6% glutaraldehyde as shown in Fig. 1. This solution was contained in a water jacketed test-tube maintained at a constant temperature ±0.2°C by circulating warm water. The thin walls of the microcapillary allowed rapid temperature equilibration. The cells were incubated for a known time and the sealed top of the microcapillary broken open with pincers. The buffer containing glutaraldehyde flowed into the microcapillary and stressed the cells, which were then fixed by the glutaraldehyde. The stress on the cells is proportional to the velocity of the interface as it moves up the microcapillary. It will be shown elsewhere [6] that in a vertical microcapillary the initial stress τ on cells at the wall is given by:

$$\tau = \tfrac{1}{2}\left[(2\gamma \cos\theta + h\rho ag)\, z^{-1} - \rho ag\right] \tag{1}$$

The stress decreases with time as z increases. In Eqn. 1, γ is the interfacial tension of the buffer (taken as $7.2 \cdot 10^{-2}$ N/m), a, is the thickness of the gap of the microcapillary, θ is the contact angle of the buffer with the glass, η is the coefficient of viscosity, z is the distance of the interface from the bottom of the microcapillary, h, is the depth of immersion of the microcapillary, g, is the acceleration due to gravity and ρ is the density of the buffer. The time required for the interface to move from its initial position z_0 to a position z at time t is given by:

$$t = \delta\xi g^{-2} \ln[(\xi - gz_0)(\xi - gz)^{-1}] - \delta(z - z_0)g^{-1} \tag{2}$$

where

$$\delta = 12\eta/\rho a^2 \text{ and } \xi = (2\gamma \cos\theta)/\rho a + gh$$

The times for the interface to move between selected points was measured and compared with predicted values. Both times agreed when $\cos\theta$ was taken as 0.6. It was found that the reproducibility of the flow time was greater in 'as received' batches of microcapillaries which were not subjected to a rigourous attempt at cleaning. The above value of $\cos\theta$ was therefore employed in Eqns. 1 and 2 to compensate for the angle of contact effects.

In some experiments cells which had been incubated at tempratures of the order of 50° C for fixed times were cooled for varying intervals before stressing. The cells were cooled by circulating water at 30° C through the jacket of the tube in which the microcapillary was immersed. Typically the temperature of the buffer fell from 52° C to 40° C in 40 s, finally falling to 30° C.

Haemoglobin-free membrane ghosts were prepared from recently out-dated transfusion blood by the method of Dodge et al. [7]. The loose white pellet of membrane ghosts obtained was suspended in an equal volume of buffer (150 mM NaCl in 5 mM sodium phosphate buffer, pH 7.4) with the addition of sodium azide to a final concentration of 0.5 mM. To reduce light scattering during CD measurement [8] 5-ml volumes of the ghost suspension were exposed to five 1.0-s pulses of 100 W, 20 kHz ultrasound (Dawe Ltd, Sonicator type 1132 B) with a 20 s interval between pulses.

CD measurements were made with a Roussel-Jouan Dichrograph MkIII spectropolarimeter using a 1 mm pathlength cell. The cell housing was heated by water circulation from a thermostatically controlled water bath. Samples were at room temperature when placed in the spectropolarimeter. The heating rate of the sample was determined in separate experiments by inserting a small bead thermistor [U23 UD, Farnell Ltd.) in a sample so that the bead touched the face of the cell which was in contact with the heated housing during measurements. The heating rate was 0.7 min^{-1}, the sample was within 0.5° C of the final equilibrium temperature after 5 min incubation. The temperature at the face were related to temperatures at the top of the sample. The latter temperatures were measured at the end of each CD measurement.

When measuring the effect on the CD measurements of cooling the sample the spectropolarimeter was cooled by water circulation from a bath at 22° C.

The relative protein content of the ghost membrane preparation was determined by dodecyl sulphate polyacrylamide gel electrophoresis as described by Fairbanks et al. [9]. The optical density profile of the stained gels were obtained with a Vitratron Universal densitometer using a 0.25 mm slit.

Results

Cells stressed as described above were examined by phase microscopy after treatment. There were usually about 100 cells in the microscope field of view. Four categories of response were defined, examples of which are shown in Fig. 2: (1) the fixed cells showed little if any distortion throughout the entire field: (2) the cells were distorted to varying degrees, generally having short tethers. Few cells had a process drawn out to a length exceeding 15 μm: (3) the tethers were generally 40 μm long being cylindrical or broken into irregularly sized and spaced beads: (4) the tethers were generally between 40—60 μm long with regularly sized and spaced beads along the length.

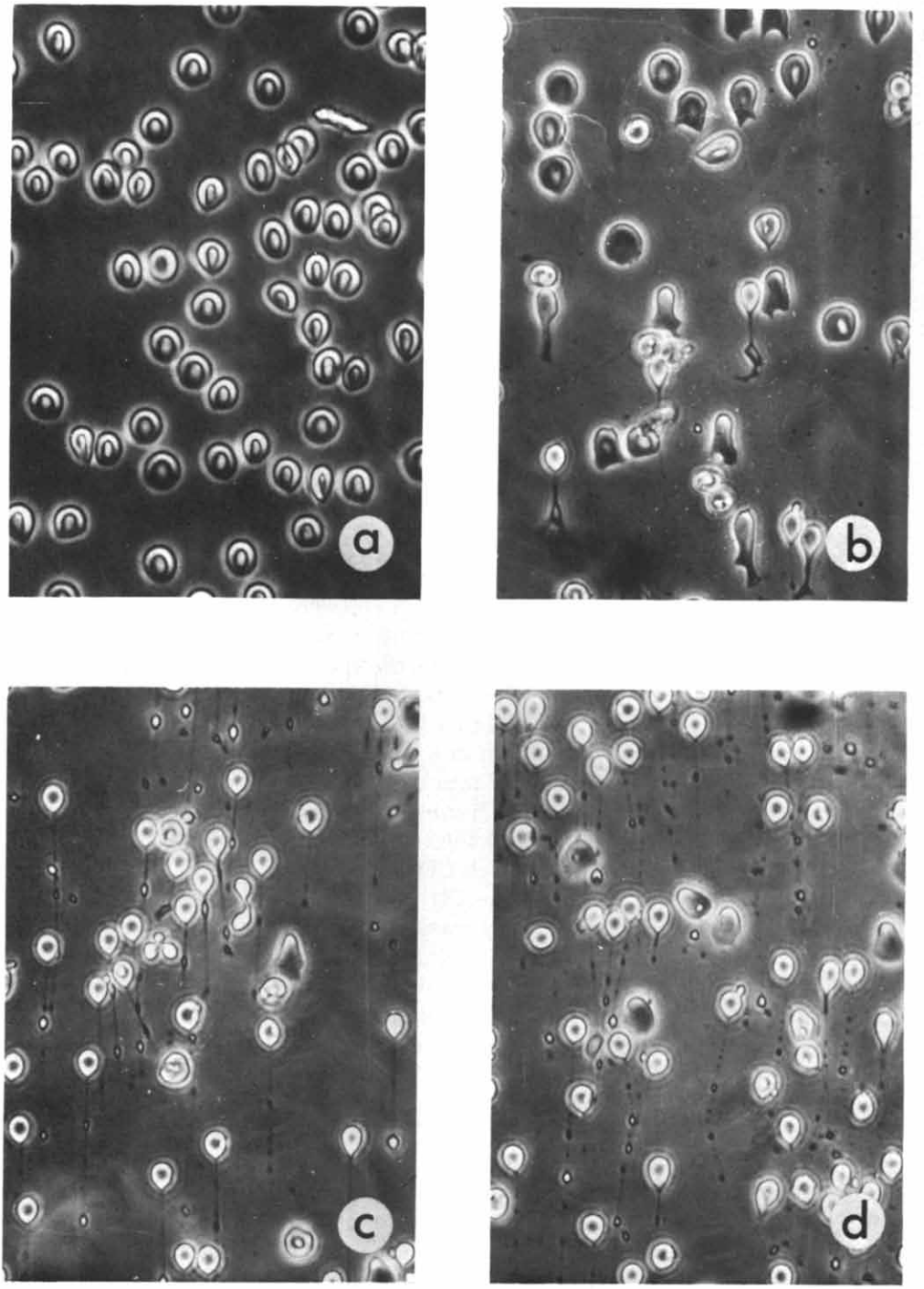

Fig. 2. Photomicrographs of different categories of cells fixed after stressing with buffered glutaraldehyde (a) Category 1; shows no process or tether formation (b) Category 2; shows unbeaded tethers on some cells (c) Category 3; beading is present but not well defined and is irregular (d) Category 4; predominance of long regularly beaded tethers.

TABLE I

DIFFERENT CATEGORIES OF RESPONSE OF CELLS TO A STRESS PULSE AFTER INCUBATION AT VARIOUS TIMES AND TEMPERATURE

The numbers refer to the different categories as illustrated in Fig. 2.

Temperature (°C)	Time (s)							
	30	60	90	120	180	300	600	900
48	1	1	1	1	1	1	1–2	1–2
49	1	1	1	1	1–2	1–2	1–2	1–2
50	1	1	1–2	2	2	2	2–3	2–3
51	3	3	3–4	4	4	2	2	1–2
52	3	3–4	3–4	4	4	3	2	1–2
53	3	3	3–4	4	4	3–4	3	1
55	3	3	3	3	2–3	1–2	1	1

The temperature-time dependance of tether/bead formation was determined using a stress regime which would give category 4 tethers under optimal conditions. The initial length of the column of cell suspension in the microcapillary was 55 mm and the cells were immersed to a depth of 30 mm. The fields of view at which the tethers were assessed was 15 mm from the open immersed end of the microcapillary. The initial stress was 0.3 N/m^2, the velocity was 1.7 $cm \cdot s^{-1}$ and the duration of stress was 7.5 s.

The results of stressing cells under the above conditions following incubation at different temperatures and times are shown in Table I. Inspection of the table shows that an incubation time of 60 s is required before category 4, evenly spaced beads are produced at the optimal temperature of 52°C. Incubation at 55°C for 120 s produced less fluid cells than incubation at 52°C. Maintaining cells at 52°C for times longer than 180 s showed a continuous decrease in the fluidity of the cell with the time of incubation. Table I shows that at a constant stress and time of incubation there is a temperature window for the production of evenly beaded tethers. Likewise at a constant temperature and stress the deformability of the cell increases with incubation time, reaches a maximum and then decreases.

To examine the effects of a time delay following incubation before exposing the cells to stress the cells were incubated at a number of temperatures for 120 s and then cooled to 30°C for various times before stressing. Table II

TABLE II

THE EFFECT OF A DELAY AT 30°C BEFORE STRESSING CELLS WHICH HAD BEEN INCUBATED FOR 120s AT DIFFERENT TEMPERATURES

Temperature (°C)	Time delay (s)							
	0	30	75	150	300	600	900	1800
51	3	3	3	2	2	1	1	1
52	4	3–4	3	2–3	2	2	2	2
54	3	3	2–3	2–3	2–3	2	2	2

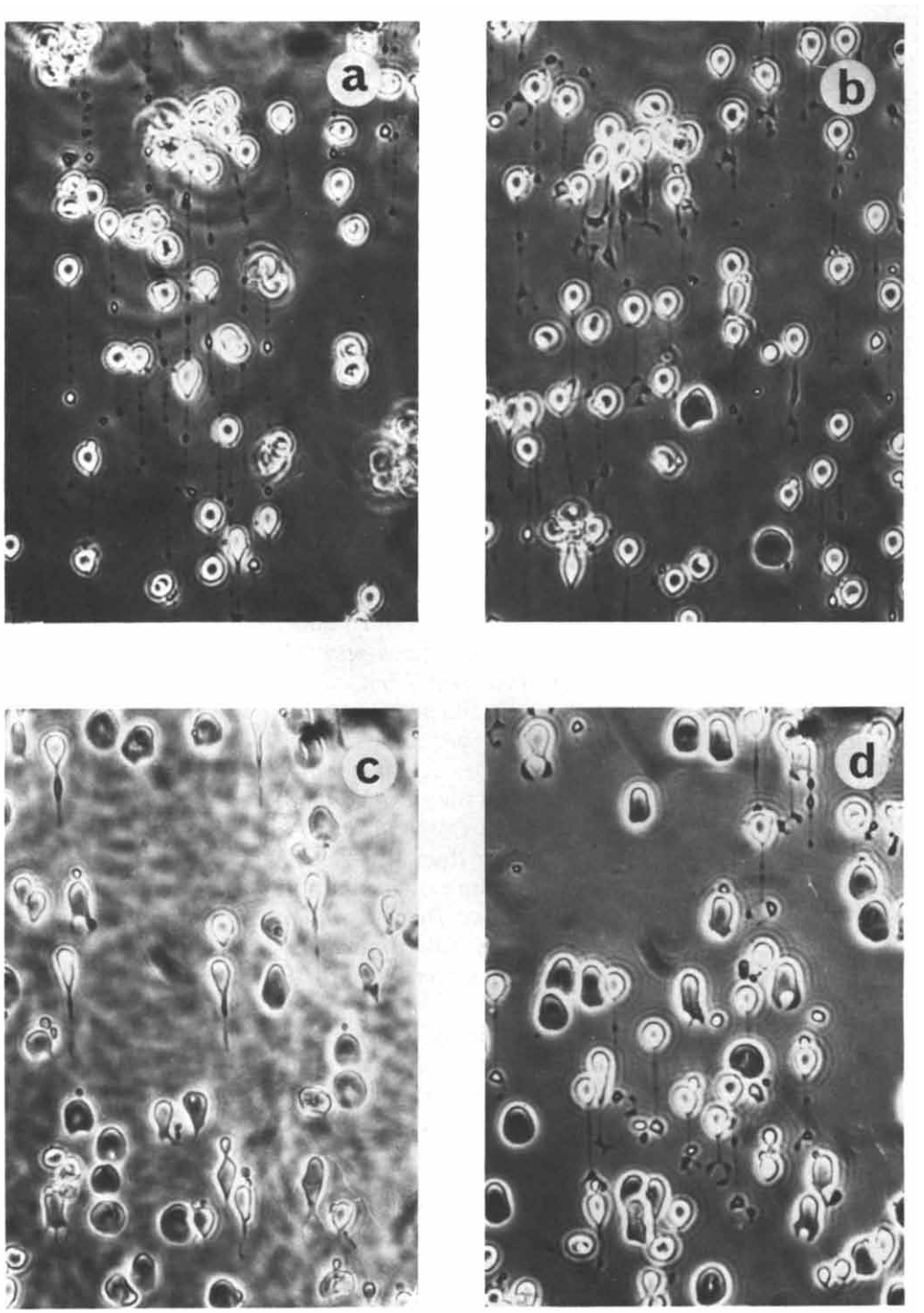

Fig. 3. Photomicrographs of stressed cells of different thermal histories. (a) Heated for 2 min at 52°C before stressing, (b) heated for 4 min at 52°C, (c) heated for 2 min at 52°C then cooled to 30°C for 10 min before stressing, (d) heated for 2 min at 52°C cooled for 10 min at 30°C and reheated to 52°C for 2 min before stressing.

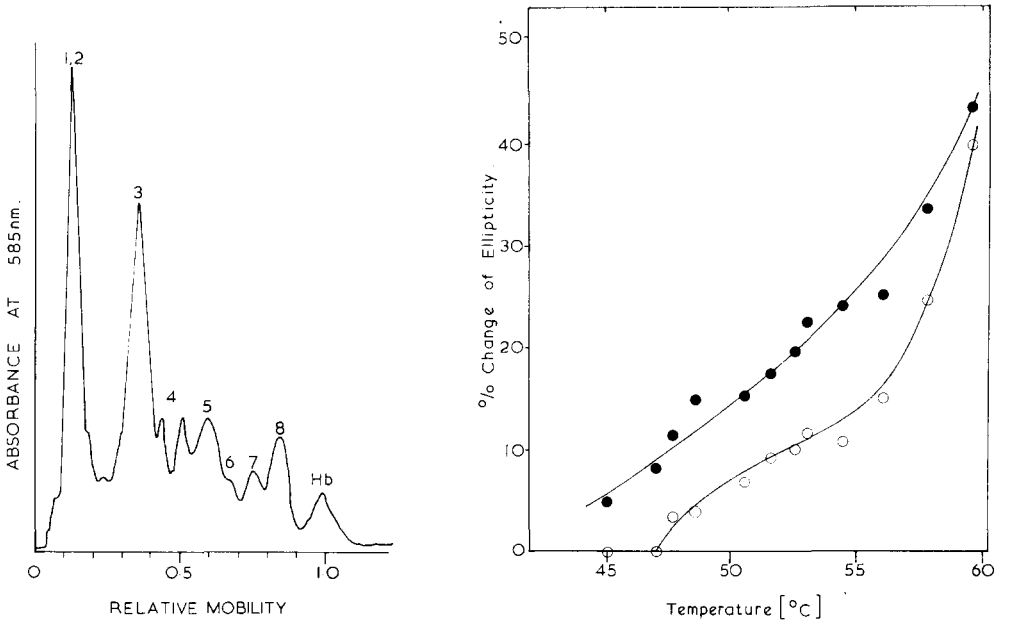

Fig. 4. Densitometric scan of the white membrane ghosts used in CD measurements. The numbering system is that of Fairbanks et al. [9].

Fig. 5. ●———●, the percentage change in ellipticity after 15 min incubation; ○———○, the percentage change in ellipticity after cooling incubated sample for 15 min to 22°C (the irreversible change).

shows that the cells became less deformable on standing and reached an apparently constant level of deformability after 10 min. The effect of reheating the cells before stressing them is shown in Fig. 3. Cells heated continuously for 2 min and 4 min show type 4 and 3—4 behaviour respectively as expected from Table I. Cells cooled for 10 min following a 2 min exposure to 52°C gave a category 2 response to stress (Fig. 3c) while cells heated for 2 min at 52°C, cooled to 30°C for 10 min and then reheated showed a category 2—3 response when stressed (Fig. 3d). Similar observations were made in three sets of experiments.

Fig. 4 shows an optical density scan of the dodecyl sulphate polyacrylamide gel electrophoretic pattern of the ghost membrane preparations used in the CD measurements. Little haemoglobin contamination is present. The ellipticity of the ghost membrane preparations decreased when the samples were incubated at elevated temperatures. The percentage decrease in ellipticity for samples incubated for 15 min at different temperatures is shown in Fig. 5. Smoothed spectropolarimeter records for incubations at 48.5°C and 57.5°C are shown in Fig. 6. The ellipticity change had reached a steady value at the end of the 15 min incubations. The percentage change in ellipticity following cooling for 15 min is also shown in Fig. 5. It can be seen that while there is some recovery of ellipticity on cooling a substantial amount of permanent change has been brought about in the membrane proteins. As the incubation temperature is increased the recovery on cooling is less.

Fig. 6 shows that membrane incubated for 15 min at 57.5°C and then cooled

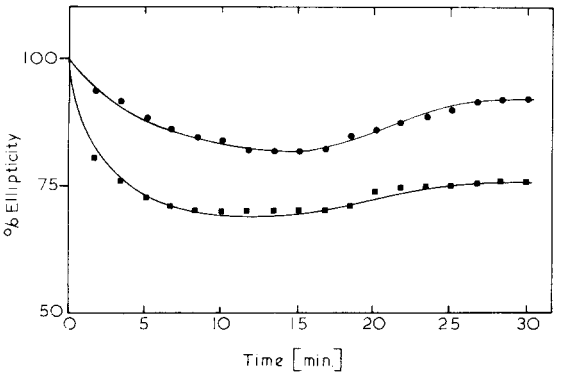

Fig. 6. Smooth spectropolarimeter records obtained from direct measurements of the CD signal during incubation. ●——●, sample incubated at 48.5° C; ■——■, sample incubated at 57.5° C.

to 22° C recover the reversibly damaged part of their ellipticity within 5 min while cells at 48.5° C do so within 10 min. The time delay in the sample reaching measurement temperature during the initial heating and the time involved in bringing about a temperature drop to 30° C make studies on the time scales involved in the earlier stages of Table I impractical.

Discussion

Four main categories of response of heated attached red cells to stress have been examined in the present work. Table I shows that when cells are subjected to the same stress conditions the most deformable state i.e. category 4 is not observed in incubations of 900 s or less until a temperature in excess of 51° C is employed. At temperatures of 51° C and above the extent of deformability increases to a maximum with incubation time and then decreases. The responses in categories 2, 3 and 4 are assessed largely by the extent and nature of beading on the tether.

The different categories were all observed in cells attached to the glass 15 mm from the open end of the microcapillary. If the temperature dependence of the viscosity of the buffered glutaraldehyde-saline solution is ignored it can be taken that the cells were exposed to the same stress pulse. In the vertical microcapillary the cells were exposed to a shear stress of decreasing amplitude (Eqn. 1) for a time of the order of 7 s (Eqn. 2). The time for the interface to move 15 mm is 1.1 s (Eqn. 2) so that the glutaraldehyde arrived in the vicinity of the cells of interest while the cells were still under stress. Cinephotomicroscopic observations of cells stressed as described elsewhere [6] in a horizontal microcapillary showed that the glutaraldehyde fixed the cells rapidly since there was little recovery of tethers, beaded or unbeaded, when the stressing buffer contained glutaraldehyde compared with the recovery of some tethers when there was no glutaraldehyde in the stressing buffer. Since the tether production and beading occurred in the presence or absence of the glutaraldehyde it follows that the beading observed in Fig. 2 is not an artifact of glutaraldehyde fixation. In the absence of glutaraldehyde some of the unbeaded or irregularly beaded tethers are recovered and the cell returns to its

original position when the stress falls to zero. Regularly beaded tethers are not reabsorbed in this manner. The chain of beads however is mechanically weak and rapidly breaks up. The glutaraldehyde was required therefore to fix the tethers when they were extended.

The types of response shown in Fig. 2 are evocative of the observations of Goldin et al. [10] on the breakup of liquid Newtonian, viscoelastic and viscoinelastic jets in air. These authors coupled their observations to an analysis which modified Weber's [11] approach to the problem of the breakup of a viscous cylinder so as to include the viscoelastic case. Their treatment did not include the viscosity of the continuous phase yet several of their results are of interest here. Regularly spaced and sized drops were observed following the growth of disturbances on jets of Newtonian and viscoinelastic jets. At the high shear rates employed in their study the shear dependent viscosity of the viscoinelastic liquid behaved as an inviscid liquid. Jets of viscoelastic solutions of sodium carboxymethyl cellulose initially propogate disturbances as a growing wave with a constant wavelength. The growth of the wave is arrested and strings of droplets connected by thinning threads are formed. The droplets are formed at regular distances from one another indicating their formation from a wave of constant wavelength. The appearance of the category 4 tethers in our work is consistent with the above description by Goldin et al. [10] of the breakup of a Newtonian or weakly viscoelastic system.

Goldin et al. [10] observed that the initial wave information on jets of diluted solutions of highly elastic liquids such as Separan or Polyox was rapidly damped out. No wave formation was observed on jets of more concentrated solutions of these materials. Instead disturbances propogate as isolated droplets connected by random lengths of threads. In many cases the threads were longer than any reasonable wavelengths that could be associated with the droplet. The droplets also varied in size. The authors considered that the irregular behaviour arose from non-linearities in the system which took the situation out of the range of their linear theory. Finally, they showed that a viscoelastic fluid which might have some inherent structure, and hence a yield value, can give rise to a stable jet if the elastic modulus $E < \gamma\ (1 - k^2\ a^2)/6a$, where k is the wave number and a the radius of the cylinder.

The development of the beading patterns on the stretched cells as a function of temperature and time are consistent with the observations of Goldin et al. [10] if the view is taken that continued heating at elevated temperatures leads to a gradual weakening of membrane elements. The initial weakened state would be that in which a tether can be pulled out but the structural elements are still sufficiently strong to suppress beading (Category 2). Further weakening of the structural elements leads to a condition where disturbances can grow but such growth is dominated by non-linear effects resulting in Category 3 behaviour. Additional weakening of structure at higher temperatures or longer incubation times results in a weakly viscoelastic behaviour which when the structural elements are destroyed would, in the case of the erythrocyte, be adequately described by the Newtonian liquid case. Table I shows that if heating continues for long times or at very high temperatures behaviour associated with an increase in membrane structure recurs. Such an occurrence is consistent with the observation of Teitel [3], Williamson et al. [4] and others that heated cells

suffer structural stiffening when the deformability of the cell is measured at lower temperatures following heating.

Our observation (Fig. 3) that previously heated cells gave a lower category response to stress on heating than did unheated control cells suggests that this technique of stressing and the end points we describe may have applications. The method may provide a simple means of testing whether erythrocyte populations have differing structural properties, possibly arising from a diseased state, other than the normal structural elements which are so weakened on incubation for 2 min at 52°C (Table I).

The analogy we draw between our work and that of Goldin et al. [10] is essentially qualitative. The latter authors deal with jets travelling at such a velocity that the time necessary to traverse a wavelength was shorter than the growth time of the disturbance. While regular beading occurs on membrane rings which remain attached to the microcapillary it has proved difficult to resolve the beading process on tethers attached to rapidly moving cells [6]. However, the concept of a real viscoelastic fluid possessing some structure and hence a yield value [10] is in our minds particularly applicable to the heated erythrocyte.

We use the general term 'structure' of Goldin et al. to describe the membrane property of the sub-reticulum which inhibits beading in the stressed unheated cell and yet allows deformability [12]. In addition we use 'structure' to describe the properties of the membrane which inhibit beading and also reduce deformability in pre-heated cells.

The CD measurements on the membrane proteins show a gradual loss of the α-helical structure with time. Part of the structure is regained on cooling, exposure to temperatures of 49°C or higher results in an ellipticity change some of which is irreversible. It has been suggested that the fragmentation of erythrocytes at elevated temperatures may result from a structural change in spectrin [5]. It is significant that spectrin associated ATPase activity of erythrocyte membranes is decreased to 50% on incubation for 3 min at 53°C [13]. Such a rapid enzyme inactivation may be responsible for the increased deformability of the erythrocyte as it changes towards Category 4 behaviour (Table I) while the recurrence of structure on continued incubation is consistent with cross-linking of parts of these membrane proteins which have been irreversibly denatured. The slightly increased deformability of stressed reheated cells compared with the non-reheated cells (Fig. 3, c and d) is consistent with the observation that part of the loss of α-helical structure in cells heated for the above times is reversible.

Erythrocyte deformability has been examined with a semiquantitative end point for the first time at temperatures in excess of 50°C. The different categories of development of beads on the tethers are reproducible features of cell incubation for different temperature-time regimes. We argue that the presence and quality of beading of tethers on stressed cells reflects the extent of the presence of structural elements in the cell membrane. The structure may arise from normal operation of the membrane subreticulium or may arise from an unnatural organization of submembrane components.

Acknowledgments

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