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TETRACAINE MODIFIES THE FRAGMENTATION MODE OF HEATED HUMAN ERYTHROCYTES AND CAN INDUCE HEATED CELL FUSION

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It is known that human erythrocytes in saline fragment by development of an unstable surface wave on the cell rim when cells are heated through the denaturation temperature of the structural protein, spectrin. Here the influence of tetracaine on the fragmentation process has been recorded and analysed by video microscopy of cells heated in rectangular glass microcapillaries. The number of waves per cell rim decreases with increasing tetracaine concentration until, at 0.5 mM tetracaine, wave growth on the cell rim is suppressed on most cells and the cells internalize membrane at the cell dimple. The rate constant for the change in the number of waves per cell with increasing tetracaine concentration is 9.6 mM^{-1} at a heating rate of 0.5 K/s. 50% of heated cells internalize membrane at 0.14 mM tetracaine. When cells are heated rapidly in suspension in test tubes the presence of tetracaine reduces the temperature for 50% haemolysis from 66°C for washed control cells to 60.5°C for cells in 2 mM tetracaine. Cells heated in microcapillaries in tetracaine concentrations of 3 mM and higher begin to swell before the spectrin denaturation temperature is reached. Cell fusion was observed at and above the spectrin denaturation temperature in cells heated in 3 and 4 mM tetracaine. It was also noted that the morphology of erythrocytes maintained in 3.6 mM tetracaine for times up to 30 min at 37°C or 20°C was strongly dependent on temperature and time.

Introduction

A wide range of amphipathic compounds can induce shape changes in human erythrocytes. Amphipaths with an anionic polar group induce crenation while those with a cationic polar group are cup formers [1–3]. Shape changes induced by amphipaths with a neutral polar group or with a quaternary ammonium group can be dependent on the temperature and duration of exposure to the amphipath [3]. The shape changes have been attributed to the action of a bending stress whereby amphipaths inducing outward curvature of membrane produce crenated cells while a bending stress inducing inward curvature results in cup formation [2].

Erythrocytes suspended in buffered isotonic saline, in the absence of amphipaths, undergo morphological changes when heated to 50°C [4,5] the thermal denaturation temperature of spectrin [6]. Two principal types of morphological change are observed [7]. In one case a wave grows rapidly on the rim of the heated erythrocyte and externalized vesicles can pinch from the crests of growing surface wave. In the second case no wave grows on the cell rim but surface wave growth on the cell dimple precedes membrane internalization at the dimple [7,8]. Wave growth on the cell surface has been treated as an example of interfacial instabil-

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

ity [7,8]. The average number of wave crests per cell rim and the proportion of heated cells internalizing membrane at the cell dimple are functions of the cell surface charge and of the sodium chloride concentration of the isotonic suspending phase [7]. Surface charge depletion and suspending phase ionic strength changes alter the cell surface potential. The stability of an interface can be influenced by changes in surface potential [9]. There is also some correlation between erythrocyte shape at 37°C and the membrane Nernst potential [10]. Theoretical treatments of the stability of membranes of finite thickness [11] including, separately, surface charge and membrane potential effects are beginning to become available [12].

In the present study the influence of a cup-forming local anaesthetic, tetracaine, on the fragmentation of heated human erythrocytes is examined. When cells are incubated at 37°C in the presence of tetracaine, it is considered that the amphipath interacts principally with the membrane bilayer [2,3]. However, the spectrin-actin cytoskeleton may act against the expression of the amphipath-induced stress and thus modify the extent of the shape change. This study uses heated erythrocytes as a model to assess the extent to which the structural proteins act against the expression of the amphipath-induced stress in the bilayer when cells are incubated at 37°C. The work is part of a programme in which the effects of amphipaths and electrical stresses [7,13] on the morphological changes in heated erythrocytes will be compared.

Materials and methods

Preparation of blood samples. The collection and washing of the human erythrocytes has been described elsewhere [8]. The cells were finally resuspended in 5 mM Hepes-buffered saline (145 mM NaCl), pH 7.4 or in buffer containing a known concentration of tetracaine hydrochloride (Sigma Ltd) at pH 7.4. The cell suspensions were allowed to stand at room temperature for the 2 h duration of a typical experiment.

Cell heating, light microscopy, video recording and morphology analysis. Precleaned rectangular cross-section microcapillaries [8] loaded with cell suspension were bathed in saline on a microscope slide and heated at a known rate by passing 20 kHz current through the external saline [8,14]. The open ends of the microcapillary were in air so that the cells were not exposed to the heating current. The differential interference contrast light microscopy, video recording of cells during heating and the scoring of wave growth on the cell rim or internalization at the cell dimple have been described [8].

Thermal haemolysis of erythrocyte suspensions containing tetracaine in test tubes. 2 ml cell suspensions were heated from room temperature in glass centrifuge tubes by immersion in a water bath set at 71°C. The rate of temperature increase in the suspension, monitored with a small thermistor, was 0.77 K/s at 50°C. Centrifuge tubes were removed at different temperatures and cooled rapidly in a cold water bath. The cell suspension was centrifuged and the haemoglobin release was measured spectrophotometrically at 412 nm. The absorbance of the supernatant was expressed as a percentage of the absorbance of a hypotonically-lysed cell sample. Haemolysis of cell suspensions in tetracaine was also measured as above, following 2 min exposures of samples in a centrifuge tube to a fixed temperature in a water bath.

Thermal haemolysis of cells in microcapillaries. The ends of microcapillaries which had been loaded with cells ($5 \cdot 10^7$/ml) were sealed with vacuum grease and the microcapillaries were placed in a constant temperature water-jacketed heated test tube. The microcapillary was withdrawn after 2 min and placed on a microscope slide. Four video fields were quickly recorded and subsequently scored for lysed cells.

Morphology of cells maintained in tetracaine/saline at 20°C and 37°C. Cells suspended ($5 \cdot 10^7$ cells/ml) for 30 min at 37°C in 1.0 ml of buffer containing drug were fixed by addition of 0.2 ml of 0.4% glutaraldehyde in saline (37°C). 40 min later the morphological status of the cells was examined with a ×100 oil immersion objective using morphological indices proposed by Fujii et al. [3]. The time course of morphological shape change at 20°C and 37°C was examined by incubating washed cells ($5 \cdot 10^8$ cells/ml) in buffer at 20°C for 30 min. Two drops of cell suspension were then added to 1.0 ml of buffer containing tetracaine. The final tetracaine concentration was 3.6 mM. The cells were held at either 20°C or

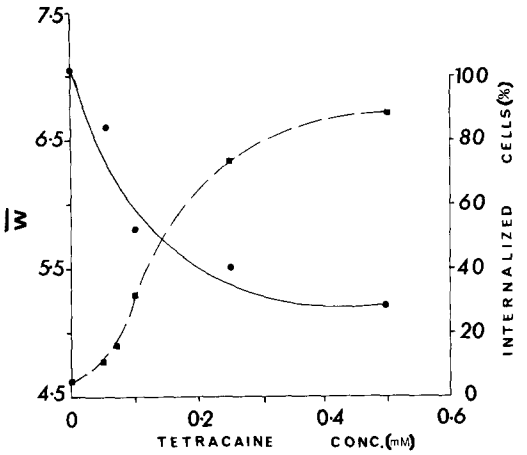

Fig. 1. The average number of waves per wavy cell ($\overline{W}$, ●———●) and the percentage of cells which internalize membrane (■— —■) for cells heated at 0.5 K/s in different concentrations of tetracaine. Between 85 and 240 cells were examined at each concentration.

37°C and fixed after a known interval by addition of 0.2 ml of 0.4% glutaraldehyde in buffer.

Results

Cells heated in tetracaine concentrations ⩽ 0.5 mM

The morphological changes in cells heated in different concentrations of tetracaine at heating rates of 1.0 K/s, 0.5 K/s and 0.15 K/s were recorded. Fig. 1 shows a decrease in the average number of waves per wavy cell ($\overline{W}$) and an increase in the percentage of cells which internalize at the cell dimple for increasing tetracaine concentrations, at a heating rate of 0.5 K/s. The continuous curve on Fig. 1 represents the relationship

$$\overline{W} - \overline{W}_B = (\overline{W}_A - \overline{W}_B) \cdot \exp(-kc) \qquad (1)$$

where $\overline{W}_A$ is the average number of waves for the control cells, $\overline{W}_B$, an asymptotic value of $\overline{W}$, is taken as 5.2, $-k$ is the rate of change of $\overline{W}$ with increasing concentration and c is the tetracaine concentration. The value of k from Fig. 1 is 9.6 mM^{-1}. The tetracaine concentration for 50% internalization was 0.14 mM (Fig. 1). 50% internalization occurred at 0.12 mM and 0.18 mM when cells were heated at 1.0 K/s and 0.15 K/s, respectively.

Fig. 2, a video sequence observed during the heating of control cells at 0.5 K/s shows the development of a wavy disturbance and cell fragmentation by membrane externalization. The membrane externalization process was complete

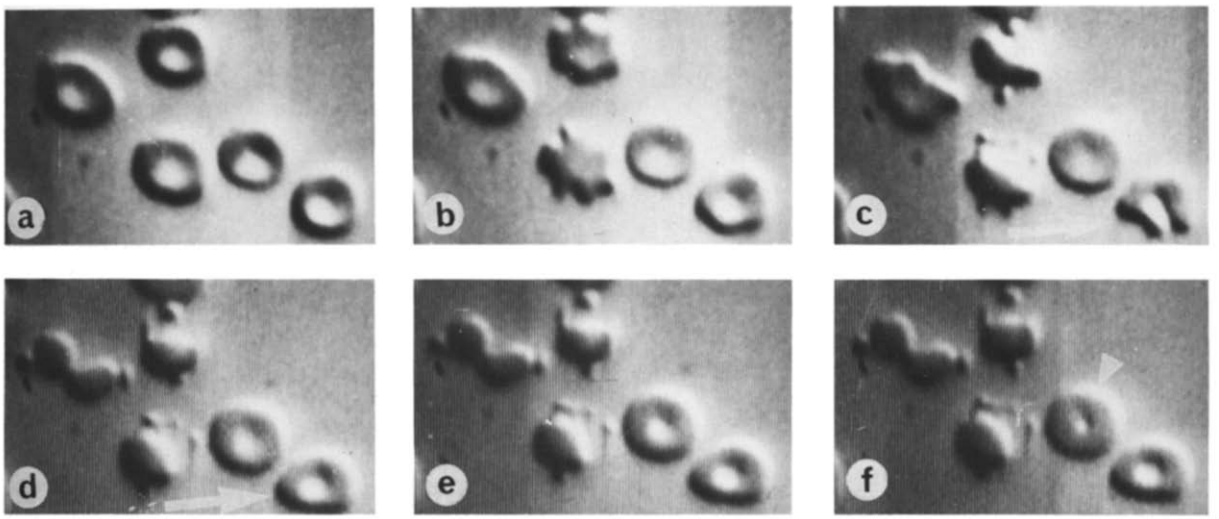

Fig. 2. Sequence of control cells heated at 0.5 K/s. (a) time zero, $t = 0$, cells 0.04 s before wave growth became discernible; (b) $t = 0.40$ s, wave growth on the rims of two cells; (c) $t = 1.22$ s external vesicle formation completed on two cells, wave growth on arrowed cell without vesicle formation; (d) $t = 6.0$ s, disturbance has subsided on the arrowed cell; (e) $t = 6.0$ s; (f) $t = 7.2$ s, cell (arrowhead) has internalized membrane at the cell dimple.

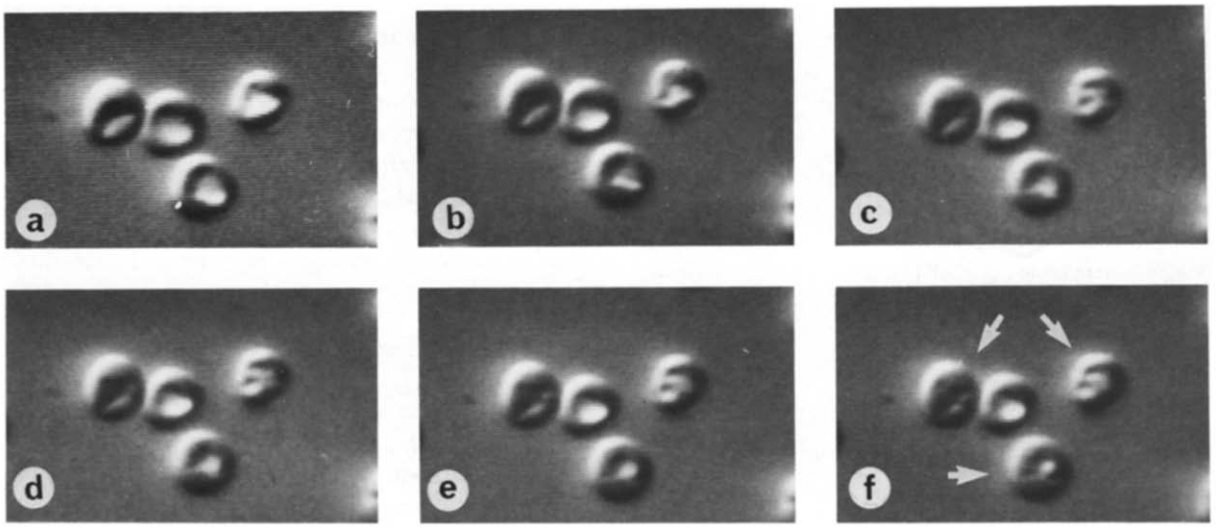

Fig. 3. Membrane internalization in cells heated at 0.5 K/s in 0.5 mM tetracaine. (a) $t = 0$, movement of membrane at the cell dimple is first detectable on the video sequences; (b) $t = 1.6$ s distortion of the dimple regions can be observed; (c) $t = 1.02$ s the shape change on the cell at the top right hand side is complete; (d) $t = 1.22$ s and (e) $t = 1.36$ s the membrane internalization process continues; (f) $t = 1.56$ s, internalized vesicles can be seen in the arrowed cells.

within 2.0 s. Two cells (arrowed and arrowhead) did not externalize membrane. One cell (arrowhead) underwent little morphological change at the spectrin melting temperature and internalized membrane at the cell dimple 7.0 s after the initiation of the sequence (Figs. 2(e–f)). The arrowed cell Fig. 2(c) developed a wavy disturbance. The amplitude of the disturbance was not sufficiently great to allow vesicles to pinch from the crests of the disturbance. The wavy outline subsided (Figs. 2(d–f)) and the cell internalized membrane at the dimple 8.0 s after the initiation of the sequence and 0.8 s after the final detail shown in Fig. 2(f). Examples of rapid internalization of membrane at the cell dimple are shown in Fig. 3 for cells heated at 0.5 K/s in the presence of 0.5 mM tetracaine. The sequence shown in Fig. 3 took place over a 1.56 s period.

TABLE I

THE AVERAGE TIMES TO VESICLE FORMATION AND MEMBRANE INTERNALIZATION FOR CELLS HEATED AT 0.5 K/s IN A RANGE OF TETRACAINE CONCENTRATIONS

Time was measured from the instant at which morphological change was observed on the first cell in the video field.

Tetracaine concn. (mM)	Average delay to vesicle formation (±1 S.E.) (s)	Average delay to internalization (±1 S.E.) (s)
0	2.37±0.1	12.7±0.9
0.1	2.39±0.22	10.0±0.7
0.25	2.85±0.34	4.8±0.6
0.5	3.13±0.3	3.1±0.5

The time course of the development of morphological changes was examined. Time zero (t_0) was taken as that instant at which the beginning of a wave outline on a cell rim or of membrane internalization at the cell dimple was detected on the first shape-changing cell in the video field of about thirty cells. For each fragmenting cell the time to the production of a vesicle was measured from the zero time point (t_0), defined above. Similarly the delay to vesicle internalization was measured from t_0 for those cells which did not produce external vesicles. Table I shows that there was a small but significant increase in the average time to external vesicle formation in fragmenting cells as the tetracaine concentration increased from 0 to 0.5 mM. The delay to internalization decreased rapidly as the drug concentration increased (Table I). A distinction was made between those cells which internalized membrane over the period when some other cells in the field were externalizing membrane and cells which, as in Fig. 1, internalized

TABLE II

THE PERCENTAGE OF CELLS WHICH FRAGMENTED BY EXTERNALIZING VESICLES, THE CELLS WHOSE DELAY TO INTERNALIZATION EXCEEDS 6 s (LATE INTERNALIZERS) AND THE PERCENTAGE OF CELLS WHICH INTERNALIZED WITHIN 6 s OF THE EARLIEST OBSERVATION OF MORPHOLOGICAL CHANGE ON ANY CELL IN THE VIDEO FIELD

The cells were heated at 0.5 K/s. The number of cells scored was 127, 76, 83 and 94 at 0, 0.1, 0.25, and 0.5 mM tetracaine, respectively.

Tetracaine concn. (mM) vesicles	Cells extern-alizing (%)	Late intern-alizers (%)	Cells internalized in < 6 s (%)
0	72	20	8
0.1	36	38	26
0.25	25	12	61
0.5	8	10	81

membrane some time after the membrane externalization process on other cells was completed. The distinction between early and late internalizing cells was formalized by considering cells which internalized membrane at a time in excess of 6 s from t_0 to be 'late internalizers'. This choice was influenced by the observation in Table I that the average delay to membrane externalization was less than 3.2 s for all samples examined. Table II shows how the percentage of cells which produced vesicles, the percentage of cells which internalized within 6 s and the percentage of 'late-internalizers' changed with tetracaine concentration. The percentages of externalizing cells and of rapidly internalizing cells decrease and increase respectively with increasing tetracaine concentration. The 'late internalizers' occur most frequently in 0.1 mM tetracaine and are the principal group at that concentration.

Cells heated in tetracaine concentrations in excess of 1 mM

No haemolysis or cell swelling was detected at or near the morphological change temperature in cells heated at 0.5 K/s in tetracaine concentrations up to 1.0 mM. However, some cells heated in 3, 4, or 6 mM tetracaine had become swollen or had lysed by the time that the temperature had reached 50°C. Some of the swollen cells externalized membrane by growth of a short wavelength disturbance on the cell rim at the spectrin denaturation temperature. Video records of cell fields 1.0 s before morphological changes occurred were examined and cells were scored as having either (i) retained their room temperature morphology (ii) become swollen or (iii) lysed. Table III shows that the percentage of cells retaining their room temperature morphology decreased with increasing tetracaine concentration for all heating rates employed. For all tetracaine concentrations the percentage of the cells retaining their room temperature shape decreased at lower heating rates.

The haemolysis of erythrocytes heated rapidly (0.77 K/s at 50°C) in bulk suspension in centri-

TABLE III

THE PERCENTAGE OF CELLS WHICH HAD (i) RETAINED THEIR ROOM TEMPERATURE SHAPE (ii) BECAME SWOLLEN OR (iii) LYSED BY THE TIME THAT THE MORPHOLOGICAL CHANGE TEMPERATURE WAS REACHED

The cells were heated at 1.0 K/s, 0.5 K/s or 0.15 K/s in different, high concentrations of tetracaine.

Heating rate (K/s)	Tetracaine concn. (mM)	Total cells scored	Unchanged morphology (%)	Swollen cells (%)	Lysed cells (%)
1.0	3	61	79	21	0
1.0	4	170	61	38	1
0.5	3	126	42	40	18
0.5	4	184	32	43	25
0.5	6	144	30	53	17
0.15	4	90	4	5	91
0.15	6	40	2	18	80

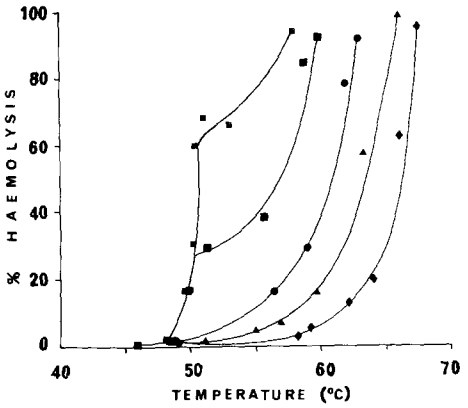

Fig. 4. Haemolysis of erythrocytes heated rapidly (0.77 K/s at 50°C) in bulk suspension in centrifuge tube in the presence of different concentrations of tetracaine: 0 (◆——◆), 1 mM (▲——▲), 2 mM (●——●), 4 mM (■——■), 6 mM (■——■).

fuge tubes, as described in Methods, is shown in Fig. 4. The presence of tetracaine reduced the temperature for 50% haemolysis. Cells in tetracaine concentrations of 2 mM and lower can survive the spectrin denaturation temperature at 50°C without

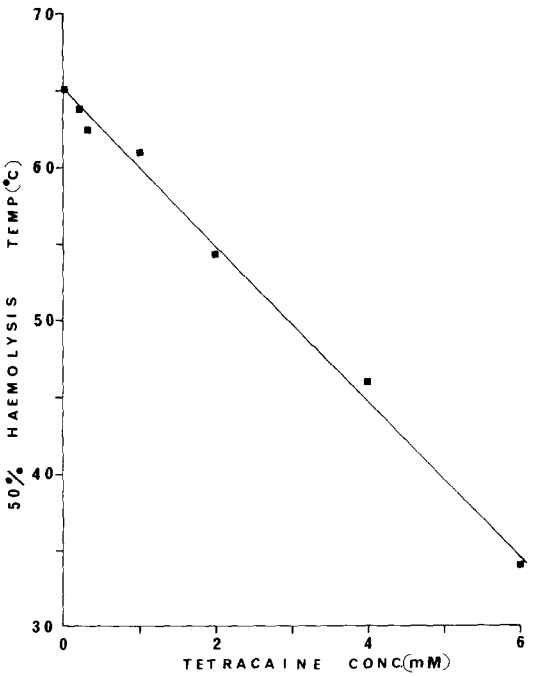

Fig. 5. The tetracaine concentration dependence of the temperature at which 50% haemolysis was observed immediately after heating cells in a microcapillary for 2 min.

significant haemolysis while cells in 4 and 6 mM tetracaine undergo significant haemolysis in the region of 50°C (Fig. 4). Little haemolysis occurred, at the tetracaine concentrations tested, below 50°C.

The haemolysis of cells in microcapillaries maintained at a fixed temperature for 2 min was estimated by immediate post-heating microscopic examination, as described earlier. The temperature at which 50% of the cells had lysed is shown in Fig. 5 as a function of tetracaine concentration. In comparison with the results of Fig. 4 the 6 and 4 mM tetracaine samples show marked lysis below 50°C (Fig. 5). The influence of a glass effect in the haemolysis of cells heated for 2 min in microcapillaries was shown as follows: The haemoglobin release when cells in suspension in buffer containing 6 mM tetracaine were heated in a centrifuge tube at 44°C for 2 min was 10%. This 10% haemoglobin release at 44°C contrasted with 50% haemolysis at 34°C (Fig. 5) and 100% haemolysis at 42°C for cells heated in microcapillaries for 2 min. Further evidence for an effect of glass on cell swelling in high tetracaine concentrations was found in the following experiment. An empty microcapillary was placed on the microscope heating slide and the heating system was assembled. Heating of the saline surrounding the microcapillary at 0.5 K/s was commenced and a drop of cell suspension in 4 mM tetracaine was brought to the end of the microcapillary. The cell suspension was drawn into the microcapillary. Microscopic examinations of the cell suspension showed that many cells were in suspension and some cells were settled on the glass. As the heating continued the cells lying on the glass or settling down on the glass swelled some time before the cells in suspension began to increase in volume.

Fusion of swollen cells occurred at or above the spectrin denaturation temperature in 16 out of a total of 19 samples heated in 3 or 4 mM tetracaine at 0.5 K/s or 1.0 K/s. No fusion was observed in six samples heated at 0.5 K/s in 6 mM tetracaine. Fusion occurred in all 7 samples heated at 0.5 K/s in 4 mM tetracaine in the presence of 5 mM EDTA. Fusion of cells heated at 0.5 K/s in 4 mM tetracaine is shown in Fig. 6. The spectrin denaturation temperature is identified by the presence of a wavy cell (Fig. 6(a)) which has frag-

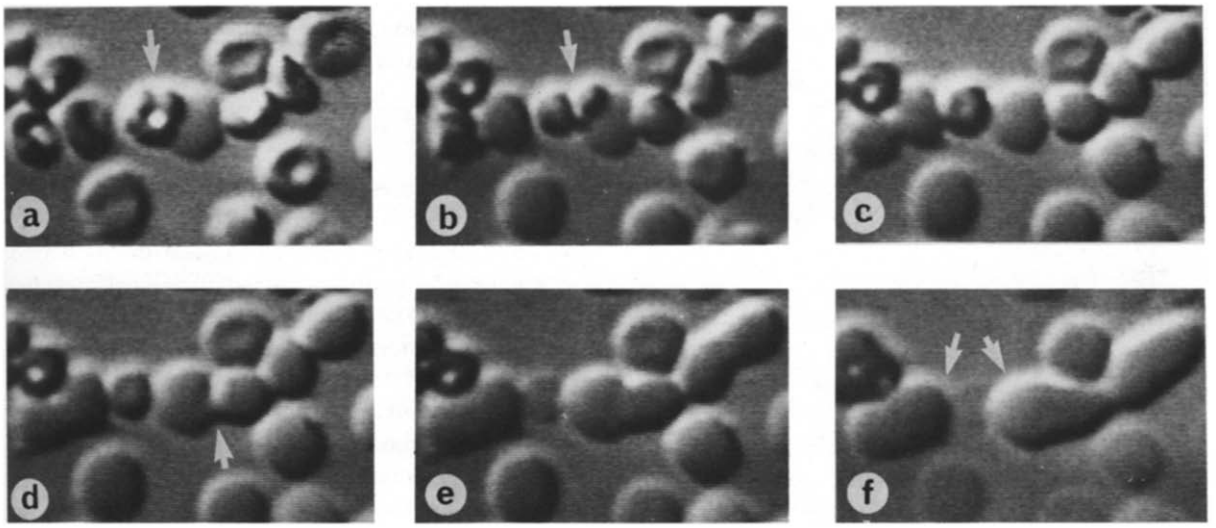

Fig. 6. Fusion of erythrocytes heated at 0.5 K/s in 4 mM tetracaine (a) $t = 0$, the spectrin denaturation temperature, a wavy disturbance is growing on the arrowed cell; (b) $t = 0.5$ s, the arrowed cell has fragmented, several cells have become swollen; (c) $t = 3.4$ s cell swelling continues; (d) $t = 9.9$ s, a contact can be seen between two cells (arrow); (e) $t = 11.5$ s and (f) $t = 17.7$ s progressive polyerythrocyte development (arrows). Haemolysis of single cells can also be seen during the sequence.

mented 0.5 s later (Fig. 6(b)). Some cells have become more swollen in the 0.5 s interval from Fig. 6(a) to Fig. 6(b). Cell swelling develops further with continued heating. The first clear sign of cell fusion is arrowed in Fig. 6(d) almost 10 s after spectrin denaturation. Polyerythrocyte formation continues to Fig. 6(f).

Influence of tetracaine on cell morphology at 20°C and 37°C

The cell suspensions from which samples were drawn for heating were held at room temperature during the two hour duration of a typical experiment. Fujii et al. [3] have noted that a transition from a marked stomatocytic form to a spherical

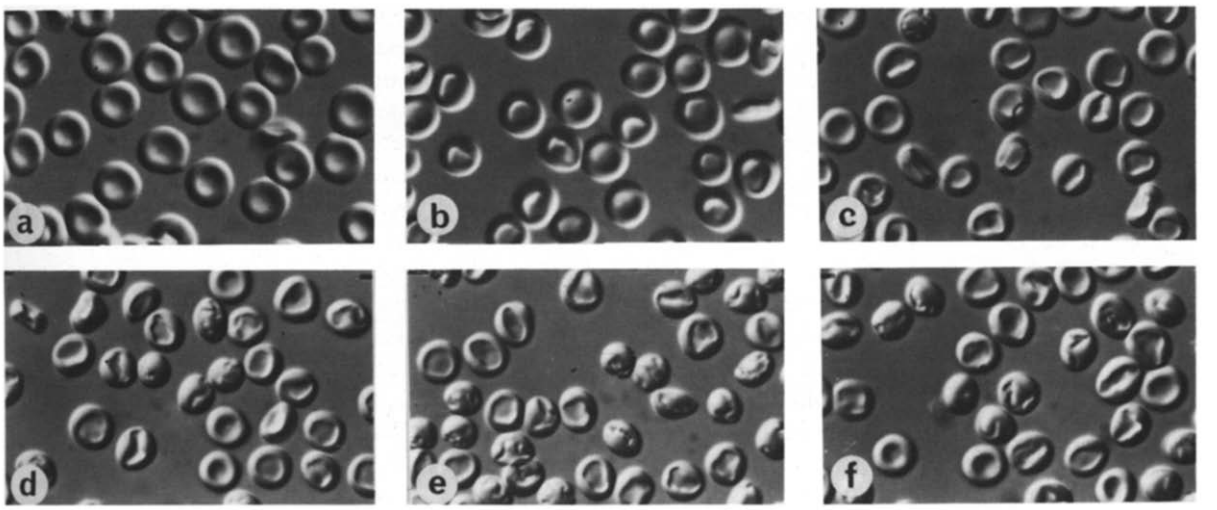

Fig. 7. Photomicrographs of cells held at 37°C for 30 min in the following tetracaine concentrations, (a) 0, (b) 0.5 mM, (c) 1.0 mM, (d) 2.0 mM (e) 4 mM and (f) 6 mM.

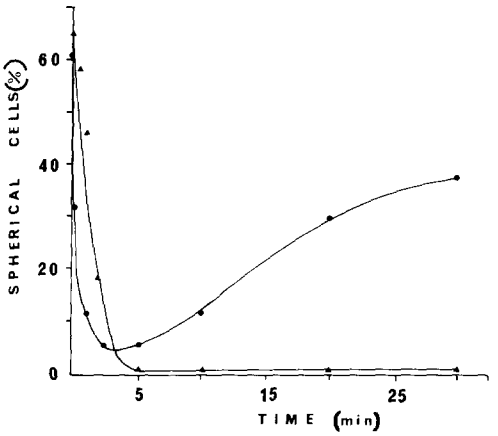

Fig. 8. The incidence of spherical membrane-internalized cells in cells suspensions held in 3.6 mM tetracaine at 20°C (▲——▲) and 37°C (●——●) for different times.

membrane-internalized cell occurred in the tetracaine concentration range 1.0 to 7.5 mM when cells were held at 37°C for 30 min. The cells in 2, 3, 5 and 6 mM tetracaine in this work showed a lower incidence of membrane internalization at room temperature than would have been expected from the work of Fujii et al. [3]. This observation led to an investigation of the influence of temperature and time on cell morphology at 20°C and at 37°C.

Typical microscopic fields of cells fixed following incubation at 37°C for 30 min in different concentrations of tetracaine are shown in Fig. 7. The cells in 0.5 mM tetracaine Fig. 7(b) show a pronounced simple. The cell shape alters progressively at higher concentrations until there is a high incidence of spherical cells with internalized membrane at 4 and 6 mM Fig. 7(e). The percentage of spherical cells with internalized membrane (stage III, Fujii et al. [3]) was scored as a function of exposure time for cells in 3.6 mM tetracaine at 20°C and 37°C (Fig. 8). The incidence of spherical cells with internalized membrane was highest immediately on adding cells to the drug solution. The incidence of spherical cells fell rapidly with time at both 37°C and 20°C. Samples at both temperatures had lost most of the spherical cells after 2 min in the drug. The cells held at 37°C subsequently showed a marked recovery of the spherical shape (Fig. 8). Cells fixed following addition to buffer containing no drug showed the normal biconcave erythrocyte shape.

Discussion

Cells incubated at 37°C for 30 min in the presence of tetracaine change from a high incidence of cup-shaped cells at 1 mM to an increased incidence of membrane internalized cells at 6.0 mM (Fig. 7). The results in Fig. 8 shows that the incidence of internalized cells in suspensions containing 3.6 mM tetracaine is a complex function of temperature and exposure time. Cells internalize membrane immediately on exposure to 3.6 mM tetracaine. The cells change shape quickly to less internalized forms during the first two minutes of exposure to the drug. Subsequently, cells held for 30 min at 20°C retain a stomatocytic shape while cells held at 37°C return slowly to a state of increased membrane internalization. The time dependence of cell shape in 3.6 mM tetracaine (Fig. 8) may be related to the polarization fluorescence observations by Salesse et al. [15] of a transient increase in internal microviscosity and lipid order in pigeon erythrocyte membranes exposed to tetracaine concentration in the range 2–5 mM. The duration of the transient change was 10 min at 37°C and 20 min at 30°C [15].

At low drug concentrations ($\leqslant 0.5$ mM) there was a gradual decrease in the average number of waves per heated cell and an increase in membrane internalization at the cell dimple with increasing drug concentration (Fig. 1). Video records of the cells during heating from room temperature to the spectrin denaturation temperature showed a small movement towards a more cupped shape as the temperature increased. The decrease in average numbers of waves per cell was reasonably well described by the exponential relationship of Eqn. 1. The concept of an asymptotic approach of $\overline{W}$ to $\overline{W}_B$ is valid because it is experimentally observed that wave growth involving only four waves per cell or less is a rare occurrence. The ability to derive a rate constant (9.6 mM^{-1}) for the decrease of the average number of waves per cell has potential usefulness in facilitating the comparison of the influence of different agents on the mode of heated erythrocyte fragmentation. The relationship between membrane internalization and drug con-

centration shows that 50% of the cells internalize membrane in 0.14 mM tetracaine (Fig. 1). The internalization curve cannot be fitted well by a simple sigmoidal relationship so that, at present the 50% internalization concentration is the most useful parameter derived from internalization measurements.

Table II shows that most heated cells in control samples fragment by membrane externalization. Most cells in 0.1 mM tetracaine do not fragment by membrane externalization, rather they internalize membrane after a delay, while most cells in 0.25 mM tetracaine internalize membrane immediately on spectrin denaturation. These observations suggest that the spectrin is part of a structure which inhibits the development of movement which would lead to membrane externalization in control cells and membrane internalization in 0.25 mM tetracaine. The high incidence of late internalization in cells in 0.1 mM tetracaine suggests that the contribution of the cytoskeleton to cell shape is minimal at that concentration. Fig. 2 includes an example of late internalization. The sudden development of the late internalization 6 s after the beginning of cell fragmentation in the field shows that the times for delay to internalization in Table I cannot be taken as a measure of the rate of membrane internalization.

The internalization of membrane in cells heated at 0.5 K/s in 0.5 mM tetracaine is shown in Fig. 3. The photomicrographs show the same features but are of a lower resolution than the $\times 100$ oil-immersion objective micrographs which previously showed the details of morphological changes and membrane internalization in erythrocytes heated in the presence of serum albumin [8].

The presence of tetracaine in the cell suspending medium acts to reduce the erythrocyte haemolysis temperature (Figs. 4 and 5). The haemolysis profile for control cells heated rapidly in bulk suspension in centrifuge tubes (Fig. 4) shows 12% and 98% haemolysis at 62 and 67°C, respectively. These results are in good agreement with the values of 17% and 100 % obtained in a similar system by Ham et al. [5] at 63°C and 65°C, respectively. Cells heated rapidly in bulk suspension at tetracaine concentrations up to 2 mM survived the spectrin denaturation temperature without appreciable haemolysis (Fig. 4). The steep increase at 50°C in haemolysis of cells heated rapidly in 4 and 6 mM tetracaine suggests that the membranes of these cells are less able to survive the loss of mechanical strength following spectrin inactivation. There was little haemolysis below 50°C (Fig. 4). Cells heated in microcapillaries in 6 mM tetracaine for 2 min showed 50% haemolysis at 34°C (Fig. 5). This finding contrasted with the observation of only 10% haemolysis in cells maintained in bulk suspension at 44°C for 2 min in a centrifuge tube and suggested that a cell-glass interaction in the microcapillaries influenced haemolysis at high tetracaine concentrations.

The 50% haemolysis temperature for cells in 1 mM tetracaine was in excess of 60°C for cells held at elevated temperatures in microcapillaries for 2 min (Fig. 5) and for cells heated rapidly in bulk suspension (Fig. 4). No cell swelling was seen on the video system as cells in 1 mM tetracaine were heated through the spectrin denaturation temperature in microcapillaries on the microscope stage. These results suggest that cell-glass interactions did not significantly affect the influence of tetracaine concentration on heated cell fragmentation at those concentrations ($\leqslant 0.5$ mM) where fragmentation has been examined in detail (Fig. 1, Table II).

Polyerythrocyte formation was observed at or above the spectrin denaturation temperature in most samples heated in 3 or 4 mM tetracaine at 0.5 K/s (Fig. 6). No polyerythrocytes were seen in a large number of control cells heated in microcapillaries on the microscope stage to the haemolysis temperature of 65°C. Polyerythrocyte formation has been recognised recently as a state which may follow the fusion of cell membranes but does not follow cell fusion in all cases [16]. It has been shown that membrane fusion without polyerythrocyte formation can be effected by 1 day old Sendai virus, while polyerythrocyte formation is observed when erythrocyte fusion is induced by 3-day-old Sendai virus. Osmotic swelling appears to be the driving force by which cells which have virally established sites of membrane fusion expand such sites to form polyerythrocytes [16]. Ahkong et al. [17] studied fusion of hen erythrocytes (observed as polyerythrocyte formation) in a mixed salts medium, containing $CaCl_2$, in the presence of glyceryl mono-oleate on a light micro-

scope heating stage. The rate of fusion was 10-times faster at 60°C than at 37°C. The authors found that all chemicals which induced cell fusion also led to swelling and they concluded that cell swelling by colloidal osmosis played an essential role in cell fusion. Cell fusion occurred in the present work in the absence of added calcium and in the presence of EDTA.

The results above (Fig. 1, Tables I and II) show that the average number of waves per cell and the percentage internalizing cells are sensitive continuous indices of stress in the erythrocyte membrane. The indices reflect changes in the membrane at tetracaine concentrations below those at which the drug-induced transient changes in lipid order and microviscosity occur [15] and below those at which cell swelling occurred. The trend of decrease of average wave number and increased incidence of internalized cells, as presented in Fig. 1, has a similar form to the changes induced in heated cell fragmentation by surface charge depletion and by membrane potential changes (in preparation).

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