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Flicker spectroscopy of erythrocytes

A sensitive method to study subtle changes of membrane bending stiffness

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Abstract. Frequency analysis of thermally excited surface undulations of erythrocytes leading to the flicker phenomenon is applied to determine biochemically and physically induced modulations of the membrane curvature elasticity. Flicker spectra of individual cells fixed to the window of a flow chamber by polylysine are taken by phase contrast microscopy, enabling investigations of the reversibility of the structural modifications. The spectra may be approximated by Lorentzian lines in most cases. By measuring the amplitude (at zero frequency) and the line width, effects of the structural changes on the curvature elastic constant, K_c, and the wavelength distribution of the undulations may be studied separately.

Effect of physically induced modifications: The temperature dependence of the flicker spectra are taken from 10 °C to 37 °C. Above 20 °C, K_c decreases with increasing temperature whereas the reverse holds below this limit. The latter anomalous behaviour is explained in terms of a conformational change associated with protein and lipid lateral phase separation. The bending stiffness increases when the cells swell osmotically, owing to surface tension effects. The dependence of the flicker spectra on the viscosity of the suspension medium agrees with the theoretical prediction.

Biochemically and drug induced modifications: 5 vol‰ of ethanol leads to a pronounced and reversible suppression of the long wavelength undulations without altering the discoid cell shape and without affecting the bending stiffness appreciably. Adsorption of dextran to the glycocalix increases K_c by a factor of 1.6 at saturation. The bending stiffness is increased by a factor of 1.3 after cross-linking the proteins with the SH-oxidizing agent diamid. Injection of Ca^{++} into the cell via ionophores evokes (within 10 min) the formation of fine – probably spectrin free – spicules. This leads to an increase in K_c by a factor of 1.3 which is explained in terms of a lateral condensation of the spectrin/actin network. The spicule formation and K_c change is completely reversible (within 2 min) after perfusion with Ca^{++}-free buffer. Cholesterol depletion leads first to a continuous increase in K_c without change of the cell shape whereas a sudden discocyte- to echinocyte transformation sets in below a critical steroid content. The latter transition is also observed in cell suspensions and is reminiscent of a phase transition. The anti-tumor drug actinomycin D evokes an increase in the bending stiffness K_c by a factor of two, suggesting that its effect is at least partially due to a modulation of the membrane structure. The α-receptor agonist leads to a remarkable increase in K_c (by about 25%) at 10^{-4} M but the effect is not reversed by the α-antagonist prazosin, suggesting that the agonist exerts a non-specific effect.

A new technique, dynamic reflection interference contrast microscopy, is introduced by which absolute values of the amplitudes of the surface undulations and therefore K_c can be determined. The value obtained: $K_c = 5 \cdot 10^{-13}$ erg is about a factor of two larger then the bending stiffness of pure lipid bilayers. We suggest that the surface undulations may also be determined by lateral fluctuations of the quasi-two-dimensional spectrin/actin network.

Key words: Erythrocyte deformability, membrane elasticity flicker spectroscopy, cell shape transitions, effects of drugs and of diseases

I. Introduction

Cell plasma membranes exhibit fascinating elastic and viscoelastic properties. By combining a remarkable low bending stiffness (Brochard and Lennon 1975; Petrov and Bivas 1984) with a low area compressibility and lateral tearing strength blood cells

may squeeze through capillaries or pores of the blood vessels with diameters small compared to the cell radius without disrupture or lysis. Simultaneously, the plasma membrane exhibits fluid-like behaviour which enables fast lateral and rotational diffusion as well as large scale hydrodynamic flow.

The remarkable combination of stability and flexibility of plasma membranes is accomplished by the coupling of the lipid/protein bilayer to the cytoplasmic network which may be two-dimensional as in the case of erythrocytes or which may extend through the whole cytoplasm.

The numerous studies of the elastic and viscoelastic properties of the red blood cell have been undertaken primarily for two reasons: firstly in order to characterize the physiological state of the cell in terms of a physical quantity (cf. Fung 1981 or Bessis and Mohandas 1975) and secondly in order to gain insight into the microscopic organization of the membrane (Evans and Skalak 1980; Fricke and Sackmann 1984).

For the achievement of the latter aim, several microscopic methods have been conceived which enable measurements of elastic and viscoelastic parameters of individual cells. These include: the shear field technique (Bessis and Mohandas 1975; Fischer et al. 1978), the micropipette technique (Evans and Skalak 1980; Kwok and Evans 1981), the electric field jump method (Gaub et al. 1984) and finally flicker spectroscopy (Fricke and Sackmann 1984). The first three methods are based on the analysis of the deformation of the cell after application of an external force. The strength of the external perturbation involved in the experiment is highest in the case of the shear field technique and smallest for the third technique. Besides this advantage, the transient deformation of the cells in a high frequency electric field enables the measurement of both the elastic and viscous parameters of the cell in the linear range of elongation.

Flicker spectroscopy is based on frequency analysis of the thermally excited surface undulations of the plasma membrane and is thus a completely non-disruptive method for the measurement of bending elastic constants (Brochard and Lennon 1975; Fricke and Sackmann 1984). An outstanding advantage of the technique is that it enables the detection of subtle modifications of the membrane structure caused, for instance, by diseases or by the aging of the cells (Sackmann et al. 1984).

The main aim of the present work was to study the effect of a variety of drugs and other agents on the bending elasticity of the red cell membrane as well as the reversibility of such biochemically induced modifications of the membrane bending stiffness. Moreover it is demonstrated in preliminary experiments that absolute values of the curvature elastic constant may be determined by application of dynamic reflection interference contrast microscopy.

II. Experimental procedure and cell preparation

II.1 Principle of flicker spectroscopy, theoretical background and evaluation of spectra

The thermally excited undulations of the membrane are visible in the phase contrast microscope owing to the pronounced flickering of the light intensity which is caused by the thickness fluctuations of the cell. The frequency spectrum of the surface undulations – called the flicker spectrum in the following – is obtained by spectral analysis of the intensity fluctuations of the light passing the cell. The details of the method have been described in a previous publication (Fricke and Sackmann 1984). The fluctuation of the phase contrast, $\delta c(x,y,t)$, in the image plane, (x,y), of the objective of the phase contrast microscope is directly proportional to the thickness fluctuation, $\delta d(x,y,t)$, of the cell

$$\delta c(x,y,t) = \frac{2(n-n')}{\lambda\sqrt{\tau}} \cdot \delta d(x,y,t)\,, \tag{1}$$

where τ is the transmission of the phase plate, λ the light wavelength and $(n-n')$ the refractive index difference between the cytoplasm of the cell and the outside medium. The fluctuation in contrast is given by

$$\delta c(x,y,t) = \frac{\delta I(x,y,t)}{I_0}\,,$$

where I_0 is the intensity of the light passing the medium and $\delta I(x,y,t)$ is the momentary fluctuation in the intensity of the light passing the cell under study. δI is determined by the thickness fluctuation at time t. The curvature elasticity can now either be determined from the time correlation function of the mean quadratic value of the thickness fluctuations $\overline{\delta d(t)^2}$ (and because of Eq. (1) from the intensity fluctuation $\overline{\delta I(t)^2}$) or from the frequency spectrum, $P(\omega)$, of $\overline{\delta d(t)^2}$ (or $\overline{\delta I(t)^2}$). $P(\omega)$, the power spectrum of the intensity fluctuation is called the flicker spectrum. In the present work the second way was chosen.

A theoretical interpretation of the flicker phenomenon was presented by Brochard and Lennon (1975) for the case of a planar membrane of infinite extension. By assuming that the thermal excitations of the membrane are determined by the bending stiffness, they derived the following expression for the frequency spectrum of an excitation of wave

vector q

$$P(q,\omega) \propto \frac{kT}{\pi\eta q S} \frac{1}{\omega^2 + (K_c q^3/2\eta)^2}, \tag{2}$$

where S is total area of the membrane, K_c its curvature elastic constant and η the viscosity of the outer medium. The viscosity of the cytoplasm does not contribute to the damping because it is higher than η by a factor of 10. In previous work (Fricke and Sackmann 1984) we provided evidence that this expression is also approximately valid for erythrocytes because the two opposing membranes of the cell are decoupled.

A major problem of the present technique is the following: one can only measure the total frequency spectrum of the intensity fluctuation consisting of a superposition of all possible modes of wave vector q:

$$P(\omega) = \int P(q,\omega)\, dq$$

and there is no way to separate these. On the other hand, it has been shown in the previous work (Fricke and Sackmann 1984) that the flicker spectrum $P(\omega)$ can be very well approximated by a superposition of at most two Lorentzian lines of somewhat different line width and in many cases it can even be represented to a very good approximation by a single such line. This is explained in terms of the fact that the number of modes contributing to the observed spectrum is restricted by the experimental boundary conditions. The maximum q value that contributes is determined by the spatial resolution of the microscope because undulations with smaller wavelengths are smeared out. By inserting an aperture in the image plane of the objective a lower bound of the wavevector is introduced (Fricke and Sackmann 1984). Experimental evidence for this is provided below. Undulations with wavelengths comparable to the diameter of the cell are probably not important for geometrical reasons, they would involve a deformation of the whole cell. From the above considerations we conclude that modes with two wavevectors ranging from $q_{\min} \approx 10^4\,\mathrm{cm}^{-1}$ to $q_{\max} \approx 5 \cdot 10^4\,\mathrm{cm}^{-1}$ can contribute to $P(\omega)$. The Lorentzian-like flicker spectra are thus characterized by a mean value $\bar{q}$ of the wavevector in the present work.

The experimental flicker spectra are analysed in the following way: First, the spectra are approximated by one or at most by two Lorentzian lines by the procedure described below. As we shall see below, one line is sufficient in most cases. From the fitted spectrum we obtain an apparent line width

$$\Delta\omega = \frac{K_c \bar{q}^3}{2\eta} \tag{3}$$

and an amplitude at $\omega = 0$

$$P(0) = \frac{4kT\eta}{\pi \bar{q}^7 S K_c^2}. \tag{4}$$

Since η is known the product $K_c \bar{q}^3$ can be determined from Eq. (3). Further information concerning a variation in $\bar{q}$ (which may be caused by a structural change of the membrane) is obtained from the product

$$P(0)\,\Delta\omega^2 = \frac{kT}{\pi\eta \bar{q} \cdot S} \tag{5}$$

provided additional information concerning the variation in the surface area (or the size) of the cell is available. For that reason phase contrast micrographs were taken together with the power spectra.

The amplitude, $P(0)$, and the line width, $\Delta\omega$, were determined by fitting a Lorentzian curve of the form

$$y(\omega) = y_0(\omega) + \frac{B}{\omega^2 + \Delta\omega^2} \tag{6}$$

to the experimental spectrum. y_0, $P(0)$ and ω^2 were determined by a least-squares-fit procedure. y_0 takes account of basal light intensity fluctuations caused by the instrument, these can be considered as white noise so that y_0 is a constant. Both B and y_0 are functions of the incident light intensity which varies with the experimental conditions. In order to account for this variation the amplitude B obtained by the fitting procedure was normalized by dividing by y_0.

If the experimental curve could not be fitted in a satisfactory way by one Lorentzian line the procedure was repeated with a superposition of two lines. However, in all cases studied in the present work a single line was sufficient within the limit of experimental accuracy. An example is shown in Fig. 1.

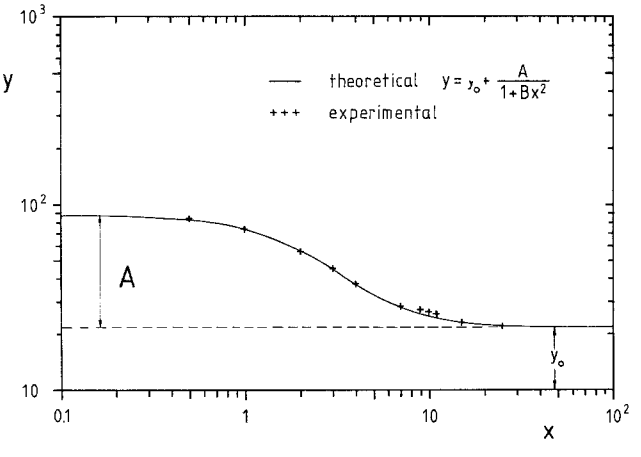


Fig. 1. Example of measured (+++) and theoretical (——) flicker spectrum of erythrocyte in NaCl/ACD buffer

II.2 Direct measurement of absolute amplitudes by dynamic reflection interference contrast technique

The reflection interference contrast technique has been introduced in order to determine static images of the surface profiles of cells adhering to glass surfaces (cf. Beck and Bereiter-Hahn 1981 for references). It is based on the evaluation of the Newtonian ring pattern formed by the interference of the light reflected from the surface of the cell and the glass plate as indicated in Fig. 2. The contour lines (or lines of equal thickness) of the cell may be obtained most accurately from the rings of destructive interference (dark lines in Fig. 2a). The optical pathway difference Δ between the rays reflected at the glass surface (1, 1′) and the cell surface (1, 2′) is related to the distance, d, between the two surfaces according to

$$\Delta = 2d\sqrt{n^2 - \sin^2\alpha} - \lambda/2 \qquad (7)$$

where n is the refractive index of the medium and α the aperture angle of the objective. The position of the dark rings is determined by the condition $\Delta = (2k+1)/2$ (k integer).

Figure 2b shows a typical interference pattern of a cup-shaped cell. If the cell flickers, the dark interference rings are statistically deformed and it is clear that the amplitudes of the membrane undulations can in principle be easily obtained from Eq. (7). H. Engelhardt and H. P. Duwe of our laboratory have developed a computer controlled image evaluation system (Engelhardt et al. 1985; Duwe 1985) which enables a rapid determination of the shape fluctuations of the Newtonian rings. Using this system, the membrane undulations along the contour lines can be recorded as a function of time. The absolute value of the curvature elastic constant, K_c, can then be determined from the mean square amplitudes of these undulations by application of the equipartition theorem (Schneider et al. 1984; Engelhardt et al. 1985).

II.3 Instrumentation and data analysis

The experimental device for the measurement of the flicker spectra has been described previously (Fricke and Sackmann 1984). The microscope was either a Zeiss Photomicroscope III equipped with a phase contrast objective Planachromat (×100/1.25 oil, 0.17, Ph3) or an inverted Zeiss Axiomat equipped with a Planapo phase contrast objective (×100/1.30 oil ∞/0.17). The frequency spectra of the intensity fluctuations were taken with a Fast Fourier Transform analyzer (HP 3582A). A circular aperture was placed in the image plane of the objective so that the flicker spectra could be taken from a small area of adjustable diameter at the centre of the cell.

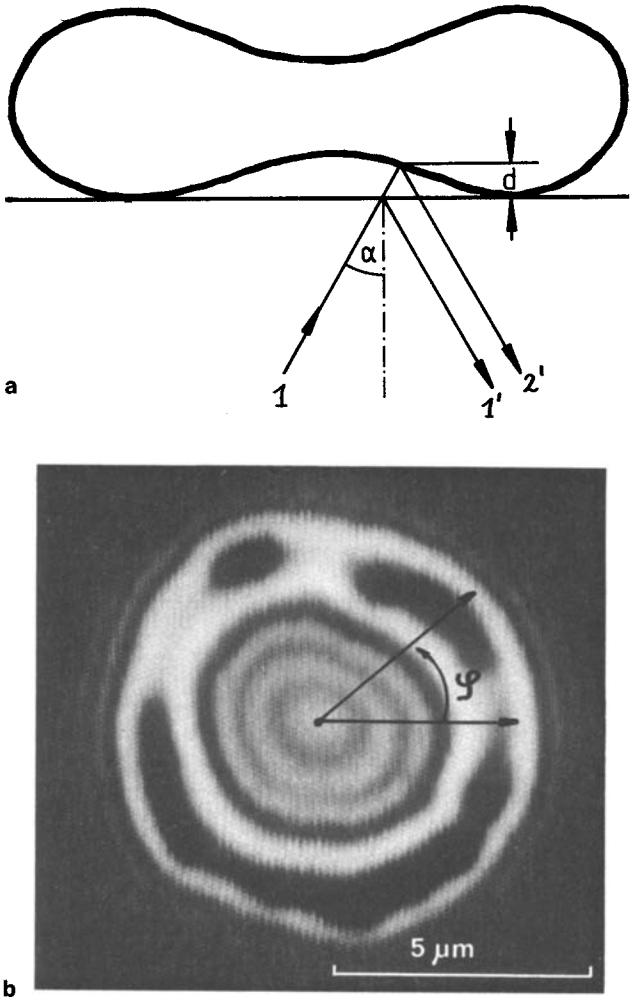


Fig. 2. a Diagrammatic representation of light path in reflection interference contrast experiment (antiflex technique). **b** Newtonian interference pattern of cup-shaped red blood cell. The dark interference rings are analysed by the image evaluation system. The cell is attached to the glass plate at the half-moon-shaped patches at the periphery

The reflection interference contrast experiments were performed with the Axiomat microscope equipped with a Zeiss Antiflex objective (Antiflex, Neofluor ×36/1.25 oil). The images were taken with a video camera (RCA/TC 1004) containing an Ultricon (4532/U) and were recorded with a Sony VO 5800 PS video recorder. For the data acquisition and analysis a home made image processing system was used (cf. Engelhardt et al. 1985). The Newtonian interference patterns were taken at a rate of 25 frames per second and stored in the recorder. The coordinates $R(\varphi)$ of the rings of destructive interference were taken as described previously (Engelhardt et al. 1985) and the deformation $u(\varphi)$ in the vertical direction was determined with the help of Eq. (7).

II.4 Preparation of cells

For each experiment cells were freshly drawn from healthy volunteers and prepared as described below:

For the storage of the cells a sterile, isotonic NaCl/ACD buffer was used, this consisted of a mixture of the following two solutions:

1) a modified physiological saline solution containing per litre of solution: 9.0 g NaCl, 17.2 mg adenine, 26.2 mg inosine, 2.5 g albumin, 10 ml Vitamine (Seromed No. 273) and 10 ml penicillin/streptomycin (Seromed No. A2212 100 I.U/ml). The pH was adjusted to pH 7.4 with NaOH.
2) an ACD buffer containing per litre of solution: 16 g sodium citrate, 25 g glucose, 17.2 mg adenine, 26.2 mg inosine, 2.5 g albumin, 10 ml Vitamine and 10 ml penicillin/streptomycin. The pH was adjusted to pH 7.4 with citric acid.

The final hematocrit was in all cases adjusted by addition of an appropriate amount of a mixture containing 4 volumes of the saline solution and 1 volume of the ACD-buffer to a cell suspension prepared in the following way: For anticoagulation, ACD buffer and the freshly drawn blood was first mixed at a volume ratio of 4:1. This suspension was centrifuged at 2,000 *g* for 5 min and the supernatant and buffy coat were removed. After adjustment of the hematocrit to 50%, 500 volumes of NaCl/ACD-buffer were added to 1 volume of the cell suspension.

For the measurement, the cells were brought into a special chamber which was fixed to the microscope stage. As described previously (Fricke and Sackmann 1984) the cells were loosely fixed to the inner surface of that window of the chamber through which the cells were observed: that is to the upper glass plate (cover glass) in the case of the Photomicroscope and to the bottom glass plate in the case of the Axiomat. In this way the membrane undulations were not superimposed by the Brownian motion of the cells. The fixation was achieved by coating the glass surfaces with polylysine of low molecular weight (3,000) by dipping them into a 3 m*M* aqueous solution of the protein. As noted previously (Fricke and Sackmann 1984) higher molecular weight polylysine leads to shape changes of the cells.

III. Effect of physically induced modification of membrane structure on flicker spectra

III.1 Influence of diameter of aperture on range of q-vectors contributing to flicker spectrum

As noted above an aperture was placed in the image plane of the objective lens which enabled as to reduce the lower limit of q-vectors (or the upper limit of wavelengths) contributing to the flicker spectrum. The amplitude and line width were determined as a function of the diameter of the aperture. A reduction in the diameter from $\varkappa = 5\,\mu m$ (70% of cell diameter) to $\varkappa = 3.2\,\mu m$ (45% of cell diameter) increases the line width $\Delta\omega$ by 30% and, according to Eq. (3), the average q-vector by 10%. This shows that the long wavelength modes are indeed eliminated by the aperture and provides at least some qualitative evidence for our above conclusion (Sect. II.1) that the number of modes contributing to the flicker spectrum are restricted by the boundary conditions.

III.2 Viscosity dependence of flicker spectrum and modification of membrane elasticity by adsorption of dextran

In order to test Eq. (2) with respect to the dependence of the flicker spectrum on the viscosity of the outer medium, the latter was varied by addition of dextran. In Fig. 3 the reduced amplitude $P(0)/v$ and the product $\Delta\omega \cdot v$ (v kinematic viscosity of medium; $v = \eta/\text{density}$) are plotted as a function of v (lower abscissa scale) or of the dextran concentration (upper abscissa scale). According to Eqs. (3) and (4) these expressions should be constant with respect to v. Figure 3 shows, however, that this is only observed

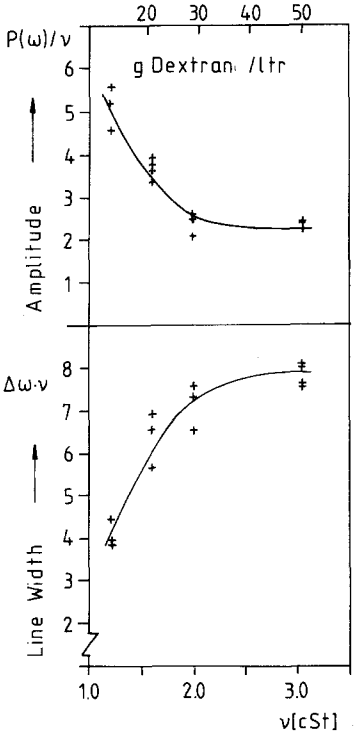


Fig. 3. Modification of flicker spectrum by addition of dextran (molecular weight: 30,000) to outer medium. *Lower abscissa scale:* Kinematic viscosity of medium ($v = \eta/\varrho$; ϱ = density). *Upper scale:* dextran concentration, c_d, in grams per litre

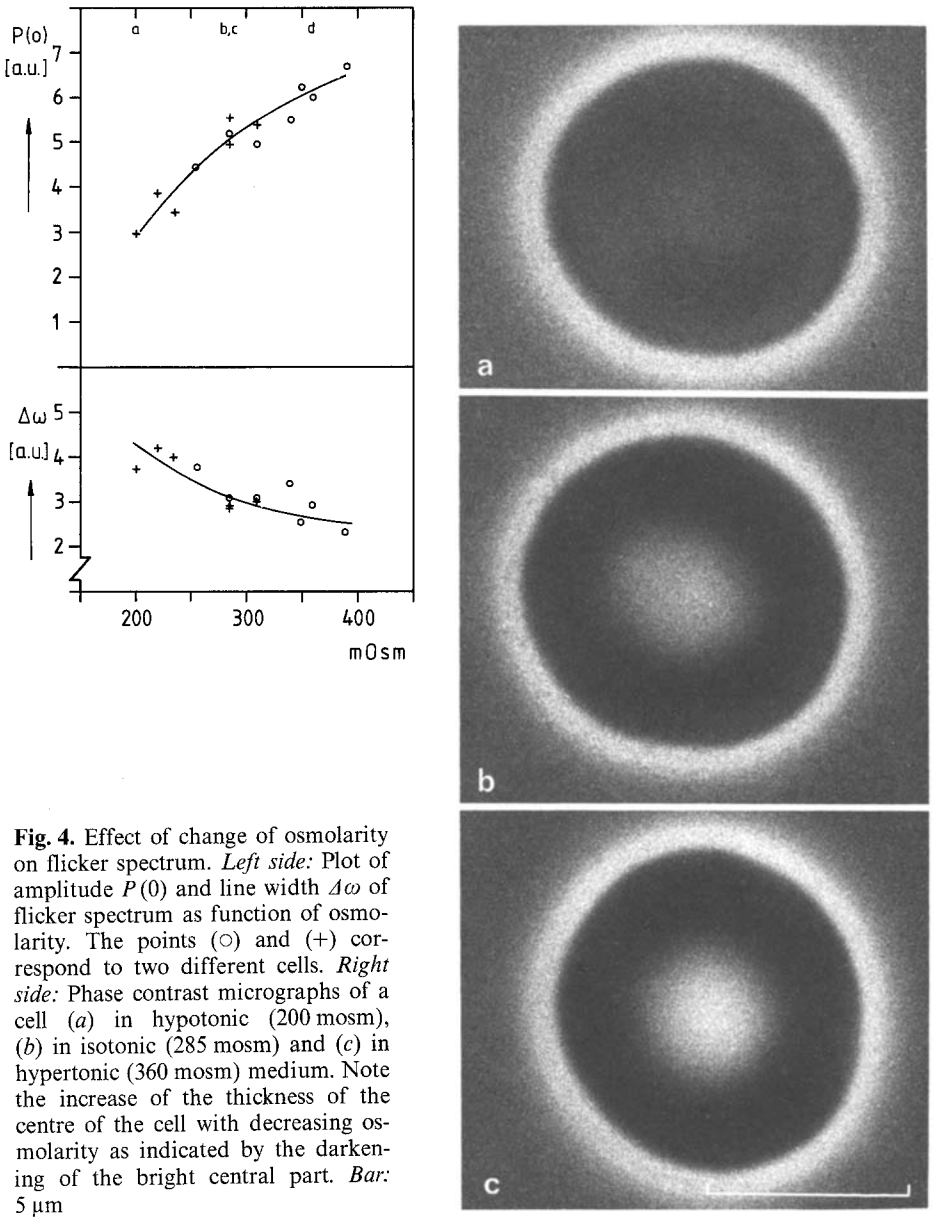


Fig. 4. Effect of change of osmolarity on flicker spectrum. *Left side:* Plot of amplitude $P(0)$ and line width $\Delta\omega$ of flicker spectrum as function of osmolarity. The points (○) and (+) correspond to two different cells. *Right side:* Phase contrast micrographs of a cell (*a*) in hypotonic (200 mosm), (*b*) in isotonic (285 mosm) and (*c*) in hypertonic (360 mosm) medium. Note the increase of the thickness of the centre of the cell with decreasing osmolarity as indicated by the darkening of the bright central part. *Bar:* 5 μm

at higher dextran concentrations ($c_d > 3$ g/l) whereas $P(0)/\nu$ decreases and $\Delta\omega$ increases strongly with c_d below this limit. This anomalous behaviour is explained in terms of a specific binding of the polysaccharide to the outer surface of the erythrocyte membrane and a subsequent modification of the membrane structure. Further evidence for a specific binding comes from the saturation behaviour of the dextran effect which is typical for the adsorption of macromolecules to interfaces (de Gennes 1981). The saturation regime is reached if the whole cell surface is covered by a monolayer of the macromolecule.

From the data of Fig. 3 it follows that the product $P(0)\Delta\omega^2$ and therefore the ratio $1/S\bar{q}$ varies by at most 10% and that the size and shape of the cells does not change appreciably. This shows that the dextran adsorption affects primarily the bending elastic constant of the membrane which is increased by a factor of 1.6 at maximum adsorption. This shows: (1) the binding of the polysaccharide to the glycocalix modifies the membrane microstructure considerably and (2) the bending elastic constant is a highly sensitive physical parameter which enables the detection of subtle alterations of the membrane structure.

III.3 Effect of osmolarity changes

Brochard and Lennon (1975) postulated that for discocytes the surface tension of the membrane does not contribute to the membrane elasticity up to second order. It was also pointed out (Brochard and

Lennon 1975; Fricke and Sackmann 1984) that this is essential for the appearance of the flickering since surface tension effects would suppress the undulations completely. The same is expected to hold for the shear elasticity of the cytoskeleton. In order to test the validity of this assumption the flicker spectrum was measured as a function of osmotic swelling. Hypertonic solutions (285 to 390 mosm) were adjusted by adding mannit and low osmolarities (285 to 200 mosm) were obtained by adding millipore water to the NaCl/ACD-buffer. The swelling of the cells at decreasing osmolarity is demonstrated by the gradual darkening of the bright central part of the phase contrast image at decreasing osmolarity (Fig. 4 right side).

According to Fig. 4 the amplitude $P(0)$ is reduced and the line width grows at decreasing osmolarity in the whole range studied. However, the variation of these parameters is considerably smaller in the hypertonic regime (> 285 mosm) than in the hypotonic one. The product $P(0)\Delta\omega^2$ (or $1/S\bar{q}$) is constant within the accuracy of the measurement between 400 and 200 mosm showing that the swelling leads to an increase in the apparent curvature elasticity. It is highly unlikely that the addition of water modifies the membrane microstructure.

One possible explanation for the reduction of the amplitude P(0) is that it is caused by the introduction of surface tension effects at the transition of the cell to a spheroid. Indeed at 200 mosm the cell appears to be nearly spherical because it bursts at 135 mosm. On the other hand one would expect that the long wavelength modes should be reduced more strongly than the undulations with large q-values, in contrast to our finding that the ratio $1/S\bar{q}$ is constant. Another explanation is therefore that the influx of water affects the coupling of the cytoskeleton to the lipid/protein bilayer in such a way that the rubber elasticity of the cytoskeleton comes into play. The shear elasticity associated with the deformation of this network would also reduce the flicker amplitude (Brochard and Lennon 1975).

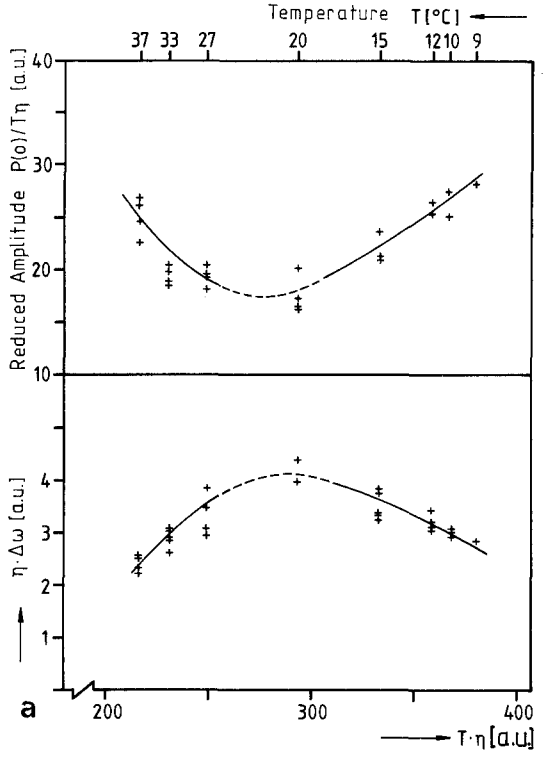
III.4 Temperature dependence of flicker spectrum

In order to test the sensitivity of the technique and to gain insight into the temperature dependence of the curvature elasticity we took flicker spectra of individual cells between 9 °C and 37 °C. A typical result is shown in Fig. 5. Since the viscosity of the medium is also markedly temperature dependent we plotted the reduced amplitude $P(0)/\eta T$ and the product $\Delta\omega\,\eta$ as a function of $T\eta$ (lower scale) or T (upper scale). According to Eqs. (3) and (4), the variation of these quantities should reflect the temperature dependence of the curvature elastic constant.

Two regimes are clearly distinguished. At $T > 20$ °C the reduced amplitude increases (and $\Delta\omega$ decreases) with increasing temperature whereas the reverse holds at $T < 20$ °C. Consider first the high temperature regime: according to Eq. (5) the expression $P(0)\Delta\omega^2/T$ is proportional to $1/S\bar{q}$. From the data of Fig. 5 it follows that $1/S\bar{q}$ decreases by about 30% between 20 °C and 37 °C. Since S does not change with temperature this corresponds to a 25% increase in $\bar{q}$ and, according to Eq. (4), a decrease in K_c by 100%. This is indeed a strong reduction of the elastic constant and is in contrast to the behaviour of pure lipid bilayers in the fluid state which exhibit a much weaker temperature dependence of K_c (Engelhardt et al. 1985). Concerning the low temperature regime, a similar consideration leads to the following conclusions. The expression $P(0)\Delta\omega^2/T$ is constant within experimental error between 20 °C and 10 °C and the change in the reduced amplitude is completely determined by a variation in the elastic constant. It follows that K_c decreases by about a factor of 1.3 between 20 °C and 10 °C and thus exhibits an anomalous temperature behaviour.

The finding of two regimes of different temperature dependence of both the amplitude and the line width provides very strong evidence for a conformational change of the membrane starting at the temperature where the two regimes meet, that is at about 20 °C. The anomalous behaviour of the elastic constant shows that this structural transition is continuous and extends into the low temperature region. A conformational change at about 20 °C has been observed previously by a variety of techniques including sedimentation measurements (Glaser and Herrmann 1980), lateral diffusion measurements (Kapitza and Sackmann 1982) and spin label studies (Galla and Luisetti 1980). The latter technique also exhibits a discontinuous change of the spin probe mobility at 8 °C. It thus appears that the discontinuity at 20 °C corresponds to the onset of lateral phase separation which is completed at about 9 °C. Evidence for this comes also from freeze fracture studies which show that the integral membrane proteins are randomly distributed above 20 °C but exhibit a more clustered lateral organization below this limit (cf. Fig. 5 b). The phase separation process is most certainly caused by crystallization of part of the lipid moiety which is also responsible for the increasing sensitivity of the red blood cell to hemolysis at decreasing temperature.

It would be interesting to extend the measurement to temperatures above 40 °C where the red blood cell membrane becomes unstable and undergoes very pronounced shape changes. Owen et al.



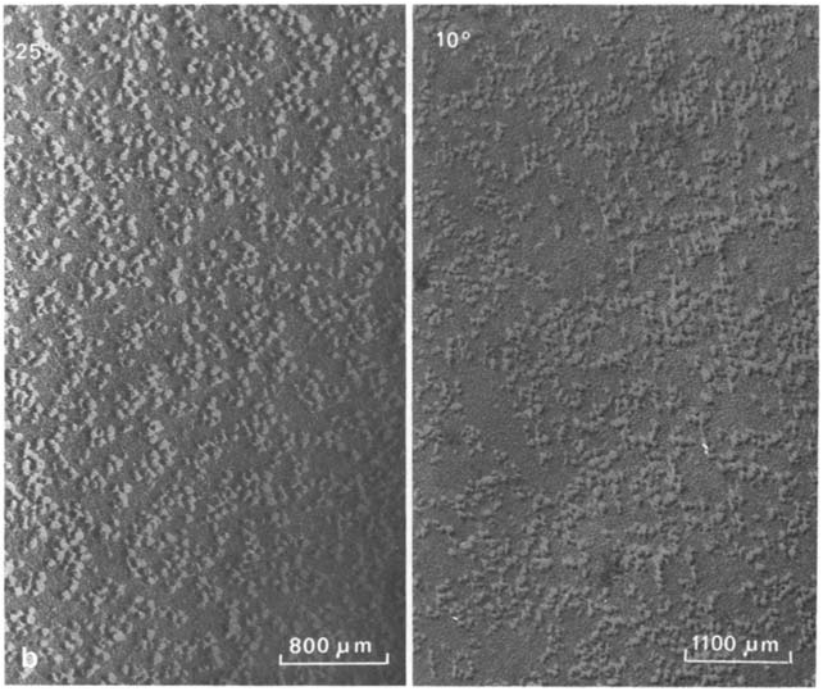


Fig. 5. **a** Temperature dependence of amplitude and line width of flicker spectrum of a single red blood cell. To account for the temperature dependence of the viscosity of the outer medium and by consideration of Eqs. (3) and (4) $P(0)/\eta T$ and $\Delta\omega\eta$ are plotted as a function of $T\eta$. The cell was perfused with PBS/ACD-buffer containing Mg^{++} and Ca^{++} (10^{-3} M). The different points given for each temperature correspond to different measurements of the same cell. The thin lines have been drawn in order to indicate the temperature dependence of the amplitude and line width. **b** Freeze fracture electron micrograph of red blood cell membrane (interior of bilayer) which was frozen from 25 °C and from 4 °C showing the transition from a random to a clustered lateral organization of the proteins (taken from T. Schlunck diploma thesis Munich 1984)

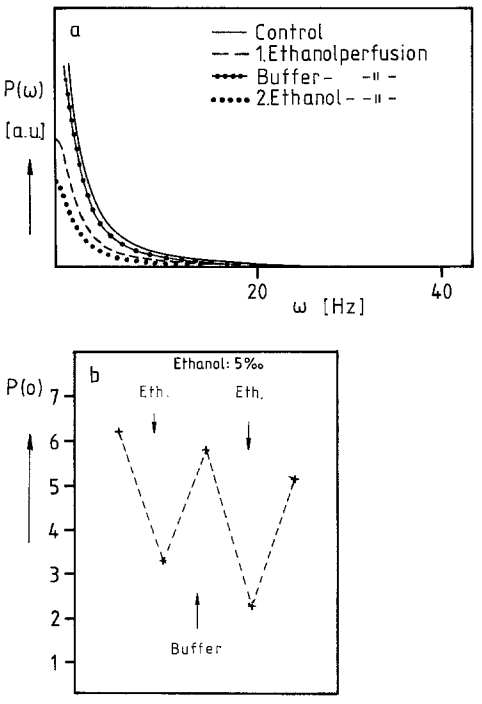


Fig. 6. Effect of ethanol on flicker spectrum. **a** Modification of spectrum of a single cell caused by alternating perfusion with 5 vol‰ of ethanol in buffer and pure buffer, respectively. The time interval between two successive perfusions was about 5 min. **b** Oscillation of amplitude caused by the alternating washing with 5‰ ethanol containing buffer and pure buffer, respectively

(1983) provided very strong evidence that this transition is associated with a conformational change of the spectrin. However, flicker spectra could not be taken above 40 °C owing to membrane instability.

IV. Effect of chemically and drug induced modification of membrane structure and elasticity on flicker spectra

IV.1 Effect of ethanol

A first test of the sensitivity of the surface undulations to chemically induced modification of the membrane structure was performed with ethanol. Figure 6 shows how the flicker spectrum $P(\omega)$ changes if a single cell is alternatingly washed with a solution of 5 vol‰ of ethanol on NaCl/ACD-buffer and pure buffer. Clearly, ethanol leads to a drastic reduction in the amplitude; but the effect is nearly completely reversible after washing the cell with pure buffer again. The changes occur within 5 min after perfusing the cell with the changed solution. As can be seen from the oscillation of the amplitudes $P(0)$ in Fig. 6 b, there is a slight irreversibility of the ethanol induced change of the membrane structure, that is $P(0)$ does not return to the original value after removing the ethanol again.

The cell studied in Fig. 6 maintained a discoid shape during the whole procedure. Most cells (about 95%), however, exhibited about three reversible discocyte-echinocyte transitions after consecutive perfusion with the ethanol containing and the pure buffer. Thereafter the cells remained as echinocytes.

The line width is not significantly changed by the alcohol which, according to Eq. (5), means that it leads primarily to an increase of the average wavevector $\bar{q}$ by about a factor of two whereas K_c is not affected. This means that ethanol suppresses the long wavelength undulations. At present we cannot explain this in terms of an ethanol induced change of the membrane microstructure.

Alcohol is decomposed into acetic aldehyde and we therefore studied the effect of this substance. No appreciable change of $P(0)$ or $\Delta\omega$ could be observed up to 4 vol‰.

IV.2 Effect of Ca^{++}-injection on the flicker spectrum and the cell shape

As noted in the introduction, the curvature elasticity of the red blood cell is expected to be substantially determined by the coupling of the lipid/protein bilayer to the cytoskeleton. In order to modify this coupling we introduced Ca^{++} into the cell via an ionophore (A 23187, Sigma). Figure 7 shows the effect of the intracellular increase in the Ca^{++} concentration on the flicker spectrum and the shape of the cell. In this experiment a cell fixed to the window of the flow chamber was perfused consecutively with different buffers and the flicker spectra were recorded after each change of the medium. The images of the cells were taken after the measurements. The cell was consecutively perfused with (1) pure NaCl/ACD-buffer, (2) buffer with 1 m*M* Ca^{++}, (3) buffer containing 1 m*M* Ca^{++} and 10 μ*M* A 23187 ionophore, (4) pure buffer, (5) buffer with ionophore alone, (6) buffer with Ca^{++} alone, (7) buffer with ionophore and Ca^{++} and (8) pure buffer.

Both the flicker spectrum and the cell shape are only changed if Ca^{++} is added together with the ionophore. As can be clearly seen, the spectral change and the shape transformation are completely reversible. Interestingly, the Ca^{++}-induced change is considerably slower (rise time about 15 min) than the recovery after re-washing with buffer (recovery time 5 min).

From the data of Fig. 7a it follows that the product $P(0)\Delta\omega^2$ (or the reverse product of the surface area and the average wave vector, $S/\bar{q}$) varies by less than 10% after Ca^{++} injection showing

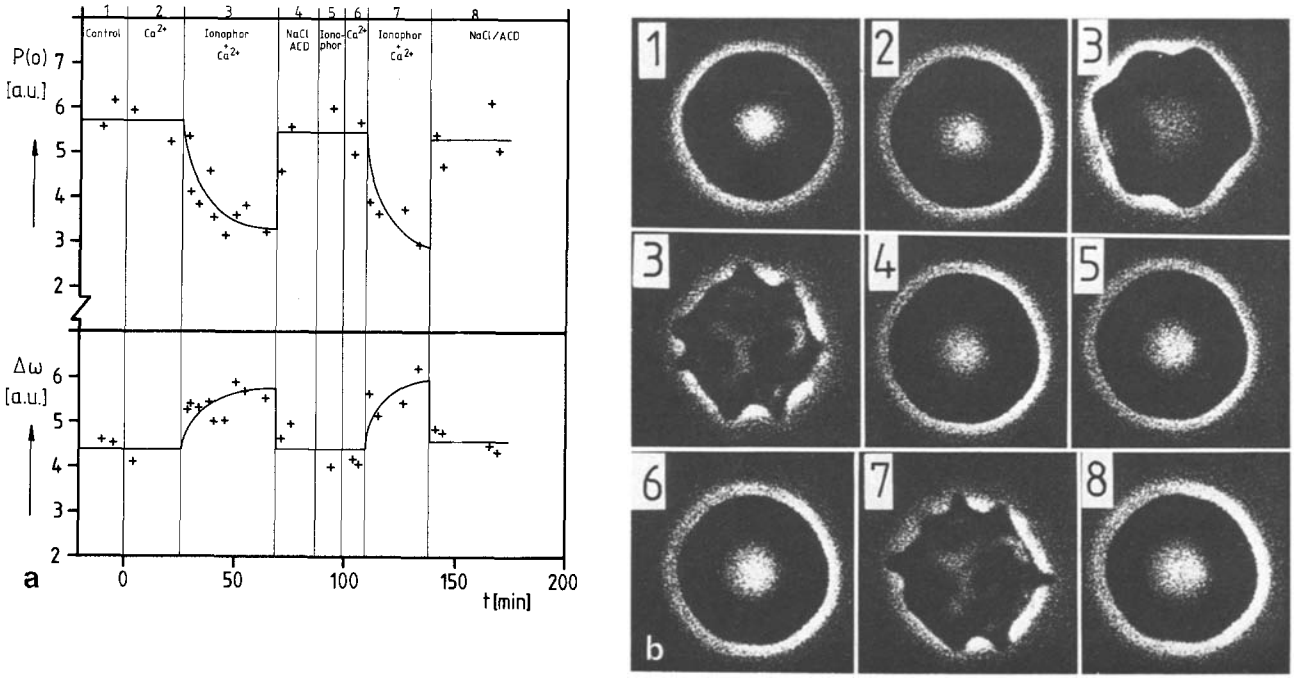


Fig. 7. Variation of flicker spectrum and the cell shape by injection of Ca^{++} into the cell via an ionophore (A 23187). **a** Change of amplitude and line width of a single cell which was consecutively perfused with (1) NaCl/ACD buffer; (2) Ca^{++} in buffer; (3) Ca^{++} and ionophore in buffer; (4) NaCl/ACD buffer; (5) Ionophore alone; (6) Ca^{++} alone; (7) Ca^{++} plus ionophore; (8) NaCl/ACD buffer. The concentration of the ionophore was 10 μM (in buffer) and of Ca^{++} 1 mM. The vertical lines indicate the time where the composition of the perfusion medium was changed. **b** Modification of cell shape caused by the consecutive perfusion procedures described in Fig. 7a. The numbers correspond to those in Fig. 7a

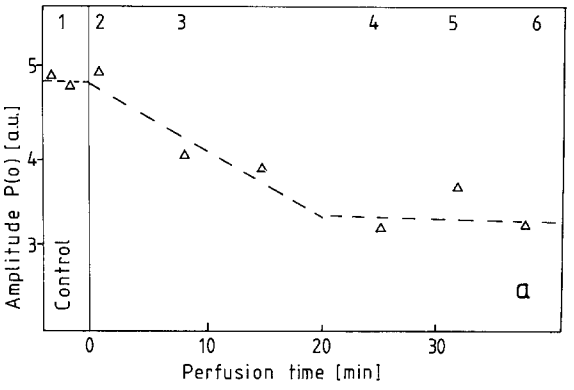
that an increase in the internal Ca^{++} concentration leads to an increase in the elastic constant K_c by a factor of 1.3. This can be explained in terms of a condensation of the cytoskeleton for the following reason:

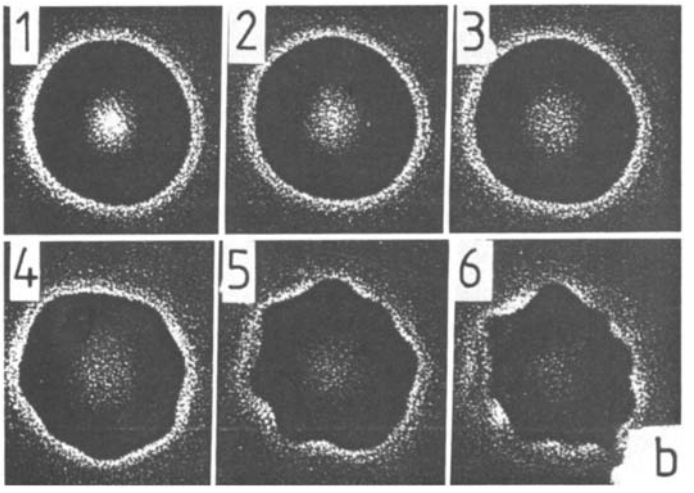
Figure 7b appears to suggest that Ca^{++} injection induces a discocyte-echinocyte transformation. However, a closer inspection shows that the spicules are much sharper as in the case of, for instance (Fig. 8b, below). Parallel freeze fracture electron microscopic studies (T. Schlunk unpublished work from diploma thesis) showed that the spicules are actually small vesicles. These are connected with the bulk of the membrane by thin necks and may eventually be detached from the cells. Elgsaeter et al. (1976) provided evidence that all agents, such as basic proteins or Ca^{++}, which precipitate spectrin in solution induce a condensation of the spectrin on the cytoplasmatic surface of the membrane and lead to the formation of protein-free vesicle blebs. Lutz et al. (1977) showed that vesicles released from red cells are spectrin-free. It is therefore most likely that the fine spicules formed by the Ca^{++} injection are spectrin free. This would lead to a higher packing density of the cytoskeleton which remains attached to the bulk of the membrane. The flicker spectrum, however, is essentially determined by the bending modes of the latter since the aperture was positioned in such a way that the regions between the spicules could be observed and analyzed.

IV.3 Effect of cholesterol depletion on flicker spectrum

A remarkable feature of the red blood cell is the well controlled cholesterol content of the membrane (0.8 mole/mole of phospholipid). Deviations from the normal concentration lead to characteristic shape changes such as discocyte-echinocyte or discocyte-stomatocyte transitions (Bessis 1973; Chailley et al. 1981). These transformations are accompanied by remarkable changes of the rheological properties of the blood (Chabanel et al. 1983). Cholesterol enrichment may also stabilize the cell shape against invaginations caused by other agents such as chlorpromazine (Lange et al. 1980).

In order to study the effect of a gradual cholesterol depletion on the membrane elasticity the following experiment was performed: The cells fixed to the window of the flow chamber were perfused with a suspension of vesicles of dimyristoylphosphatidylcholine (lipid conentration 10^{-3} M) containing 5 mole% of cholesterol. Then the flicker spectrum of an individual cell was recorded approximately every 10 min. Simultaneously phase contrast micrographs





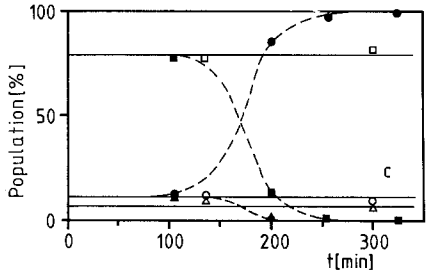


Fig. 8. **a** Time dependence of amplitude $P(0)$, of individual red blood cell which was perfused with a suspension of dimyristoylphosphatidylcholine vesicles containing 5 mole% of cholesterol starting at time $t = 0$. Measuring temperature 20 °C. **b** Phase contrast micrographs of cell taken at various times after starting the perfusion: (*1*) control (0 min); (*2*) 3 min; (*3*) 11 min; (*4*) 23 min; (*5*) 30 min; (*6*) 35 min. The numbers are also indicated in **a**. **c** Variation of relative number of discocytes (■), echinocytes (●) and stomatocytes (▲) with time in a cell suspension which was incubated with vesicles of dimyristoylphosphatidylcholine (10^{-2} M lipid) containing 20 mole% of cholesterol at 20 °C. The points (□, ○, △) show the result for a non-incubated cell suspension. The cells were analysed in a Fuchs-Rosenthal counter chamber

were taken after each measurement. The result is shown in Fig. 8.

The amplitude, $P(0)$, decreases markedly during the first 20 min after starting the incubation and remains constant thereafter. From the micrographs it may be seen that the cell maintains its discoid shape for the first 20 min and is suddenly transformed in an echinocyte after this time. The ratio $1/S\bar{q}$ does not change significantly so that the elastic constant is increased by about 10% during the first 20 min.

The most interesting result of this experiment is the following: The continuous decrease of the amplitude $P(0)$ with time shows that cholesterol is continuously removed from the cell during the first 20 min of perfusion whereas the shape does not change appreciably. After this time, however, a discocyte- to echinocyte transformation sets in abruptly. This leads to the conclusion that the shape-transformation occurs if the cholesterol content decreases below a critical value. The shape transformation thus has typical features of a phase transition.

Further evidence for such a phase-transition-like behaviour comes from parallel experiments in which we determined the relative population of discocytes and echinocytes in a cell suspension which was incubated with vesicles containing 20 mole% of cholesterol. The relative population of echinocytes was determined as a function of time and the result is shown in Fig. 8c. Obviously, cell shape transformations are not observed up to 100 min after incubation. Discocyte-echinocyte transformation sets in abruptly between 100 and 200 min and is completed after 250 min. Some residual stomatocytes also vanish at 150 min after incubation.

IV.4 Effect of the cross-linking agent diamid

Diamid is a cross-linking substance which oxidizes SH-groups and leads to a drastic reduction of the deformability of the cell in shear fields (Haest et al. 1980). Figure 9a shows the effect of this agent on the amplitude $P(0)$ of the flicker spectrum of a single cell. The amplitude decreases with a response time of 30 min. The cell shape is only slightly changed by the agent and the effect is completely irreversible (cf. Fig. 9a) as would be expected for a cross-linking substance. From the measurement of $P(0)\Delta\omega^2$ it follows that the ratio $1/S\bar{q}$ is not changed within experimental error, leading to the conclusion that the cross-linking by diamid leads to an increase in the bending stiffness by a factor of 1.3.

IV.5 Drug-induced changes of the surface undulations

The effect of numerous drugs on the flicker spectrum was studied (K. Fricke doctoral thesis, Munich

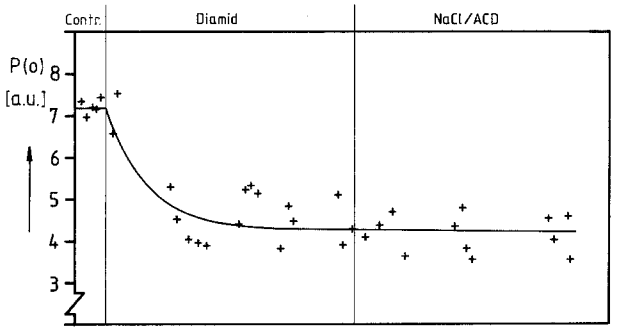


Fig. 9. Effect of perfusion of a cell with 1 m*M* diamid in the NaCl/ACD buffer on amplitude of flicker spectrum and demonstration of irreversibility of effect after re-washing with buffer

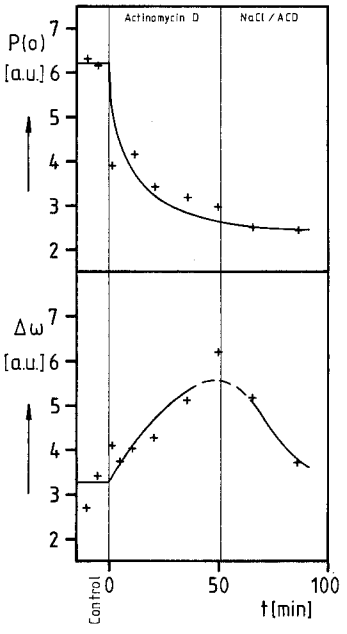


Fig. 10. Change of the amplitude $P(0)$ and the line width of the flicker spectrum of a cell induced by perfusion with 10^{-6} M of actinomycin D. Note that the effect on the amplitude is irreversible while that on the line width is reversible

1985). Only about 20% of the drugs studied changed the flicker spectrum to a remarkable extent. Two examples are given below:

a) Effect of actinomycin D. This antitumor drug is a well known inhibitor of the RNA synthesis owing to its strong tendency to bind to DNA. Figure 10 shows that it can also substantially modify the membrane structure even at 10^{-6} M. The amplitude and the line width are changed by more than a factor of two. The ratio $1/S\bar{q}$ is increased by about 20% which means that the drug leads to an increase of the elastic constant by a factor of two. According to Fig. 10 the amplitude $P(0)$ is irreversibly decreased

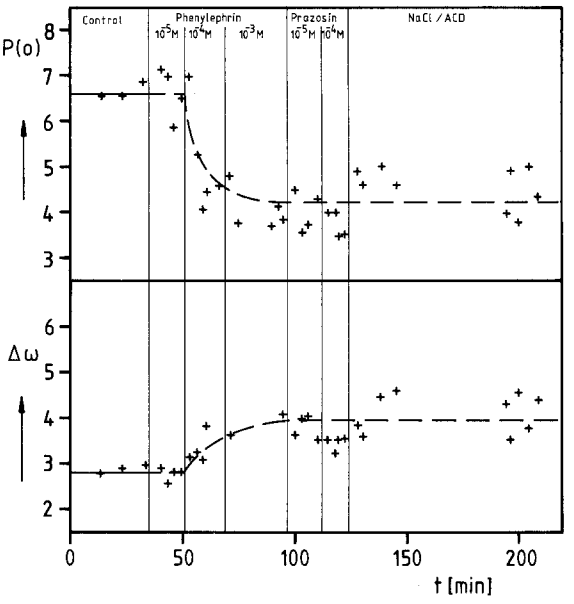
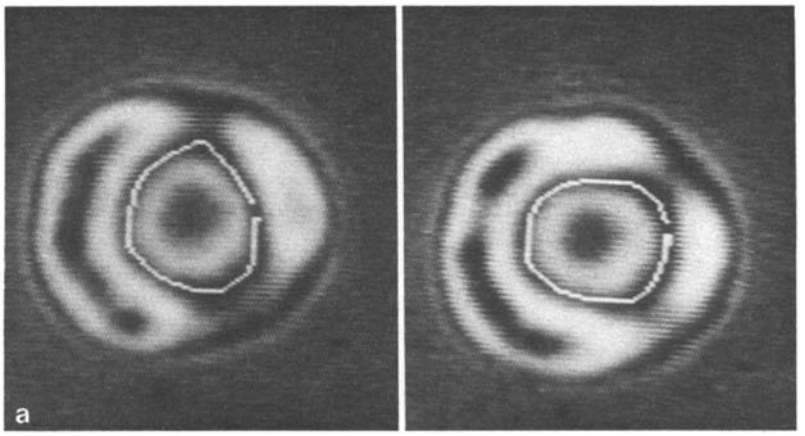


Fig. 11. Effect of various concentration of an α-agonist (phenylephrin) on the flicker spectrum. Check of reversibility of effect by α-antagonist (prazosin) and/or perfusion with pure buffer

whereas the line width is reduced again after perfusion with pure buffer. This behaviour has been verified with another cell studied but cannot be explained yet.

Sinha and Chignell (1979) reported that actinomycin as well as other antitumor drugs with a strong tendency to intercalate into DNA lead to a change of the mobility of probes in erythrocyte membranes. These experiments provide evidence that these drugs may also exert their biological effects by modifying the membrane function owing to their tendency to bind to membrane proteins, such as transport proteins (Sinha and Chignell 1979). The interesting finding in the present work is that actinomycin D exerts a remarkable effect at micromolar concentrations.

b) Effect of α-agonist (phenylephrin) and α-antagonist (prazosin). As a second example, the effect of a subsequent addition of the agonist and the antagonist are shown in Fig. 11. The agonist leads to a strong decrease in the amplitude $P(0)$ above concentrations of 10^{-4} M. In order to see whether this is a specific effect due to the binding of phenylephrin to α-receptors which can be reversed by the inhibitory action of an antagonist, the latter was added after saturation of the effect caused by the agonist. Obviously, the effect is not reversible even after perfusion with pure buffer. This leads to the conclusion that the change of the surface undulations is due to non-specific binding of the agonist.



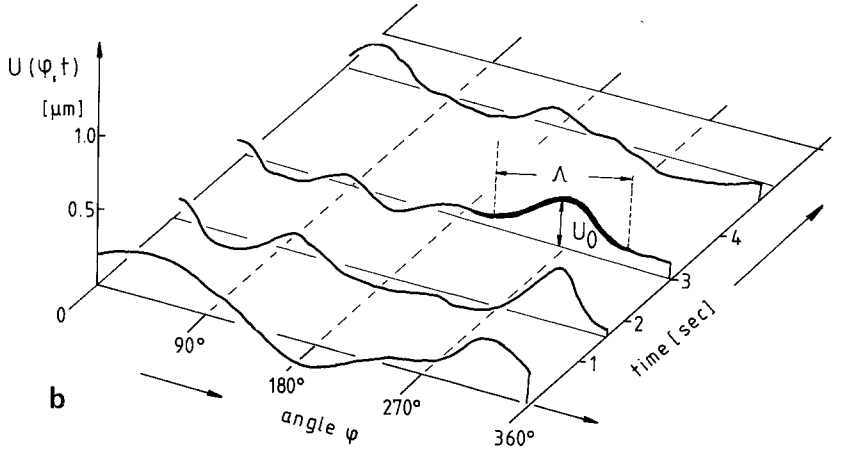


Fig. 12. a Reflection interference contrast micrograph of discocyte taken at different times (t and $t+1$ s). The ring of destructive interference is marked by a white line which was drawn by the computer along the line of minimum intensity. **b** Transient deformations $u(\varphi, t)$ of membrane in vertical direction measured along a ring of destructive interference of radius $R = 7$ μm recorded at different time intervals. The angle φ is defined in Fig. 2b. Λ is the wavelength and u_0 the amplitude of the wave of wave vector $q = 2\pi/\Lambda$

V. Measurement of absolute amplitude and curvature elastic constant by the reflection interference contrast technique

In a first preliminary application of the reflection interference contrast technique, the transient deformation of the membrane in the direction of the rotational axis of the cell was measured along a ring of destructive interference at time intervals of 1 s. Figure 12a shows two of the deformation patterns observed at time intervals of 2 s.

In principle, the curvature elastic constant could be determined accurately by decomposing the deformation pattern $u(\varphi, t)$ at time t_i into Fourier components of wave vector q and amplitude u_q according to

$$u(\varphi, t_i) = \sum u_q(t_i) \exp\{i q \varphi\} .$$

The curvature elastic constant could then be determined by application of the equipartition theorem from the mean square amplitudes $\langle u_q^2 \rangle$ from the following equation (Brochard and Lennon 1975; Engelhardt et al. 1985):

$$\langle u_q^2 \rangle = \frac{kT}{K_c q^4 S} .$$

In the present and still preliminary application of the technique we are only able to give a first estimate of the mean square amplitudes $\langle u_q^2 \rangle$. From the transient deformations of Fig. 12b we estimate a value of the mean square amplitude of $\langle u_q^2 \rangle = 10^{-10}$ cm^2 for a q-vector of $q = 5 \cdot 10^3$ cm^{-1}. The total area of the cell is $S \approx 1.3 \cdot 10^{-6}$ cm^2 which leads to

$$K_c = 5 \cdot 10^{-13} \text{ erg} .$$

This value agrees within a factor of two with the elastic constant reported by Brochard and Lennon (1975) for human erythrocytes: $K_c = 3 \cdot 10^{-13}$ erg. It is only by less than a factor of two larger than the curvature elastic constant measured for bilayers of dimyristoylphosphatidylcholine well above the phase transition: $K_c = 3.5 \cdot 10^{-13}$ erg (Engelhardt et al. 1985).

VI. Concluding discussion

The evaluation of surface undulations by flicker spectroscopy is a non-disruptive and sensitive technique to establish subtle changes of the membrane curvature elasticity induced (1) by variations of the external conditions (temperature, osmolarity) (2) by changes in the lipid (e.g. cholesterol) composition and (3) by chemical agents or drugs.

The absolute values of the amplitude $P(0)$ and line width $\Delta\omega$ of 80% of the different cells studied agree within $\pm 10\%$. Only about 20% show deviation up to $\pm 20\%$ from the average values of $P(0)$ and $\Delta\omega$. The same degree of scattering of the data points is obtained if the spectra of the same cell are taken several times. The above differences of the $P(0)$- and $\Delta\omega$-values are therefore most probably due to systematic errors either in the evaluation procedure or because of background scattering and can therefore be reduced by improving the technique. In a parallel experiment we separated old and young cells in a density gradient and compared the flicker spectra of these populations. $P(0)$ and $\Delta\omega$ agreed within experimental error. This shows that the curvature elasticity of red blood cells of healthy donors is a very well controlled property. Owing to this high reproducibility, the red blood cell is an ideal biological object for physical studies.

A disadvantage of the present method of flicker spectroscopy is that the spectra cannot be resolved with respect to the wave vector q. This difficulty can most certainly be circumvented by the interference contrast technique by which the surface undulations may be analysed in terms of the space-time correlation function. With this technique it should also be possible to determine the mean square amplitudes of individual modes which would enable the most accurate measurements of the curvature elastic constant (Engelhardt et al. 1985). This will be a topic of future research. The advantage of the present technique is that it is very simple and highly sensitive to structural changes of the membranes.

The main problem for future research would be to understand the membrane elasticity in terms of the microstructure of the membrane. The astonishing finding that the value of the curvature elastic constant of erythrocytes ($K_c = 7 \cdot 10^{-13}$ erg) is only by about a factor of two larger than that of pure lipid bilayer vesicles (Engelhardt et al. 1985) suggests that the spectrin-actin meshwork is only loosely coupled to the lipid/protein bilayer. This is an agreement with the present view that the coupling occurs by binding of part of the cross-links of the network to Band III protein via the coupling protein ankyrin. Further evidence for a relatively loose coupling comes from the finding that the lateral mobility of most of the Band III proteins is still rather high (Schindler et al. 1980). The low value of K_c also strongly suggests that the spectrin molecules are not stretched but are rather folded so that the rubber-elasticity does not restrict the membrane undulations appreciably.

Evans suggested (private communication) that the thermal excited surface undulations of the red cell membrane could also be caused by lateral fluctuations of the spectrin/actin-meshwork. Indeed, a contraction of the network would lead to a protrusion towards the outside and an expansion towards the inside of the cell. For a protrusion with the form of a spherical cap the amplitude, u, of an undulation of diameter Λ would be related to the relative change of the mesh size, $\delta l/l$, of the network and the thickness, Δ, of the bilayer according to

$$u = \frac{1}{8} \frac{\Lambda^2}{\Delta} \frac{\delta l}{l}.$$

The ratio Λ^2/Δ provides a large amplification factor. For typical wavelengths of 1 μm, and for $\Delta \approx 5 \cdot 10^{-7}$ cm the observed amplitudes of 0.1 μm could be produced by a relative expansion (or contraction) of the network by $\delta l/l = 4 \cdot 10^{-3}$.

In recent work we showed that spectrin exhibits a remarkable electrostatic interaction with phosphatidylserine (Maksymiw, Diploma Thesis, Munich 1985), a major lipid component of the inner monolayer of erythrocyte membranes. A fluctuation in the electrostatic coupling of the spectrin to the lipid moiety of the inner monolayer could also lead to a fluctuating expansion and compression of the latter. According to Svetina and Zeks (1983) this would lead to a fluctuation in the spontaneous curvature of the membrane. Thus a thermally induced fluctuation in the electrostatic coupling of the spectrin to the membrane could provide another driving force for the membrane undulations.

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