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# Influence of Temperature and Cholesterol on the Rotational Diffusion of Band 3 in the Human Erythrocyte Membrane†

Erich A. Nigg and Richard J. Cherry*

ABSTRACT: Band 3 rotation in the human erythrocyte membrane is measured by observing flash-induced dichroism of eosin probes. The decay of the absorption anisotropy is found to be strongly dependent on temperature. The results are analyzed on the assumption that rotation of band 3 only occurs about the membrane normal. It is deduced that both fast and slowly rotating forms of band 3 coexist in the membrane. The equilibrium between these forms is temperature dependent, the slowly rotating species becoming increasingly dominant as the temperature is reduced. Plots of the fractional distribution of the different species against temperature show a marked change of slope at around 37–40 °C. The effects are essentially reversible over the range 1–45 °C and independent of the presence of the spectrin–actin network. The results could be due to temperature-dependent protein–protein associations mediated either by a protein conformational change or by lipid phase segregation. In further experiments, the cholesterol content of the erythrocyte membrane is varied by incubation with lipid vesicles. No significant changes in the rotational diffusion of band 3 are observed following variation of membrane cholesterol/phospholipid mole ratios over the range 0.34–1.66. This is a surprising result in view of the well-known effects of cholesterol on lipid fluidity.

The rotational diffusion of proteins in biological membranes may be investigated by using a flash photolysis technique developed in recent years (Razi Naqvi et al., 1973; Cherry et al., 1976a; Cherry & Schneider, 1976; Lavalette et al., 1977). The method exploits the long triplet lifetime of probe molecules which are covalently coupled to membrane proteins. Rotational diffusion is measured by observing the decay of dichroism of flash-induced absorption transients of the triplet probe.

When intact human erythrocytes are labeled with the triplet probe eosin 5-isothiocyanate[1] (eosin-NCS), most of the label is found to be associated with band 3, a major constituent of the erythrocyte membrane (Cherry et al., 1976b, 1977). Band 3 comprises a class of membrane spanning proteins which are involved in anion transport across the red cell membrane [for a recent review, see Cabantchik et al. (1978)]. Previously we have measured the rotational diffusion of eosin-labeled band 3 by the flash photolysis method and used these measurements to investigate protein–protein interactions in the membrane (Cherry et al., 1976b). We showed that the spectrin–actin network has little or no effect on band 3 rotation, throwing doubt on the proposal that these components are physically linked (Pinto da Silva & Nicholson, 1974; Elgsaeter et al., 1976). More recently we combined diffusion measurements

† From the Eidgenössische Technische Hochschule, Laboratorium für Biochemie, ETH-Zentrum, CH 8092 Zürich, Switzerland. *Received March 12, 1979.* This work was supported in part by the Swiss National Science Foundation, Berne.

[1] Abbreviations used: eosin-NCS, eosin 5-isothiocyanate; eosin-MA, eosin-5-maleimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; *C/P*, cholesterol/phospholipid mole ratio.

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with chemical cross-linking to demonstrate the dimeric state of band 3 in the red cell membrane (Nigg & Cherry, 1979). These latter studies used a new eosin derivative, eosin-5-maleimide (eosin-MA).

Here we report improved measurements of rotational diffusion of band 3 which permit a more detailed analysis than was hitherto possible. We have used these improved measurements to investigate two further properties of band 3 rotation, namely, its dependence on temperature and on the level of cholesterol in the membrane. Previously, several studies have shown that the cholesterol content of the red cell membrane may be increased or decreased by incubation with cholesterol–lecithin vesicles of appropriate composition (Bruckdorfer et al., 1968; Cooper et al., 1975, 1978). The influence of cholesterol on lipid fluidity is well documented (Oldfield & Chapman, 1972; Vanderkooi et al., 1974; Cooper et al., 1978), and it is therefore of particular interest to examine its effect on protein diffusion.

## Materials and Methods

*Labeling of Erythrocytes and Ghost Preparation.* Fresh human blood ($O^+$) was obtained from the Swiss Red Cross Blood Transfusion Service and used within 12 h. The blood was centrifuged, and the plasma and white cells were removed by aspiration. Some of the plasma to be used in the subsequent incubation was stored at 4 °C. The erythrocytes were washed 2–3 times in 5 mM $NaH_2PO_4$–$Na_2HPO_4$, 150 mM NaCl, pH 7.4. They were then given a further wash in isotonic phosphate buffer, pH 7.4.

Eosin derivatives (either eosin-MA or eosin-NCS from Molecular Probes) were first dissolved in the same buffer at a concentration of 0.5 mg/mL. One milligram of label was added per 5 mL of packed erythrocytes, and the reaction was allowed to proceed in the dark at room temperature. After 30–60 (eosin-MA) or 180 (eosin-NCS) min, the reaction was stopped and unreacted label was removed by two washes of the cells in a 40–50-fold volume of 5 mM $NaH_2PO_4$–$Na_2HPO_4$, 150 mM NaCl, pH 7.4. To prepare ghosts, we hemolyzed the cells in 40–50 volumes of 5 mM $NaH_2PO_4$–$Na_2HPO_4$, pH 7.4, and washed them 3–4 times with the same buffer until they were essentially free of hemoglobin. All operations except the labeling step were carried out at 0–4 °C. Ghost suspensions used for flash photolysis contained ~1 mg/mL of protein and were 2–5 μM with respect to eosin. Prior to measurements, oxygen was displaced from the samples by a stream of argon as described previously (Cherry, 1978).

*Preparation of Lipid Dispersions.* Cholesterol–lecithin dispersions of varying composition were prepared essentially following a procedure described by Cooper et al. (1978). Egg lecithin (40–80 mg) (grade I, Lipid Products Ltd.) was mixed with up to 80 mg of cholesterol (Merck, puriss) in 3:2 chloroform–methanol (v/v). Solvent was completely evaporated under vacuum, and the dried lipids were dispersed in 10 mL of 155 mM NaCl. The lipid suspension was then sonicated under nitrogen in a metal container placed in a water bath either at 4 °C for cholesterol-depletion or at 45 °C for cholesterol-loading experiments. The sonifier was a Branson B 30 operated at 90–110 W, using a standard tip. Sonication was intermittent with on-off periods of about 0.5 s duration and a total time of 100 min. After sonication, 4 mL of plasma which had previously been heated for 30 min at 56 °C was added and the lipid–plasma dispersion was centrifuged for 30 min at 30000*g*.

*Incubation of Red Cells with Lipid Dispersions.* Eosin-labeled erythrocytes were given a final wash in the following incubation medium: 50 mM $NaH_2PO_4$–$Na_2HPO_4$, 64 mM NaCl, 5 mM KCl, 10 mM glucose, 16 mM inosine, 0.6 mM adenine, 0.08 mg/mL penicillin G (Sigma, 1667 units/mg), 0.2 mg/mL streptomycin sulfate (Serva, 745 EU/mg), pH 7.4. A 2.5-mL amount of packed erythrocytes was diluted with incubation medium up to 14 mL and added to the lipid–plasma dispersion. The suspension was then placed in a dialysis bag and dialyzed against incubation medium in a gently shaking water bath at 37 °C for up to 40 h. After the incubation, the erythrocytes were separated from the liposomes by centrifugation and washed three times in 5 mM $NaH_2PO_4$–$Na_2HPO_4$, 150 mM NaCl, pH 7.4, before ghosts were prepared. Prior to flash photolysis experiments and lipid extractions, the ghosts were given a final wash in 5 mM Hepes, 7 mM NaCl, pH 7.4. As control samples for mobility measurements, we used ghosts from erythrocytes which had been incubated either in the absence of lipid or in the presence of cholesterol–lecithin dispersions with a mole ratio of 0.8–1.0.

*Analytical Methods.* The amount of bound eosin was determined spectrophotometrically as described previously after first solubilizing ghosts with sodium dodecyl sulfate (Cherry et al., 1976a). Protein was determined by using the method of Lowry et al. (1951), phospholipid phosphorus was measured according to Chen et al. (1956), and cholesterol was determined as described by Courchaine et al. (1959).

*Characterization of Eosin-MA Binding.* Ghost membrane proteins were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis as described by Fairbanks et al. (1971). Gels were cast from a 5.6% acrylamide solution; bis(acrylamide) was 3.61% of the acrylamide monomer concentration. Cylindrical gels of 8.5-mm diameter were loaded with up to 800 μg of protein. Eosin fluorescence was photographed through an orange filter following excitation of the unstained gels with UV light. The fluorescent bands were marked with an ink-wetted needle, and the gel was subsequently stained with Coomassie blue. Eosin fluorescence was quantified from scans of the photographic negative by using an Integraph CH gel scanner. Selective extractions of proteins, glycoproteins, and lipids from ghosts were performed according to the following procedures: release of bands 1, 2 (spectrin), and 5 (actin) was achieved as described by Fairbanks et al. (1971); glycoproteins were extracted according to Hamaguchi & Cleve (1972); and lipids were extracted by the method of Rose & Oklander (1965). A functional characterization of the eosin binding to band 3 was achieved by measuring sulfate exchange under equilibrium conditions in intact erythrocytes as described previously (Nigg et al., 1979).

*Flash Photolysis Measurements.* The flash photolysis apparatus used in these experiments is described in detail elsewhere (Cherry, 1978). The application of the technique to investigate the rotational diffusion of band 3 has also been previously reported (Cherry et al., 1976b). Briefly, protein-bound eosin probes are excited at 540 nm by a linearly polarized laser pulse of duration 1–2 μs. Transient absorbance changes at time $t$ after the flash arising from ground-state depletion are simultaneously measured at 520 nm for light polarized parallel [$A_{\parallel}(t)$] and perpendicular [$A_{\perp}(t)$] with respect to the polarization of the exciting flash. Data are analyzed by calculating the anisotropy parameter $r(t)$, defined by the expression (Jablonsky, 1961)

$$r(t) = \frac{A_{\parallel}(t) - A_{\perp}(t)}{A_{\parallel}(t) + 2A_{\perp}(t)} \quad (1)$$

$r(t)$ is independent of the signal lifetime and depends only on rotational motion, provided the absorption transient exhibits a single exponential decay.

Table I: Triplet Lifetimes of Eosin Derivatives Bound to Band 3[a]

| | eosin-MA | | | eosin-NCS | | |
|---|---|---|---|---|---|---|
| temp (°C) | $\tau_1$ ($\mu$s) | $\tau_2$ ($\mu$s) | % $\tau_2$ | $\tau_1$ ($\mu$s) | $\tau_2$ ($\mu$s) | % $\tau_2$ |
| 18 | 2640 (91) | 343 (33) | 4.2 (0.1) | 1880 (32) | 323 (39) | 26.2 (1.0) |
| 25 | 2517 (71) | 246 (49) | 4.6 (1.9) | 1813 (9) | 321 (37) | 29.8 (3.8) |
| 30 | 2499 (76) | 275 (18) | 5.0 (0.6) | 1625 (9) | 290 (39) | 35.0 (1.1) |
| 37 | 2368 (66) | 192 (2) | 5.2 (0.1) | 1445 (15) | 257 (13) | 38.1 (4.2) |
| 45 | 2276 (52) | 186 (8) | 6.5 (0.8) | 1342 (7) | 209 (5) | 42.9 (1.1) |

[a] The absorption transient $s(t) = A_{\parallel}(t) + 2A_{\perp}(t)$ was calculated from the data. $s(t)$ was fitted to a double exponential decay having time constants $\tau_1$ and $\tau_2$. % $\tau_2$ represents the fractional contribution of the short-lived component. Each number is the average of two independent measurements; the numbers in parentheses denote 1 SD. Lifetimes are for deoxygenated samples (see Materials and Methods).

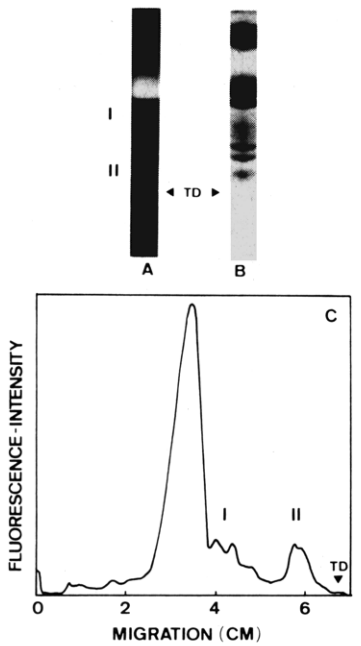


FIGURE 1: Fluorescence distribution in polyacrylamide gels of eosin-MA labeled membranes. Ghosts were labeled with 1.3 × $10^6$ molecules of eosin-MA/cell. (A) Eosin fluorescence in unstained gel loaded with 600 $\mu$g of protein; (B) Coomassie blue stained gel loaded with 300 $\mu$g of protein; (C) scan of the eosin fluorescence in gel of part A. TD denotes the position of the tracking dye.

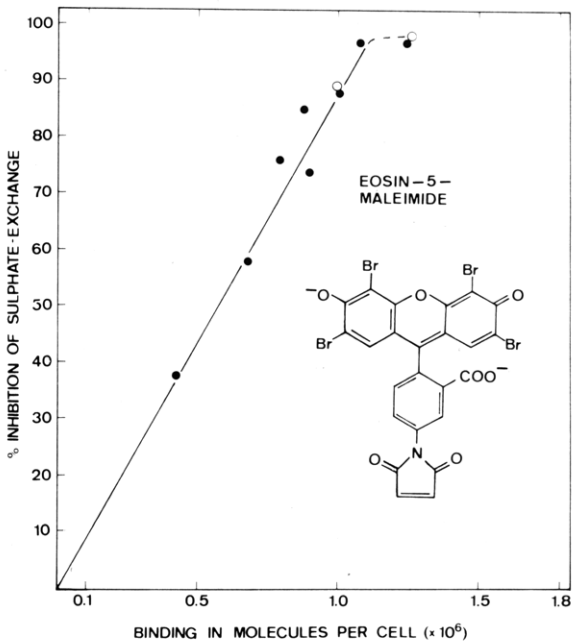


FIGURE 2: Inhibition of sulfate exchange in eosin-MA labeled erythrocytes. Sulfate equilibrium exchange was measured following tracer efflux from intact erythrocytes preloaded with radioactive sulfate as described previously (Nigg et al., 1979). Red cells were labeled with eosin-MA either at pH 7.4 (●) or at pH 6.9 (○). Bound eosin was determined spectrophotometrically in ghosts prepared from aliquots of the cells.

All results reported here were obtained by averaging 32 signals with a Datalab DL 102A signal averager. Data analysis was accomplished by a Hewlett-Packard HP 9825A desk-top computer interfaced to the signal averager. The computer was also used to fit the experimental $r(t)$ by exponential decays by using an iterative nonlinear least-squares program.

## Results and Analysis

*Binding of Eosin-MA to Band 3.* Flash photolysis measurements are typically performed on ghosts labeled with (1.0–1.3) × $10^6$ molecules of eosin-MA/cell. This corresponds to ~1 eosin molecule/band 3 monomer. The component(s) which bind eosin-MA were characterized by polyacrylamide gel electrophoresis by using eosin fluorescence for detection (Figure 1). In order to see possible minor components, we relatively heavily labeled the erythrocytes (1.3 × $10^6$ eosin molecules/cell) and the gels were deliberately overloaded. As estimated from scans of fluorescence intensity, 75–85% of the eosin fluorescence runs together with the characteristic zone of band 3, which shows a sharp leading edge and a trailing end. The faint fluorescent bands running ahead of band 3 (labeled I and II in Figure 1) do not correspond to major Coomassie blue positive bands in the gel. The components in region I which comprise 5–10% of the total fluorescence could be due to minor glycoproteins or proteolytic products of band 3. The remaining fluorescence found in region II may in part be lipid associated. However, most of it probably corresponds to split off or free eosin-MA since eosin-MA coelectrophoresed with unlabeled ghosts appears in the same region of the gel. Certainly, this fraction of the label is not protein-associated and therefore is not expected to contribute to dichroism observed in the microsecond–millisecond time range.

Using selective extraction procedures, we could confirm the above results. We found that less than 8% of the label was removed by low salt extraction of spectrin and actin, less than 5% was associated with glycoproteins, and only trace amounts with lipids. The binding of eosin-MA to band 3 is further reflected by its extremely powerful action as an inhibitor of the anion transport system associated with this protein. Figure 2 shows that sulfate equilibrium exchange in intact erythrocytes is virtually completely inhibited after binding of 1.1 × $10^6$ molecules of eosin-MA/cell. Thus, eosin-MA is as effective as the most powerful known inhibitors of the anion transport system in erythrocytes, the disulfonic acid stilbene inhibitors (Lepke et al., 1976; Ship et al., 1977).

*Triplet Lifetimes of Eosin Probes.* Some differences are detectable between results obtained with eosin-NCS and

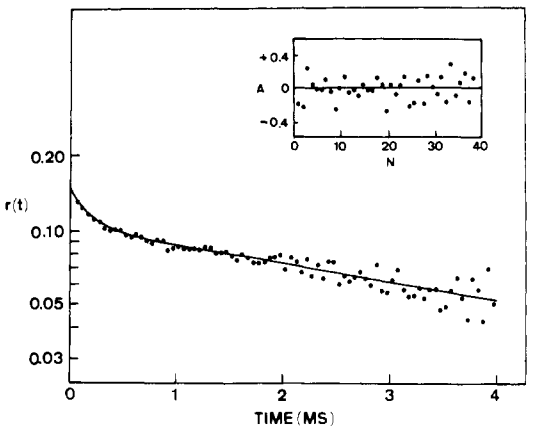


FIGURE 3: Time dependence of absorption anisotropy for eosin-MA labeled erythrocyte membranes at 37 °C. Flash-induced transient dichroism of the eosin probe was measured, and $r(t)$ was calculated as described in Materials and Methods. The solid line fitting the experimental points was obtained by fitting the data by eq 3. The constants describing this curve are $\alpha_1 = 159$ μs, $\alpha_2 = 5740$ μs, $B_1 = 0.0444$, and $B_2 = 0.1036$. The insert shows the corresponding autocorrelation function ($A$) (Grinvald & Steinberg, 1974). The measurement was made on ghosts suspended in 5 mM phosphate buffer, pH 7.4.

eosin-MA. For both probes we observed that when bound to band 3, the triplet state lifetime exhibits a double exponential decay (Table I). However, the short-lived component contributes only 5% in the case of eosin-MA, whereas for eosin-NCS it is ~40% at 37 °C. These multiple lifetimes suggest the existence of different binding sites. Even if all sites are on band 3, interpretation of $r(t)$ curves can in principal be complicated in this situation (Rigler & Ehrenberg, 1973). However, in the case of eosin-MA the decay is so close to monoexponential that such complications can safely be neglected. In addition, the time range of the flash photolysis measurements with eosin-MA is extended by the longer triplet lifetime of this probe.

*Comparison of Rotation Measurements with Eosin-NCS and Eosin-MA.* Some quantitative differences result from the analysis of the mobility data obtained when using the two probes. $r(t)$ curves recorded when using eosin-MA tend to exhibit a more pronounced decay compared to those obtained with eosin-NCS. Differences in selectivity toward band 3 as well as differences in orientation of the bound labels may account for the somewhat different numerical results. However, it is important to emphasize that the same qualitative information about band 3 mobility is obtained from flash photolysis measurements using either of the two probes to label erythrocytes. In the following sections we therefore include only the data obtained when using eosin-MA. More detailed measurements were made with this probe since it offers several advantages compared to the previously used eosin-NCS. These are a faster reactivity and a higher specificity for band 3 together with a longer triplet lifetime and nearly monoexponential decay in the protein-bound state.

*Interpretation of Flash Photolysis Data Obtained at 37 °C.* A typical result obtained from flash photolysis experiments with eosin-MA labeled erythrocyte membranes is shown in Figure 3. To interpret this result, we consider a model in which protein rotation only occurs about an axis normal to the plane of the membrane [see Cherry (1978) for a detailed discussion]. Such a model is reasonable since thermodynamic considerations indicate that the hydrophilic moiety of band 3 will prevent appreciable rotation about axes lying in the plane of the membrane. The model is further supported by chemical labeling and proteolysis experiments (Bretscher, 1973; Steck, 1974; Morrison et al., 1974; Shin & Carraway, 1974) and by the detection of protein-bound carbohydrate moieties exclusively on the outer surface of the cell (Tanner & Boxer, 1972; Steck & Dawson, 1974).

For rotation about the membrane normal, the time dependence of the absorption anisotropy $r(t)$ is given by eq 2,

$$r(t) = \frac{r_0}{A_1 + A_2 + A_3}[A_1 \exp(-D_{\|}t) + A_2 \exp(-4D_{\|}t) + A_3] \quad (2)$$

where $A_1 = (6/5)\sin^2\theta\cos^2\theta$, $A_2 = (3/10)\sin^4\theta$, $A_3 = (1/10)(3\cos^2\theta - 1)^2$, $D_{\|}$ is the rotational diffusion coefficient, $\theta$ is the angle between the transition dipole moment for absorption and the normal to the plane of the membrane, and $r_0$ is the anisotropy at zero time. It is assumed that the same transition dipole moment is used for excitation and measurement. In this case $r_0$ has the theoretical value of 0.4, but in practice is lower due to various factors. These may include instrumental effects, excitation of a nonnegligible fraction of molecules, and rapid but restricted independent motion of the eosin probe (Cherry & Schneider, 1976). Qualitatively the decaying part of $r(t)$ in eq 2 is due to randomization of the transition dipole moments by Brownian rotation about the membrane normal. The constant term $A_3$ (referred to as the residual anisotropy) arises because complete randomization is prevented by the inability of the protein to rotate about axes lying in the plane of the membrane. Of course $r(t)$ will eventually go to zero due to rotation of the whole erythrocyte ghost; the relaxation time of this process is, however, in the order of seconds.

Examination of the experimental $r(t)$ in Figure 3 shows that it is not a single exponential decay. However, the anisotropy does not appear to reach a time-independent value as predicted by eq 2. In fact, an excellent fit to the data is obtained with the double exponential equation

$$r(t) = B_1 \exp(-t/\alpha_1) + B_2 \exp(-t/\alpha_2) \quad (3)$$

where $B_1$, $B_2$, $\alpha_1$, and $\alpha_2$ are constants (see Figure 3). No improvement in fit is obtained by adding further terms.

A possible explanation of the above finding is that the model leading to eq 2 is incorrect. Either the slowly or rapidly decaying part of $r(t)$ might be due to rotation about axes lying in the plane of the membrane. This, however, would contradict the many investigations which strongly indicate that band 3 maintains a constant orientation with respect to the sidedness of the membrane (Bretscher, 1973; Singer, 1974; Steck & Dawson, 1974). Moreover, the validity of eq 2 for describing membrane protein rotation has been partially confirmed with model systems (Heyn et al., 1977) which also indicate that even a rocking motion (i.e., partial rotation about axes in the plane of the membrane) is minimal. A calculation based on the theory of Kinosita et al. (1977) indicates that band 3 rocking motion would have to be of large amplitude (total cone angle >70°) to account for either of the two components.

We therefore consider a second explanation far more probable, namely, that the results reflect the existence of at least two populations of band 3 proteins with different rotational mobilities. A sufficiently slow rotation of a fraction of band 3 would in fact explain two features of the data. Firstly, it would give rise to the slow decay in $r(t)$ observed at long times. Secondly, complete randomization about the normal axis would not be achieved during the time of the experiment, so that the predicted residual anisotropy would not be observed.

In previously reported measurements (Cherry et al., 1976b) the slowly decaying component was not distinguishable from

Table II: Analysis of Transient Absorption Anisotropy over 4 ms at 37 °C[a]

| residual anisotropy (%) | coefficients (%) | | time constants (ms) | | |
|---|---|---|---|---|---|
| | $B_1$ | $B_2$ | $\alpha_1$ | $\alpha_2$ | $\alpha_2/\alpha_1$ |
| 0 | 26.8 (3.3) | 73.2 (3.3) | 0.17 (0.04) | 6.46 (0.90) | 38 |
| 25 | 23.6 (3.2) | 51.4 (3.2) | 0.15 (0.04) | 3.38 (0.58) | 23 |

[a] Five independent sets of data were analyzed by assuming the residual anisotropy to be either 0 (eq 3) or 25% as described in the text. Numbers in parentheses denote 1 SD.

a constant anisotropy. This distinction is possible in the present experiments because of greater accuracy arising from the use of signal averaging.

To make a quantitative analysis of our experiments, we have adopted the following procedure. Firstly we fit the data by eq 3; i.e., we assume that the residual anisotropy is zero. This is a possible value since $A_3 = 0$ when $\theta \simeq 55°$. We then make a further fit by assuming that there is a residual anisotropy of 25% (of $r_0$). Measurements at 45 °C, discussed in the following section, show that this number is an upper limit. In this way we can show that the conclusions which we draw from the data are independent of the precise value of the residual anisotropy. Table II shows the results of this procedure when applied to data obtained at 37 °C over a time range of 4 ms. It can be seen that irrespective of the choice of residual anisotropy, the ratio of the time constants $\alpha_2/\alpha_1$ is very much greater than the factor 4 predicted by eq 2. This is inconsistent with a single rotating species but compatible with multiple components. Since there may be any number of components, each of which contributes two exponential terms, the numbers $\alpha_1$ and $\alpha_2$ do not have precise physical significance. At best they may be regarded as approximate average relaxation times for respectively rapidly and slowly rotating species of band 3 in the membrane. The procedure used previously to obtain a single diffusion coefficient (Nigg & Cherry, 1979) results in an averaging of all components, both fast and slow.

*Effects of Glutaraldehyde and Glycerol.* As a control, ghosts were fixed for 30 min in 1% glutaraldehyde at 22 °C. This procedure abolishes the decay of the anisotropy consistent with immobilization of band 3. $NaDodSO_4$ gel electrophoresis demonstrates that all of the membrane proteins are cross-linked into high molecular weight aggregates by the fixation. In a further experiment, no effect on rotation of band 3 was observed when the medium contained 70% glycerol, demonstrating that band 3 rotation is independent of the viscosity of the aqueous phase.

The above findings are relevant to a possible complication in the interpretation of the data, namely, that part of the decay in $r(t)$ could be due to independent segmental motion of a portion of the band 3 protein. The absence of any influence of the aqueous viscosity on rotational motion rules out the involvement of independent motion of the hydrophilic moiety of band 3. Immobilization by glutaraldehyde cross-linking suggests, though does not prove, that the measurements relate to the rotation of the whole protein. This is further reinforced by the previous observation that noncovalent aggregation of band 3 also abolishes rotation (Cherry et al., 1976b). Finally, the observed decay times of the anisotropy are longer than 100 μs, corresponding to Brownian rotation of a large particle in a viscous medium. We therefore consider it very unlikely that independent segmental motion contributes to the observed rotation.

*Temperature Dependence of Band 3 Rotational Mobility.* Figure 4 shows the time dependence of the absorption anisotropy over the temperature range from 1 to 45 °C. The

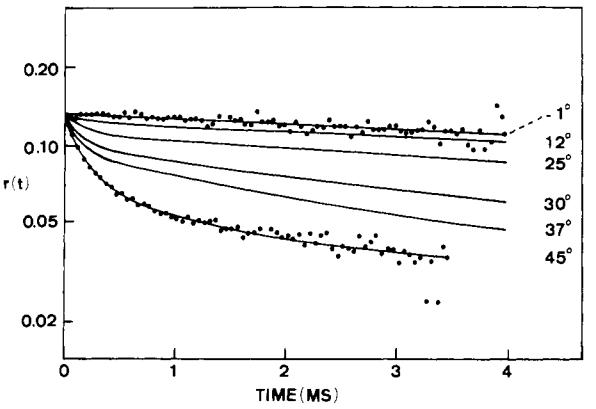


FIGURE 4: Temperature dependence of transient absorption anisotropy. $r(t)$ curves for ghosts in 5 mM phosphate buffer, pH 7.4, were measured at different temperatures. The solid line fitting the experimental points at 1 °C was obtained from a single exponential equation. For all other temperatures the data were fitted by a double exponential equation by assuming the residual anisotropy to be 25% as described in the text. For the purpose of clarity, experimental points are omitted at intermediate temperatures. The curves have been normalized to the same initial anisotropy. The variation in initial anisotropy (±10%) is within the range produced by instrumental factors.

curves become flatter on lowering the temperature, although even at 1 °C a slight decay is still observed.

A possible explanation of this effect is that it is due to a temperature-dependent change in $\theta$ (eq 2). The virtual lack of decay observed at 1 °C might then be due to alignment of the transition dipole moment along the rotation axis. This, however, seems to us to be a highly unlikely interpretation since it requires not only a rather dramatic conformational change of the protein resulting in an alteration of $\theta$ by about 35° but also that this change should accidently produce the particular orientation of $\theta \simeq 0°$. Moreover, this would have to be true for each of two eosin probes with chemically different reactive groups since qualitatively similar results are obtained with both eosin-NCS and eosin-MA. We therefore conclude that the temperature effect is almost certainly due to changes in band 3 mobility. This interpretation is supported by data of Fowler & Branton (1977), who observed a strong temperature dependence for the lateral diffusion of intrinsic proteins in the erythrocyte membrane.

As already discussed, $r(t)$ at 37 °C does not reach the constant residual anisotropy predicted by eq 2. Figure 4 shows that this is true for lower temperatures too. Above 37 °C, the high noise at times longer than 3.5 ms (due to decreasing anisotropy and signal lifetime) makes it impossible to ascertain whether a constant level has been reached. To provide a quantitative analysis of the temperature effect, we pursue the argument that the failure to attain a residual anisotropy is due to a slowly rotating fraction of band 3. We follow the data-fitting procedure described above and again assume upper and lower limits for the residual anisotropy; the results are summarized in Figure 5.

The data in Figure 5 were obtained by analyzing 2-ms scans in order to obtain more data points at short times. This is justified because the time constants of the two components are such that both can be obtained from data collected over 2 ms. In fact, analysis of 4-ms scans gives essentially similar results to those presented in Figure 5 (compare Figure 5 and Table II).

The analysis reveals several interesting features. Considering first the coefficients $B_1$ and $B_2$, the results indicate that the equilibrium between fast and slowly rotating species is temperature dependent (Figure 5A,B). As the temperature

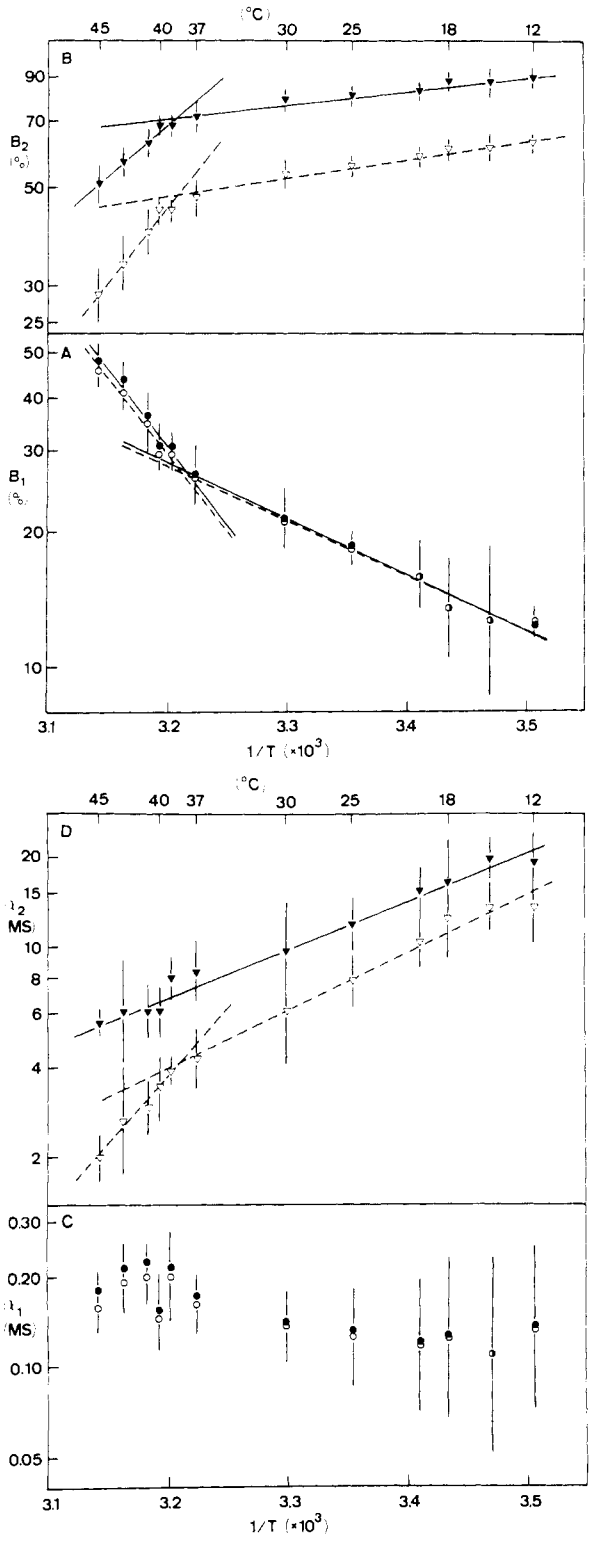


FIGURE 5: Constants describing the temperature dependence of transient absorption anisotropy. Parts A and B show the temperature dependence of the coefficients $B_1$ and $B_2$ and parts C and D of the time constants $\alpha_1$ and $\alpha_2$. Each point is the average of 2–10 independent measurements: (filled symbols and solid lines) results obtained by fitting the experimental data by eq 3, i.e., by assuming that the residual anisotropy is equal to zero; (open symbols and dashed lines) results obtained by assuming the residual anisotropy to be 25%. The lines represent linear regressions through points below and above 37 °C, respectively. The solid line in part D is a single linear regression over the whole temperature range.

is decreased, the contribution from slowly rotating species increases. Moreover, we observe a marked change of slope at ~37–40 °C. The equilibrium between the different species shifts more rapidly with temperature above this point than below it.

The time constant $\alpha_1$ for the fast component (Figure 5C) appears to be relatively insensitive to temperature. (The values at lower temperatures are rather inaccurate because the fast component is less than 20%.) In contrast, $\alpha_2$, the time constant characterizing the slow component, is strongly temperature dependent, decreasing by at least a factor of 5 between 12 and 45 °C (Figure 5D).

It should be emphasized that the particular choice of residual anisotropy does not qualitatively affect the above conclusions and has little quantitative effect on the parameters of the fast component. As would be expected, the choice of the residual anisotropy does considerably affect the absolute values of $B_2$ and $\alpha_2$. The temperature dependence of $\alpha_2$ exhibits a change in slope at 37–40 °C when the residual anisotropy is 25%, correlating with that seen for the coefficients $B_1$ and $B_2$. This is no longer observable when the residual anisotropy is assumed to be zero.

The procedure we have presented for analyzing the data, though not unique, is probably the simplest. To show that the conclusions are not simply a property of the curve-fitting, we have used alternative analyses. For example, the residual anisotropy may be allowed to vary with temperature, corresponding to a varying contribution from an "immobile" component. In all cases we assume that eq 2 correctly describes the rotation of any single species of band 3 in the membrane. With this assumption, all analyses lead essentially to the same conclusion, namely, that there is a temperature-dependent equilibrium in the membrane between rapidly rotating species and slowly rotating forms of band 3. The slow component may also include a fraction which is "immobile" on the time scale of the experiment.

The temperature effect on band 3 mobility is essentially reversible in both directions, indicating that no irreversible structural changes occur in the protein over the temperature range investigated. The same temperature dependence of band 3 mobility is observed after low-salt extraction of spectrin and actin from the cytoplasmic surface of the membrane. The bulk of these extrinsic proteins cannot therefore be responsible for the reduced mobility of band 3 at low temperatures.

*Influence of Cholesterol on Band 3 Mobility.* The cholesterol/phospholipid mole ratio ($C/P$) in normal erythrocyte membranes is ~0.9. By incubating red cells with cholesterol–lecithin dispersions of varying composition, we have varied the cholesterol/phospholipid mole ratio in the membrane from 0.34 to 1.66. We then measured the rotational diffusion of band 3 as a function of the cholesterol content in the ghost membranes. A representative set of data is shown in Figure 6, from which it may be seen that very little alteration in the shape of $r(t)$ is produced either by depletion of membrane cholesterol down to 38% of the normal value or by incorporation of exogenous cholesterol up to 185%. This is the case at both 15 and 37 °C, indicating that variations in cholesterol content have little influence on the temperature dependence of band 3 rotation over the range of 15–37 °C.

We analyzed the data as before, choosing 25% for the residual anisotropy. The results of this analysis for a range of different $C/P$ values at 37 °C are shown in Figure 7. The straight lines are linear regression curves through the experimental points. They show that both coefficients $B_1$ and $B_2$ and time constants $\alpha_1$ and $\alpha_2$ vary by less than 10% over the $C/P$ range investigated, and it is doubtful whether these minor changes are significant. Analysis with zero residual anisotropy gives exactly the same conclusions. Thus, the

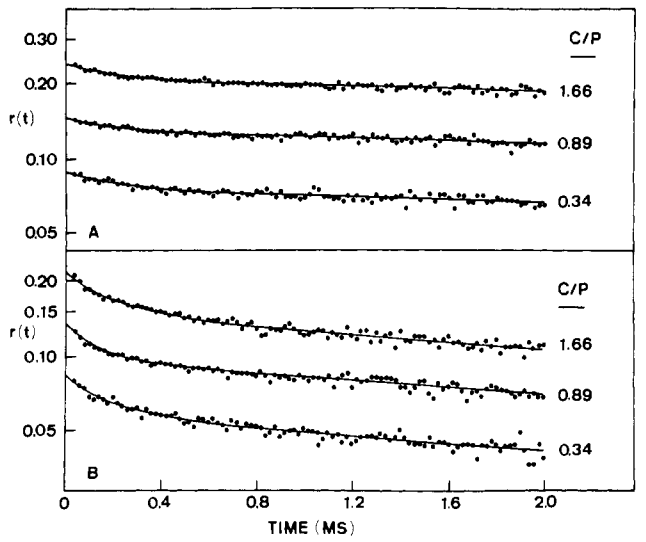


FIGURE 6: $r(t)$ at 15 and 37 °C in ghosts with low and high cholesterol. The cholesterol/phospholipid mole ratio ($C/P$) in ghost membranes was varied as described in Materials and Methods. $r(t)$ curves for ghosts in 5 mM Hepes and 7 mM NaCl, pH 7.4, were measured at 15 (A) and 37 °C (B). The lines fitting the experimental points were obtained as described in the text, with the residual anisotropy equal to 25%. For illustrative purposes the three curves in each panel have been artificially separated by vertical displacements of upper and lower curves by 20%. Otherwise the data almost exactly superimpose.

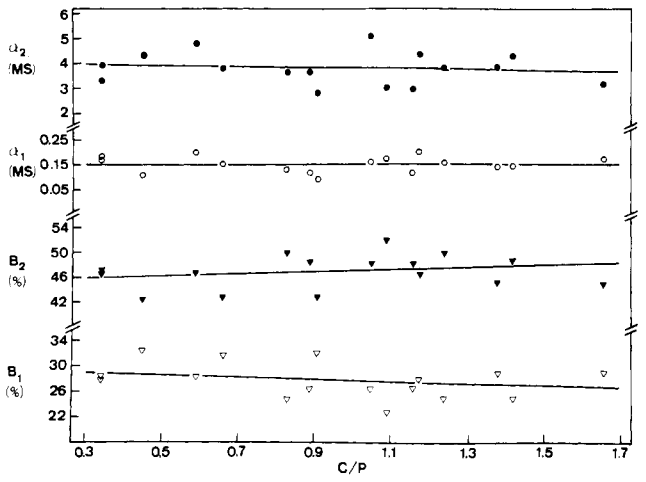


FIGURE 7: Dependence of band 3 mobility at 37 °C on membrane cholesterol content. The constants $B_1$, $B_2$, $\alpha_1$, and $\alpha_2$ were obtained as in Figure 5, taking the residual anisotropy equal to 25%. The solid lines are linear regressions through the calculated constants. Ghosts were in 5 mM Hepes and 7 mM NaCl, pH 7.4.

rotational diffusion of band 3 is remarkably independent of membrane cholesterol over the $C/P$ range investigated.

## Discussion

*Temperature Effects.* In this study we demonstrate that the decay of absorption anisotropy of eosin-labeled band 3 proteins in the human erythrocyte membrane is strongly dependent on temperature. We conclude that over the range of 12–45 °C at least two populations of band 3 proteins may be distinguished by their different mobilities. These populations exist in a temperature-dependent equilibrium in the membrane, the slowly rotating component being dominant at low temperatures. We emphasize that this conclusion is independent of any particular curve-fitting procedure used to analyze the experimental data. The underlying assumption is that we are observing rotation of the whole band 3 protein which occurs only about an axis normal to the plane of the membrane. We have already argued against alternative models involving rotation about axes in the plane of the membrane or independent segmental motion of band 3.

We now go on to consider the possible structural implications of different populations of band 3 in the membrane. In general terms, the existence of a slowly rotating fraction of band 3 implies that the mobility of this fraction is restricted, either by protein–protein interactions or by a lipid phase of high viscosity. We propose that the following models could explain the temperature-dependent equilibrium between populations of band 3 proteins with different mobilities: (I) temperature-dependent association of band 3 with other membrane proteins; (II) distribution of band 3 between lipid domains of different fluidity; (III) temperature-dependent self-aggregation of band 3. Possibilities I and III may be mediated either by a protein conformational change or by changes in the physical state of lipids surrounding band 3.

Model I seems less likely since extraction of most of the peripheral proteins, spectrin and actin, does not abolish the mobility restriction exerted on band 3 at low temperatures. The major sialoglycoprotein also is unlikely to be involved since treatment of intact cells with trypsin or neuraminidase has no effect on band 3 rotation (E. Nigg and R. J. Cherry, unpublished experiments). Any quantitatively minor component of the membrane could hardly provide a sufficient anchor to strongly restrict rotation of the bulk of band 3.

Model II visualizes that band 3 proteins are distributed among lipid domains with different fluidities. In this case, regions of extremely high viscosity, probably corresponding to a gel phase, would have to be envisaged to explain the slow component. Below 20 °C most of band 3 would have to exist in such gel-phase regions. However, X-ray studies indicate that there is negligible gel-phase lipid present in the erythrocyte membrane above 0 °C (Gottlieb & Eanes, 1974). Moreover, a variety of studies show that intrinsic proteins are excluded from crystalline lipid domains (Verkleij et al., 1972; Hong & Hubbell, 1973; Kleemann & McConnell, 1974, 1976; Wunderlich et al., 1974; Höchli & Hackenbrock, 1976; Chapman et al., 1977; Cherry et al., 1978). We thus consider that model II is unlikely to be the correct explanation of the data.

Model III, involving self-aggregation of band 3 dimers, appears to us to be the most attractive of the three possibilities. The temperature dependence of the time constant $\alpha_2$ is easily explained by this model since it would simply reflect the formation of increasingly large aggregates with decreasing temperature. A possible difficulty is that, to our knowledge, no temperature-dependent aggregation of the band 3 associated intramembrane particles has been reported for the human erythrocyte membrane. However, the ratio of fast and slow time constants is such that aggregates of only 5–10 particles in diameter are required to explain the observed results. It is an open question to what extent such "microaggregation" of erythrocyte membrane proteins can be detected by freeze-fracture electron microscopy since the high density of intramembrane particles makes a quantitative analysis extremely difficult.

The factors which could cause temperature-dependent aggregation of band 3 are largely a matter of speculation at the present time. Conceivably a conformational change of band 3 could be involved. The existence of such a conformational change is suggested by the temperature dependence of anion transport (Brahm, 1977), although there is no direct evidence.

Alternatively, aggregation of band 3 could be mediated by changes in the physical state of membrane lipids. Studies using

differential thermal calorimetry (Ladbrooke & Chapman, 1969) and X-ray diffraction (Gottlieb & Eanes, 1974) failed to detect any phase transition below 40 °C in the erythrocyte membrane. This is hardly surprising in view of the high cholesterol content and the heterogeneous lipid composition of the membrane. However, lipid phase segregation occurring over a broad temperature range and/or involving a small fraction of the lipids is not ruled out. In fact, several groups using different techniques such as Raman spectroscopy (Verma & Wallach, 1976a,b), viscosity measurements (Zimmer & Schirmer, 1974), and $^{31}P$ NMR (Cullis, 1976; Cullis & Grathwohl, 1977) have collected evidence in support of a possible transition (which may be the beginning or end point of a phase segregation) in the temperature range 15–20 °C. Gottlieb & Eanes (1974) observed a phase segregation of extracted membrane lipids over the range 2–20 °C, but only after removing virtually all of the cholesterol. Verma & Wallach (1976a), using Raman spectroscopy, have also reported a transition at ~38 °C, which appeared to involve apolar amino acid side chains of membrane proteins. The transition was irreversible above 42 °C and exhibited a strong pH dependence. A very similar transition was also observed by Bieri & Wallach (1975) by using paramagnetic quenching of protein fluorescence.

The situation concerning lipid phase segregation in the erythrocyte membrane is thus far from clear. In the present studies there is no well-defined low-temperature end point for the distribution of band 3 between fast and slowly rotating components. The temperature dependence of this distribution does, however, show a marked change of slope around 37–40 °C. It may well be that different techniques, including our own, monitor different features common to a very broad phase segregation for which an exact definition of starting and end points is very difficult.

The temperature effects we observe could be relevant to the very large activation energy of the erythrocyte anion transport system, which is in the range 26–37 kcal/mol (Lepke & Passow, 1971; Dalmark & Wieth, 1972; Chow et al., 1976; Brahm, 1977). Ross & McConnell (1978) found that this activation energy is not observed when band 3 is reconstituted into liposomes consisting principally of egg phosphatidylcholine. They suggest that in the erythrocyte there may be an equilibrium between active and inactive forms of band 3 which is sensitive to temperature-dependent lipid fluidity. The present observation that there is a temperature-dependent equilibrium between states of band 3 with different mobilities is clearly relevant to this proposal. Furthermore, Thomas & Hidalgo (1978) have recently drawn attention to possible correlations between protein mobility and function. Thus, the less mobile form of band 3 may have reduced anion transport activity. This does not of course imply that transport occurs by a rotational mechanism but simply that the same factors which restrict protein rotation may also restrict intramolecular motions which are essential for transport.

As already pointed out, the time constants characterizing the fast and slow components cannot be directly related to diffusion coefficients. It may, however, be remarked that the time constant of the fast component, which is about 100–200 $\mu$s, is of reasonable order of magnitude for the relaxation time of the band 3 dimer. This may be seen by comparison with rhodopsin, which has a relaxation time of 20 $\mu$s at 20 °C (Cone, 1972). Slower rotation is expected for band 3 dimers because of their considerably greater size (the relaxation time is proportional to the square of the cross-sectional diameter in the plane of the membrane).

*Effects of Cholesterol.* To further investigate the influence of membrane lipids on band 3 mobility, we varied the membane cholesterol content in vitro from 38 to 185% of the normal value. Interestingly, we found no significant effect on band 3 rotation. Neither the distribution nor the time constants of the fast and slow moving components were appreciably dependent on cholesterol content in the range investigated. This finding is surprising at first sight since many reports have demonstrated the influence of cholesterol on membrane fluidity both in the erythrocyte membrane (Vanderkooi et al., 1974; Cooper et al., 1978) and in other systems (Oldfield & Chapman, 1972). A possible explanation is that band 3 occurs in lipid domains from which cholesterol is preferentially excluded. Other authors have previously proposed that cholesterol is excluded from the immediate environment of band 3 (Bieri & Wallach, 1975; Cullis, 1976), although a monolayer study suggested a preferential interaction between these components (Klappauf & Schubert, 1977).

An alternative explanation is that changes in lipid fluidity following alterations in the membrane cholesterol level do not strongly influence the mobility of band 3 proteins. It should be remembered that "fluidity" is usually measured by detecting the molecular motions of the lipid hydrocarbon chains, either directly by using techniques such as NMR or indirectly by the use of small lipophilic probe molecules. The exact relationship between such molecular motions and diffusion is not at all clear. It is of interest to note that in myotube plasma membranes both lipid and protein lateral diffusions are rather insensitive to alterations in the lipid fatty acid composition (Axelrod et al., 1978).

The possibility that the effect of cholesterol on diffusion may be less marked than its effect on lipid chain motion is suggested by previous measurements of lipid lateral diffusion. It was found that lipid diffusion coefficients in egg phosphatidylcholine bilayers change by only a factor of 2 over the $C/P$ range of 0–1 (Fahey et al., 1977; Wu et al., 1977). In the present experiments we were not able to deplete erythrocyte membranes completely of cholesterol, the lowest $C/P$ ratio being 0.34. Recent deuterium NMR studies (Haberkorn et al., 1977) show that quadrupole splittings of deuterated dipalmitoylphosphatidylcholine change relatively little with cholesterol content above this ratio. Deuterium quadrupole splittings are a measure of order, which, as emphasized by Seelig & Seelig (1974), cannot necessarily be identified with fluidity. However, Kawato et al. (1977), using time-resolved fluorescence probe techniques, have deduced that, above the lipid phase transition, the effects of cholesterol are principally on the amplitude of lipid chain reorientation, while the rate is almost unaffected.

The above considerations suggest that variation in cholesterol content above $C/P$ ratios of 0.34 could conceivably have little effect on band 3 rotation. Thus, it is not necessary to conclude from the results that band 3 occurs in cholesterol-free regions of the membrane. Whatever the true explanation, the findings are clearly relevant to the proposed role of membrane "fluidity" in a variety of physiologically important phenomena. In particular, our data demonstrate that it may be dangerous to conclude a significantly altered mobility of transmembrane proteins based on "fluidity" changes reported by small lipophilic probe molecules.

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