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Transbilayer asymmetry of pyrene mobility in human spherocytic red cell membranes

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The diffusion-dependent formation of pyrene excimers (excited dimers) was studied in normal and spherocytic red cell membranes. Pyrene emission was alternatively quenched in either bilayer half by non radiative energy transfer to haemoglobin. Pyrene excimer to monomer fluorescence intensity ratio, I'/I, was 0.35 ± 0.03 (S.E.) in washed red blood cells obtained from normal donors ($n = 8$) and 0.45 ± 0.03 ($n = 13$) in the corresponding isolated, haemoglobin-free resealed membranes ($P < 0.02$). In the spherocytic condition the respective values were 0.28 ± 0.01 ($n = 9$) and 0.53 ± 0.03 ($n = 9$), $P < 0.001$. In contrast to the decrease of I'/I in red cells as compared to isolated membranes, being 22% in normal cells and 47% in spherocytic ones, haemoglobin added to the exofacial side of isolated membranes, respectively, reduced I'/I by 18% and 5%. In normal red cell membranes, pyrene mobility appears to be higher in the inner monolayer than in the outer one. In spherocytic membranes our results indicate an enhanced transmembrane asymmetry in lipid monolayer fluidity, probably due to a defect of the membrane protein skeleton organization.

Introduction

In human erythrocytes [1–3], and presumably also in other cells [3–5], the inner and the outer halves of the plasma membrane differ in their protein and lipid composition. Membrane transverse asymmetry also refers to mobility of membrane components [6–10].

Lipid matrix fluidity, a physiologically most prominent feature of biological membranes combines different types of motional freedom whithin the lipid matrix. The enhanced mobility of fatty acid chain ends, as compared to that of phospholipid headgroups, implicates a maximum of fluidity at the midplane of the bilayer [11]. Rotational mobility of membrane components, on the other hand, does not necessarily correlate with their lateral diffusion [12].

Membrane lipid content and distribution appear to be controlled by proteins, particularly aminophospholipid translocases [13] and by the protein skeleton beneath the red blood cell membrane (RBC) [14–16]. In normal RBC membranes depleted of spectrin and protein 4.1, an increase in transmembrane phospholipid movement has been described [14,17].

Genetically defective interactions between components of the membrane protein skeleton [18,19] and between the latter and the lipid bilayer [20] seem to constitute the molecular basis of hereditary spherocytosis (HS). The mobile fraction of labelled phosphatidylethanolamine (PE) localized in the internal monolayer was found by Rimon et al. [6] to be significantly diminished at low temperature in HS as compared to normal membranes. This was attributed to the abscence of a stabilizing effect of the membrane protein skeleton against structural rearrangement. In its natural, nonfluorescent form, PE is mostly constrained

Abbreviations: RBC, red blood cells; HS, hereditary spherocytosis; Py, pyrene; I', Py excimer (excited dimer) fluorescence intensity maximum; I, Py monomer fluorescence intensity maximum; Hb, haemoglobin.

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to the inner monolayer of the red cell membrane [3]. The localization of exogenously applied labelled PE can, however, not fully be defined under the conditions used by Rimon et al. [6].

On the other hand, Williamson et al. [15] reported external monolayer to be less fluid than the internal one, both in the normal and in the spherocytic RBC as long as humans are considered. Whether corresponding fluidity differences between both leaflets are more distinct in one or the other case cannot be resolved by the method used by these authors [15].

Lateral diffusion of fluorescent lipid analogues, as measured by photobleaching recovery in the membrane of intact RBC does not appear to be perturbed by spectrin depletion [21]. Assessment of lateral mobility by fluorescence photobleaching recovery, however, considers diffusion lengths in the order of μm [22]. Depending on the diffusion lengths considered, lateral mobility within the lipid matrix may be obstructed by integral proteins [22]. In a shorter range (nm), lipid matrix fluidity can be measured on the basis of diffusion-limited excimer formation [22–24]. Intramembrane fluidity distribution was correspondingly studied, in the present work on the basis of the diffusion dependent formation of excimers by pyrene (Py). This small hydrophobic probe distributes rather uniformly in the membrane lipid matrix [23]. Non radiative energy transfer from Py to heme acceptors localized on either side of the membrane evidenced that in HS membranes, as compared to normal ones, the fluidity difference between the inner and the outer monolayer is enhanced.

This work has been presented to a joint meeting of the Deutsche Physiologische Gesellschaft and the Physiological Society [25].

Materials and Methods

Erythrocyte membranes. Venous blood samples were obtained from healthy donors and splenectomized HS patients. RBC were separated from the heparinized samples by usual centrifugation and washing procedures. Isolated resealed RBC membranes and isolated unsealed RBC membranes were, respectively, prepared according to Bjerrum [26] and to Dodge [27].

Cholesterol and phospholipid content of RBC membranes was determined by spectrophotometric techniques [27,29]. Cholesterol/phospholipid ratio was 0.997 ± 0.117 ($n = 4$) and 0.983 ± 0.102 ($n = 7$) for HS and normal RBC, respectively. Moreover, no difference was found between the phospholipid pattern of normal and HS RBC as studied by two-dimension thin-layer chromatography. The percentage of fatty acid methyl ester as determined by gas chromatography, was also similar in both cases (unpublished results).

Probe incorporation. Py obtained from Sigma was purified by sublimation and dissolved in ethanol (10^{-3} mol/l), before being added to RBC (hematocrit 3%) or RBC membranes (protein concentration 0.25 mg/ml) suspended in phoshate buffered saline. Final Py and ethanol concentrations were respectively 10^{-5} mol/l and 1% (v/v). In paired experiments RBC and RBC membranes suspensions were concomitantly incubated with the probe for 18 h (4°C), while being gently shaken. Stirring was avoided. The haemolysis occurring during the incubation, expressed as per cent of total haemoglobin, was 0.1% and 3.0% for normal and HS RBC, respectively. Py microaggregates were removed either by osmotically coupled sedimentation [30] or by double centrifugation. All chemicals were of spectroscopic or analytical grade quality.

Fluorescence measurements. Py molecules in excited state can react, within their life-time, with the same probe molecules present in the ground state, thus, leading to diffusion-controlled excited dimer (excimer) formation. Excimers exhibit an unstructured fluorescence band shifted to the red with respect to the monomer band. Diffusion controlled rate of Py excimer formation, as measured through the excimer to monomer fluorescence intensity ratio I'/I, expresses translational mobility of the probe in a given medium [22–24,31,32]. Fluorescence spectra were recorded on a Spex Fluorolog spectrofluorometer with front face excitation capability or on an I.S.S. GREG 200, spectrofluorometer both provided with photon counting electronics and thermostated sample holder. All spectra were recorded at 25°C. Excitation wavelength was 335 nm. Fluorescence intensities were evaluated at 375 nm for I and at 475 nm for I'. Values were corrected for contribution of monomer emission at excimer maximum and excimer emission at monomer maximum. Control suspensions without probe were used to correct background light scattering. Absorption spectra were recorded on a Cary Varian 219 spectrophotometer.

Energy transfer experiments. Py fluorescence may be quenched, due to the presence of Hb, by trivial energy transfer or by resonance energy transfer. For examining the influence of trivial energy transfer on the I'/I ratio Py spectra of resealed RBC membranes were recorded in the presence of RBC separately intercalated in the emission path, a double-compartment cuvette being used for this purpose. Py fluorescence emission of these membranes (excited at 90°) was thus measured after passing through a suspension of unlabelled RBC.

Resonance energy transfer from Py within lipid matrix to haemoglobin (Hb) localized at the inner side of the membrane was examined by relating Py fluorescence intensity of RBC to that of isolated RBC membranes. Considering the Förster distance (R_0) for the

Py-Hb donor-acceptor system being about 4.3 nm [22], it is possible to assume that resonance energy transfer from Py in the lipid matrix to cytosolic Hb will preferently quenche the fluorescence originating from the probe localized within the inner half of the bilayer. Correspondingly, Py fluorescence originating in the outer half of the membrane should preferently be quenched by resonance energy transfer when Hb is added to a suspension of Py-labelled isolated resealed RBC membranes. Addition of Hb to Py-labelled unsealed membranes, on the other hand, provides a model for quenching Py fluorescence from both sides of the membrane. Control experiments ($n = 2$) were performed for studying the difference in the interaction of Hb with sealed and unsealed membranes during these measurements. When membranes were exposed to Hb (3.5 mg/ml in saline buffer) at 25°C for 30 min, the increment in the membrane Hb content expressed as Hb/membrane proteins (w/w), was 1.5- and 20.7-times in sealed and unsealed membranes, respectively. 'Wash-out' curves of Hb of these membranes were followed for 60 minutes. Hb concentration in the corresponding supernatant decreased in the case of unsealed RBC membranes and remained constant in that of resealed RBC membranes.

Estimation of energy transfer efficiency. Non radiative, resonance energy transfer is dependent on the distance between donors and acceptors as well as acceptor concentration for a given donor concentration. Defining k as the donor decay rate in the absence of acceptors and R_0, the Förster distance, as the separation at which the rate of resonance energy transfer equals the donor decay rate k, the inverse sixth power dependence of the energy transfer rate, k_T, on the donor acceptor separation R can be expressed as

$$k_T = k(R_0/R)^6 \quad (1)$$

while the efficiency of transfer is defined by

$$T = k_T/(k_T + k) \quad (2)$$

Estimation of the efficiency of the energy transfer from Py to Hb, in terms of distance and concentration of acceptors, was done using a semi-empirical model proposed by Eisinger and Flores [33]. The acceptors heme groups are assumed to be uniformly and continuosly distributed in a semi infinite continuum space beyond the membrane interface. Donor-acceptor and donor-interface distances R and d, respectively, are expressed in terms of R_0, $r = R/R_0$ and $x = d/R_0$, and acceptor density ρ as the number of heme in a volume of magnitude R_0^3. The analytical expression for resonance energy transfer obtained with this model is

$$T = \pi\rho/(\pi\rho + 6x^3) \quad (3)$$

which is an expression of the dependence of the efficiency, T, on the dimensionless acceptor concentration ρ and x, the separation between donors and the boundary plane of the acceptors continuum. The fraction of light emitted by Py embedded in each leaflet of the bilayer was estimated from the integral of $(1 - T)$ evaluated between the limits of x of each leaflet (0 to 0.5 for the inner leaflet and 0.5 to 1.0 for the outer one) and normalized to the total emission of the whole bilayer as unity. In this evaluation the value of 4.3 nm was used for of R_0 [22]. For intact RBC a value of 0.98 was used for ρ, considering the cytosol Hb concentration being 34 g/dl. When Hb was added to a suspension of Py labelled membranes, at 0.21 g/dl, the corresponding ρ value of 0.006 was used. For statistical evaluation the Kruskal-Wallis ranking sum test was used.

Results

At a comparable amount of membranes, Py fluorescence of RBC appears to be quenched with respect to that of resealed RBC (Fig. 1) and unsealed membranes. Py monomer fluorescence band overlaps with the Soret absorption band of Hb heme group. Py fluorescence in RBC may, thus, be quenched both by long range resonance energy transfer from lipid matrix Py to cytosolic heme group and also by trivial energy transfer. Fig. 2 shows Py fluorescence of isolated resealed RBC membranes when unlabelled RBC were separately intercalated in the optical path, a condition where only trivial energy transfer can occur. Note the increase of I'/I under this condition. In RBC, on the contrary, I'/I appears to be lower than in isolated

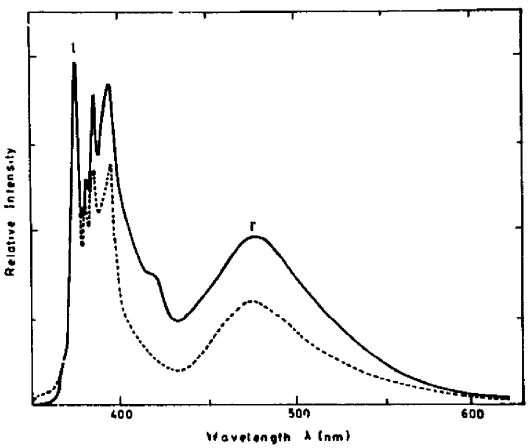

Fig. 1. Fluorescence spectrum, with front face excitation, of Py in IRM (———), and RBC (- - - -). The latter curve is shown amplified (23.4×). I correspond to monomer and I' to excimer maxima.

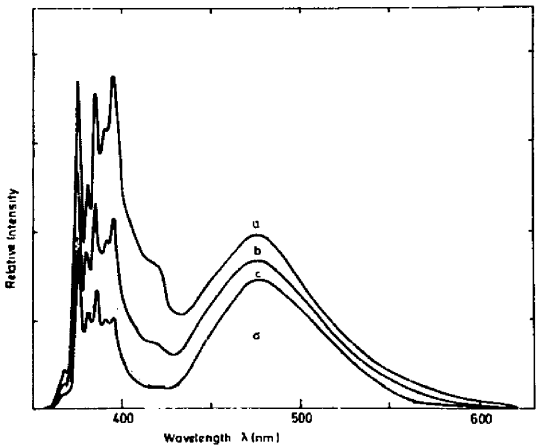

Fig. 2. Fluorescence spectrum, with right angle excitation, of Py in IRM (———). Effect of Hb (RBC) when only trivial energy transfer is possible. Initial conditions (a) and the effect of separately intercalating an increasing amount of RBC in the optical path (b and c). For comparison the spectrum, with front face excitation, of Py in RBC is included (d). (- - - - - -).

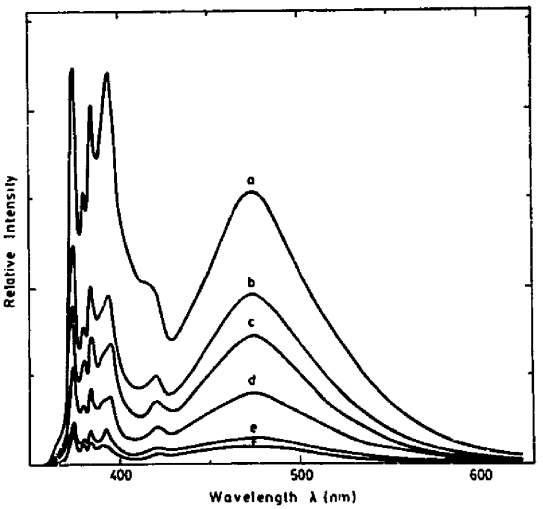

Fig. 3. Fluorescence spectrum, with front face excitation of Py in IRM. Effect of Hb when also resonance energy transfer can occur. Initial condition (a) and Hb (mg/ml) addition (b = 0.19; c = 0.32; d = 0.72; e = 1.44; f = 2.11).

TABLE I

Excimer to monomer fluorescence intensity ratios of pyrene in normal and hereditary spherocytosis erythrocyte membranes

Normal (N) and hereditary spherocytosis (HS) red blood cells (RBC), isolated resealed membranes (IRM) and isolated unsealed membranes (IUM) were labelled with pyrene as described under Materials and Methods. Fluorescence intensity of pyrene excimer (I') and monomer (I) were determined at 475 nm and 375 nm, respectively, at 25°C. Data represent mean values ± S.E. for n samples

Sample	n	I'/I
Normal		
RBC	(8)	0.35 ± 0.03
IRM	(13)	0.45 ± 0.03
IUM	(8)	0.37 ± 0.05
HS		
RBC	(9)	0.28 ± 0.01
IRM	(9)	0.53 ± 0.03
IUM	(9)	0.39 ± 0.04

N RBC vs. N IRM: $P < 0.02$; HS RBC vs. HS IRM: $P < 0.001$; N RBC vs. HS RBC: $P < 0.04$; N IRM vs. HS IRM: $P < 0.08$.

TABLE II

Percentages by which pyrene excimer to monomer intensity ratio changes upon the presence of hemoglobin at either side of the red cell membrane

Pyrene labelling of normal and hereditary spherocytosis red cell membranes is as described in Table I. Human hemoglobin (Hb) was added to isolated membranes (final concentration 2.11 mg/ml)

Presence of hemoglobin	N	HS
Inner side (IRM vs. RBC)	−22	−47
Outer side (IRM vs. IRM + Hb)	−18	−5
Inner side (IUM vs. RBC)	−5	−28
Both sides (IUM vs. IUM + Hb)	+3	−2

membranes both in the normal as well as in the HS case (Tables I and II).

Py fluorescence in resealed membranes is quenched by addition of Hb (Fig. 3). I'/I first increases and then decreases as the concentration of Hb is progressively raised (Fig. 3). The I'/I ratio also increases when RBC are lysed at a high hematocrit and subsequently resealed in the presence of a correspondingly lowered Hb concentration (unpublished results). The initial increase of I'/I can be related to trivial energy transfer (Fig. 2). The subsequent decrease of I'/I, as the concentration of Hb raises, can be attributed to a resonance energy transfer mediated suppression of Py fluorescence originating in the outer half of the IRM. Differences between normal and HS conditions appear both in I'/I values (Table I) and in the variation of these values upon the presence of Hb (Table II).

Estimation of the fraction of light emitted by Py embedded in each leaflet of intact RBC gave 11% of the light emitted from the inner leaflet and 89% of the light emitted from the outer one. The same estimation for the case when Hb was added to a suspension of Py labelled resealed membranes gave 40% of the light emitted from the outer leaflet and 60% of the light emitted from the inner one.

Discussion

Membrane related Py fluorescence was quenched by Hb either already present in RBC or externally applied to isolated resealed RBC membranes. Resonance en-

ergy transfer from Py to Hb, allowed to discern between the fluidity of the inner and the outer side of the membrane. Transbilayer asymmetry in lipid matrix fluidity seems to be enhanced in the HS membrane, possible as a consequence of a defective protein skeleton.

Py fluorescence quenching, occurring in RBC versus isolated resealed membranes (Fig. 1) or when Hb is added to Py-loaded resealed membranes (Fig. 3), may be due to long range energy transfer from Py to heme group of Hb. The Förster distance (R_0), for the Py-Hb donor acceptor system (4.3 nm), is within the order of magnitude of the membrane bilayer thickness [22]. In the intact RBC, resonance energy transfer from membrane Py to cytosol Hb, should quench the fluorescence generated mainly within the inner half of the bilayer. Conversely, addition of Hb to resealed RBC membranes may, thus, quench mainly the fluorescence originating in the outer half of the membrane. If Py fluorescence recorded in RBC mainly refers to probe molecules localized in the outer monolayer of the red cell membrane, I'/I in RBC reflects translational mobility in that leaflet. In fact, estimation done about the Py to Hb resonance energy transfer efficiency (see Materials and Methods) allow to assign almost a 90% of the emitted light to the outer leaflet. Information on the bilayer as a whole may be derived, on the other hand, from Py fluorescence of resealed membranes in the abscence of Hb. In contrast to the Hb induced increase of I'/I in the condition of trivial energy transfer (Fig. 2), a decrease of the ratio is observed in RBC as compared to isolated membranes (Table I and II). Trivial energy transfer not being responsible for the observed Hb dependent decrease of I'/I appears, on the contrary, to mask it. Moreover, when 1-pyrenedodecanoic acid is used as a probe, a larger value of I'/I is observed in normal RBC as compared to isolated membranes [34]. Being the Py moiety located at a given depth of the bilayer, the quenching due to the resonance energy transfer would not affect I'/I. The corresponding increase is adscribed only to the trivial energy transfer, due to the stronger absorption RBC have at the monomer emission wavelength range than at the excimer emission range. Thus, in our experiments where Py is distributed across the membrane matrix, the lower value of I'/I observed in RBC as compared to isolated membranes, can thus be related to a reduced mobility of unquenched probe molecules in the outer leaflet as compared to the whole probe population.

Accepting a role of resonance energy transfer in Hb-dependent quenching of Py fluorescence, the lower value of I'/I in RBC as compared to resealed membranes in the normal and in the HS membranes, indicates the mobility of the probe being higher in the inner leaflet than in the outer one in both conditions, (Table I). The lower value of I'/I in HS RBC than in normal cells, indicates a lower fluidity of the outer monolayer in the former than in the latter condition. Above mentioned differences and the higher I'/I value of the HS resealed membranes indicate a higher fluidity of the inner leaflet of the HS membrane. Consequently the fluidity asymmetry of HS cell membranes appears to be enhanced.

Addition of Hb to resealed membranes also lowers I'/I both in the normal and in the HS membranes. The differences in the fraction of light emitted from both monolayers (60% from the inner leaflet and 40% from the outer one) are not so big however as in RBC, where Hb is present at higher concentration near the inner leaflet. This effect may be an expression of the fluidity being highest at the midplane of the bilayer [11]. By addition of Hb the HS membrane showed the lowest decrease (Table II). This is in accordance with the enhancement of the asymmetry in the transbilayer fluidity of the HS membrane. Py fluorescence should also be examined when Hb is added to RBC. However, the already low Py fluorescence intensity in RBC is quenched down to the limit of measurement.

Although the amount of resealed membranes obtained by the Bjerrum method has not been quantified in the present work, a different interaction of Hb with resealed and unsealed isolated membranes under these experimental conditions was observed; more Hb appears to be trapped in unsealed membranes than in resealed membranes (see Materials and Methods). Resealed membranes show a quite distinct response to Hb addition as compared to unsealed membranes (Table II). Isolated resealed membranes thus seem to be sealed in a proportion adequate to visualize differences in I'/I with respect to RBC. Membrane lipid asymmetry [8] and bilayer configuration [35] can be preserved during osmotic lysis by the presence of Mg ion, as should be the case in resealed membranes prepared by the Bjerrum method. Transmembrane asymmetry characteristics in resealed membranes, correspondingly may be comparable to those of RBC. In fact, when unsealed membranes are compared with RBC I'/I actually decreases in a lesser extent, as compared to resealed membranes (Table II), this may be a possible expression of a reduction of transmembrane asymmetry in unsealed membranes with respect to RBC. Transmembrane asymmetry on the other hand, appears to be conserved in the Bjerrum preparation, even after the prolonged incubation period applied in the present work for probe inclusion. Changes in membrane fluidity may also be the consequence of a lipid loss to which HS cells appears to be very suceptible in vitro [36]. Such a possibility cannot be excluded in the present case where lipid losses have not been measured. The membrane damage occurring during the incubation with Py, however, could not have been excessive since

haemolysis under these conditions did not exceed 3% (see Materials and Methods).

Similar as well as contradictory conclusions have been reported in previous studies, in relation to transmembrane fluidity asymmetry of normal RBC membrane. Several authors find the inner monolayer to be more fluid than the outer one [7,8,15]. Cogan and Schachter reported the reverse to be true [9,10]. Dynamic properties of phopholipids may not change independently in either monolayer. Together with alterations of the fluidity in one monolayer, changes in the other one could also occur. Accordingly, our data indicate that a higher mobility of lipids in the inner leaflet implicates a lower one in the outer leaflet (Table I). Lipid composition [37] and phospholipid distribution [15] of the RBC membrane do not appear to be grossly altered in splenectomized HS patients (see also Materials and Methods).

Spectrin deficiences, possibly occurring as assembly disorders in the membrane skeleton [38] may affect intramembrane lipid dynamics. Thus, spectrin/actin depleted inside-out vesicles prepared from HS cells, bind less spectrin/actin extracted from either normal or abnormal cells [39]. Alterations of protein bands 2.1 and 4.1 may alter the assembly of spectrin into the membrane skeleton [39] and, thus, disturb the interaction of the latter with intramembrane lipids. Spectrin depletion, correspondingly, increases rotational movements of phospholipid heads [40]. Deficiences of the protein skeleton organization, moreover, may increase the translocation of lysophosphatidylcholine and other phospholipids [14,17]. Membrane skeleton interaction with the bilayer thus may influence intramembrane lipid dynamics, although not being necessarily the mayor determinant of membrane phospholipid asymmetry in human erythrocytes [41]. Phospholipid asymmetry, seems in fact primarily be mantained by an aminophospholipid translocase which may involve an endofacial protein [42].

The present work indicates an enhanced fluidity difference between the lipid monolayers of the HS RBC membranes. This enhanced transmembrane asymmetry in lipid monolayer fluidity is probably due to a defect of the membrane protein skeleton.

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References

1 Van Deenen, L.L.M. (1981) FEBS Lett. 123, 3–15.
2 Sleight R.G. (1987) Annu. Rev. Physiol. 49, 193–208.
3 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71.
4 Drenthe, E.H.S., Klompmakers, A.A., Bonting, S.L. and Daemen, F.J.M. (1980) Biochim. Biophys. Acta 603, 130–141.
5 Miljanich, G.P., Brown, M.F., Mabrey-Gaud, S., Dratz, E.A. and Sturtevant, J.M. (1985) J. Membr. Biol. 85, 79–86.
6 Rimon, G., Meyerstein, N. and Henis, Y.I. (1984) Biochim. Biophys. Acta 775, 283–290.
7 Seigneuret, M., Zachowski, A., Hermann, A. and Devaux, P.F. (1984) Biochemistry 23, 4271–4275.
8 Tanaka, K.-I. and Ohnishi, S.-I. (1976) Biochim. Biophys. Acta 426, 218–231.
9 Cogan, U. and Schachter, D. (1981) Biochemistry 20, 6396–6403.
10 Schachter, D., Cogan, U. and Abbott, R.E. (1982) Biochemistry 21, 2146–2150.
11 Tilley, L., Thulborn, K.R. and Sawyer, W.H. (1979) J. Biol. Chem. 254, 2592–2594.
12 Kleinfeld, A.M., Dragsten, P., Klausner, R.D., Pjura, W.J. and Matayoshi, E.D. (1981) Biochim. Biophys. Acta 649, 471–48.
13 Seigneuret, M. and Devaux, P.F. (1984) Proc. Natl. Acad. Sci. USA 81, 3751–3755.
14 Dressler, V., Haest, C.W.M., Plasa, G., Deuticke, B. and Erusalimsky, J.D. (1984) Biochim. Biophys. Acta 775, 189–196.
15 Williamson, P., Bateman, J., Kozarsky, K., Mattocks, K., Hermanowicz, N. Choe, H.-R. and Schlegel, R.A. (1982) Cell 30, 725–733.
16 Haest, C.W.M. (1982) Biochim. Biophys. Acta 694, 331–352.
17 Mohandas, N., Rossi, M., Bernstein, S., Ballas, S., Ravindranath, Y., Wyatt, J. and Mentzer, W. (1985) J. Biol. Chem. 260, 14264–14268.
18 Lux, S.E. (1983) in Metabolic Basis of Hereditary Diseases (Stanbury, J.B., Wyngaarden, J.B., Fredrickson, D.S., Goldstein, J.L. and Brown, M.S., eds.), pp. 1573–1605, McGraw Hill, New York.
19 Becker, A.U. and Lux, S.E. (1985). Clin. Haematol. 14, 15–43.
20 Minetti, M., Ceccarini, M., Di Stasi, A.M.M., Petrucci, T.C., Scorza, G. and Di Nucci, G. (1984) J. Submicrosc. Cytol. 16, 183–184.
21 Bloom, J.A. and Webb, W.W. (1983) Biophys. J. 42, 295–305.
22 Eisinger, J., Flores, J. and Petersen, W.P. (1986) Biophys. J. 49, 987–1001.
23 Galla, H.-J. and Sackmann, E. (1974) Biochim. Biophys. Acta 339, 103–115.
24 Galla, H.-J. and Luisetti, J. (1980) Biochim. Biophys. Acta 596, 108–117.
25 Behn, C., Celedón, G., Montalar, Y., Bagnara, M. and Sotomayor, C.P. (1988) Pflügers Arch. Eur. J. Physiol. 411, R65.
26 Bjerrum, P.J. (1979) J. Membr. Biol. 48, 42–67.
27 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
28 Roeschlau, P., Bernt, T. and Gruber, W. (1974) Z. Klin. Chem. Klin. Biochem. 12, 403–427.
29 Bartlett, G.R. (1959) J. Biol. Chem. 234, 446–471.
30 Galembeck, F., Robilotta, P.R., Pinheiro, E.A., Joelkes, I. and Bernardes, N. (1980) J. Phys. Chem. 84, 112–115.
31 Hresko, R.C., Sugar, I.F., Barenholz, Y. and Thompson, T.E. (1986) Biochemistry 25, 3813–3823.

32 Fal, R., Barenholz, Y. and Wagner, R.R. (1988) Biochemistry 27, 30–36.
33 Eisinger, J. and Flores, J. (1983) Biophys. J. 41, 987–1001.
34 Eisinger, J. and Flores, J. (1985) Biophys. J. 48, 77–84.
35 Lew, V.L., Hockaday, A., Freeman, C.J. and Bookchin, R.M. (1988) J. Cell Biol. 106, 1893–1901.
36 Reed, C.F. and Swisher, S.N. (1966) J. Clin. Inv. 45, 777–781.
37 Zail, S.S. and Pickering, A. (1979). Br. J. Haematol. 42, 399–402.
38 Bennet, V. (1990) Physiol. Rev. 70, 1029–1065.
39 Hill, J.S., Sawyer, W.H., Howlett, G.J. and Wiley, J.S. (1984) Biochem J. 201, 259–266.
40 Yeagle, P. (1982) Biophys. J. 37, 227–239.
41 Gudi, S.R.P., Bhakuni, V., Gokhale, S.M. and Gupta, C.M. (1990) Biochim. Biophys. Acta 1023, 63–72.
42 Connor, J. and Schroit, A.J. (1990) Biochem. 29, 37–43.