Biochimica et Biophysica Acta 856 (1986) 212–218
Elsevier

BBA 73010

Temperature-dependent specificity of *cis-trans* isomeric fatty acid interaction with the erythrocyte membrane

Adam Csordas [a,*] and Konrad Schauenstein [b]

[a] *Institute of Medical Chemistry and Biochemistry, and* [b] *Institute of General and Experimental Pathology, University of Innsbruck, School of Medicine, Fritz-Pregl-Strasse 3, A-6020 Innsbruck (Austria)*

(Received November 12th, 1985)

Key words: Membrane stabilization; Fatty acid isomer; Hemolysis; Configuration dependence; (Erythrocyte membrane)

Stabilization of red cells against hypotonic haemolysis by *cis-trans* isomeric free C_{18} fatty acids occurs with pronounced specificity which is strongly temperature-dependent, but in a distinctly different manner for the two configurational isomers. Oleic acid (*cis*-18:1) stabilizes very efficiently at 0°C, even at the highest concentrations. Elaidic acid (*trans*-18:1) causes neither stabilization nor haemolysis at this temperature. At room temperature (23°C), elaidic acid acquires the ability to protect, without turning haemolytic at high concentrations. At 37°C elaidic acid also becomes haemolytic. The protecting effect of oleic acid at 0°C is the result of a rapid reaction. The characteristic, temperature-dependent specificity of *cis-trans* isomeric C_{18} fatty acid interaction with the red cell membrane appears to be a general phenomenon, since it was observed alike with erythrocytes of different species.

Introduction

The osmotic resistance of the erythrocyte membrane is altered by a number of endogenous [1–5] and exogenous [6–9] factors. Free fatty acids are known to promote haemolysis at higher concentrations, but in a certain lower concentration range they stabilize the erythrocyte membrane against osmotic stress [10,11]. The molecular mechanism of stabilization is not known. The promotion of haemolysis at higher concentrations is generally attributed to the so-called detergent-like effect [12]. However, the exact mechanism of detergent-mediated rupture of the erythrocyte membrane is not elucidated.

In the present study we set out to investigate the mechanism of these apparently paradoxical effects. The exact nature of fatty acid interaction with the plasma membrane is of considerable interest, since elevated levels of circulating free fatty acids have been implicated in ischemic damage of the myocardium [13]. Therefore, we investigated the temperature-dependence of (a) protection against osmotic rupture over a well-defined lower concentration range and (b) haemolysis at higher concentrations of fatty acids. The striking result of the present study is that pronounced configuration-dependent specificities are revealed by examining these fatty acid effects at different temperatures. Furthermore, progress has been made in elucidating the mechanism of stabilization, which was found to be most efficient at 0°C, but only with fatty acids of *cis*-configuration, while the *trans*-isomer showed no effect whatsoever at this temperature.

Unsaturated C_{18} *cis*-fatty acids exhibit especially strong effects of stabilization, but it should be emphasized that the biphasic behaviour is encountered also with other fatty acids [12] and certain detergents [14,15]. In a previous study,

* To whom correspondence should be addressed.

performed at room temperature with chicken and sheep erythrocytes, we reported the structure-dependence of stabilization and configuration-dependence of haemeolysis promotion by free unsaturated C_{18} fatty acids [11]. In another study [10] similar effects were found with human erythrocytes, but the question of configuration-dependence was not investigated. All previous studies were carried out at room temperature. As we observed that elaidic acid turns haemolytic with increasing temperatures, we decided to investigate the effects of free fatty acids on the osmotic resistance of red cells at 0°C, room temperature (23°C) and 37°C. In the present report we show that the specific effects exerted by oleic acid (*cis*-18 : 1) and elaidic acid (*trans*-18 : 1) on the erythrocyte membrane change drastically as a function of temperature.

Materials and Methods

Erythrocyte preparations

Heparinized blood was drawn from an adult female sheep, from female chickens of the White Leghorn strain, human erythrocytes were from a male volunteer (A. Cs.). After three washings with Hanks' solution and removal of the buffy coat the suspensions were adjusted to a 50 : 50 (v/v) ratio of pellet to isotonic buffer.

Isotonic and hypotonic buffer solutions

The isotonic buffer was 155 mM NaCl/50 mM sodium phosphate (pH 7.4). In order to prepare the hypotonic haemolysis buffer for sheep erythrocytes, the isotonic buffer was mixed in a ratio of 1 : 2 with the same sodium phosphate which had no NaCl. Incubation of sheep, chicken and human erythrocytes in 10 mM sodium phosphate (pH 7.4) without NaCl resulted in instant and total lysis and was used as 100% haemolysis control. Because of the higher osmotic resistance of chicken [11] and human [10] erythrocytes, the standard isotonic sodium phosphate saline buffer (155 mM NaCl/50 mM sodium phosphate (pH 7.4)) was diluted with distilled water until the degree of haemolysis was above 90% at 0°C. At 37°C, in this buffer solution, the degree of haemolysis was below 10% but still accurately measurable.

Determination of osmotic resistance and treatment with fatty acids

The effect of unsaturated free C_{18} fatty acids on the osmotic fragility of erythrocytes was determined as described previously [10,11]. The two *cis-trans* isomeric C_{18} fatty acids, oleic acid (*cis*-18 : 1) and elaidic acid (*trans*-18 : 1), were dissolved in methanol and the appropriate molar amounts were applied to the buffered hypotonic NaCl solution just prior to the addition of red cells. Volumes of 5, 10 and 15 μl of the methanolic solution of fatty acids were added to 5 ml of the haemolysis buffer. Addition of these volumes of methanol did not affect the osmotic fragility of erythrocytes. 50-μl aliquots of the erythrocyte stock suspension were then mixed with the 5 ml of the hypotonic sodium phosphate/saline buffer (pH 7.4) (haemolysis buffer), containing various concentrations of fatty acids. The hypotonic buffer solution was equilibrated at 0°C, room temperature (23°C) and at 37°C, respectively, before addition of the free fatty acids and the erythrocyte suspension. The same results were obtained when the fatty acids were added at room temperature to the hypotonic buffer and then equilibrated at the respective temperatures before addition of erythrocytes. After an incubation time of 30 min, the samples were centrifuged (5 min at 2000 rpm) in a Hettich, Roto Silenta/K centrifuge and the absorbance of the supernatant was determined at 540 nm. It is essential that the centrifugation step follows without delay and at the proper temperature. The centrifuge was used at 0°C and at room temperature, respectively.

Reagents

The fatty acids were obtained from Sigma; salts used for buffer solutions were from Merck. All the reagents were of the highest purity available.

Results

Temperature-dependence of cis-trans isomeric free fatty acid effects on sheep erythrocytes

In the present study we compared the *cis-trans* isomeric oleic (*cis*-18 : 1) and elaidic (*trans*-18 : 1) acid, as to their protecting effects against hypotonic haemolysis at different temperatures, in order to detect possible temperature-induced changes in

the membrane at the level of configurational specificity.

Fig. 1 shows the osmotic resistance of sheep erythrocytes examined at 0°C, room temperature (23°C) and 37°C, with different concentrations of oleic and elaidic acid. The differences in the degree of haemolysis observed at the lowest fatty acid concentration ($1 \cdot 10^{-7}$ M) mark the effect of temperature on osmotic resistance and are identical with control values without addition of fatty acids. The osmotic resistance of erythrocytes per se, without exogenous fatty acids, being strongly temperature-dependent, increases markedly from 0 to 37°C. For this reason, at 0°C, hypotonic buffers were used to give a degree of haemolysis reaching almost 100%. With these buffers at 37°C, haemolysis was below 20%. Thus, with the same buffer, measurable values of haemolysis were obtained at 0, 23 and 37°C. There are three striking results of this experiment. First, at 0°C there is a complete lack of protection against hypotonic haemolysis by elaidic acid (*trans*-18:1). Second, the most efficient stabilization is exhibited by oleic acid (*cis*-18:1) at 0°C, even at the highest concentrations tested. These higher concentrations of oleic acid lead to rapid haemolysis at room temperature. Therefore it is essential to use a cooled centrifuge and put the samples from the ice-bath straight into the cooled centrifuge. Otherwise, haemolysis occurs instantly and the remarkable protection by oleic acid at this temperature cannot be detected. Third, elaidic acid (*trans*-18:1), which shows neither protection nor haemolysis promotion at 0°C, becomes strongly haemolytic at 37°C. At room temperature, elaidic acid does stabilize against hypotonic rupture, but, even at the highest concentrations used, does not become haemolytic beyond the degree caused by the hypotonic buffer.

Temperature-dependence of the C_{18} cis-trans isomeric free fatty acid interaction with chicken and human erythrocytes

In order to test how general is the temperature-dependence of specific effects exhibited by *cis-trans* isomeric C_{18} fatty acids, human erythrocytes and nucleated avian (chicken) erythrocytes were also examined. As shown in Fig. 2, the lack of protection by the *trans*-isomeric elaidic acid at 0°C appears to be a general property of the red-cell membrane. This common property of sheep, chicken and human red blood cells is demonstrated by comparing the effects of oleic and elaidic acid at 0°C and 37°C: (i) Elaidic acid causes neither stabilization nor haemolysis at 0°C, while oleic acid exhibits a remarkable stabilization at this temperature (note the decrease of haemolysis from 98% to 0% with human erythrocytes). (ii) The haemolytic effect of oleic acid is not expressed at 0°C. Even the highest concentrations of oleic acid lead to a strong stabilization at this temperature. (iii) The haemolytic effect of elaidic acid, which is not expressed at room temperature [11] and at 0°C, becomes very pronounced at 37°C. Thus, the initial observations made with sheep erythrocytes (Fig. 1) are fully confirmed by similar experiments using human and nucleated chicken erythrocytes.

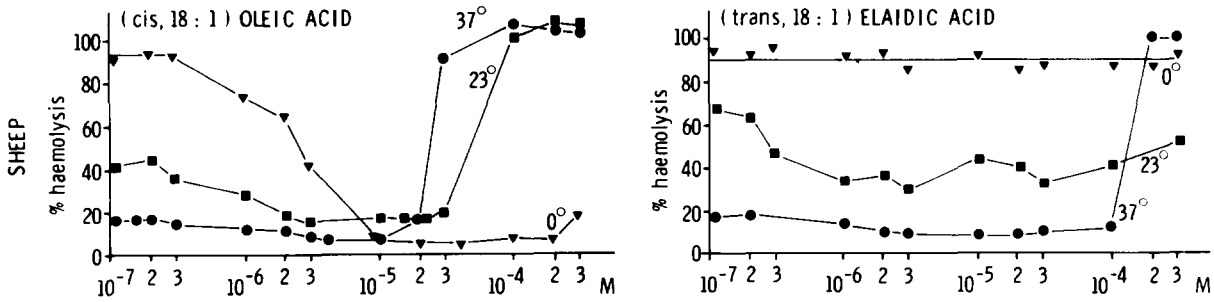

Fig. 1. Temperature-dependent effects of oleic acid (*cis*-18:1) and elaidic acid (*trans*-18:1) on the osmotic fragility of sheep erythrocytes. Effects of the *cis-trans* isomeric C_{18} fatty acids in hypotonic buffer solution (0.30%) at different temperatures. Stabilization against osmotic rupture over a certain concentration range and promotion of haemolysis at higher concentrations. The degree of haemolysis was determined after incubation for 30 min at the respective temperatures in presence of the indicated concentrations of fatty acids. ▼, 0°C; ■, 23°C; ●, 37°C.

The effects of oleic and elaidic acid and of other unsaturated C_{18} fatty acids, at room temperature, on the chicken and sheep erythrocyte membrane were reported previously [11]. However, it should be pointed out that, in the previous study, the haemolysis buffer contained K^+, while the present experiments have been done with buffers containing exclusively Na^+ as a cation. As was shown in our previous work, performed at room temperature, elaidic acid does stabilize chicken and sheep erythrocytes against hypotonic haemolysis without causing haemolysis, even at the highest concentrations, contrary to the *cis*-isomers. This protection against hypotonic haemolysis by elaidic acid was especially pronounced in the case of chicken erythrocytes [11]. For all unsaturated C_{18} fatty acids, the stabilizing effect was shown to persist through several washings and centrifugations [11]. This shows that elaidic acid did interact with the chicken and sheep erythrocyte membranes at room temperature, far below its melting point (44.5°C). Since these basic observations, at room temperature, were published already [10,11], they have been omitted from Fig. 2. However, in the present study, using the buffer without K^+ and at pH 7.4, the previous observations, which were made under slightly different conditions, are reproducible (data shown only for sheep erythrocytes, Fig. 1).

Persistent interaction of elaidic acid with the erythrocyte membrane at 0°C

The question arises as to whether the different melting points of oleic acid (16.3°C) and elaidic acid (44.5°C) are responsible for the lack of effects at 0°C by elaidic acid (neither stabilization nor haemolysis promotion) and the lack of haemolysis promotion (but efficient stabilization) by oleic acid at 0°C. Such a correlation appears unlikely, considering the strong effects exhibited by elaidic and

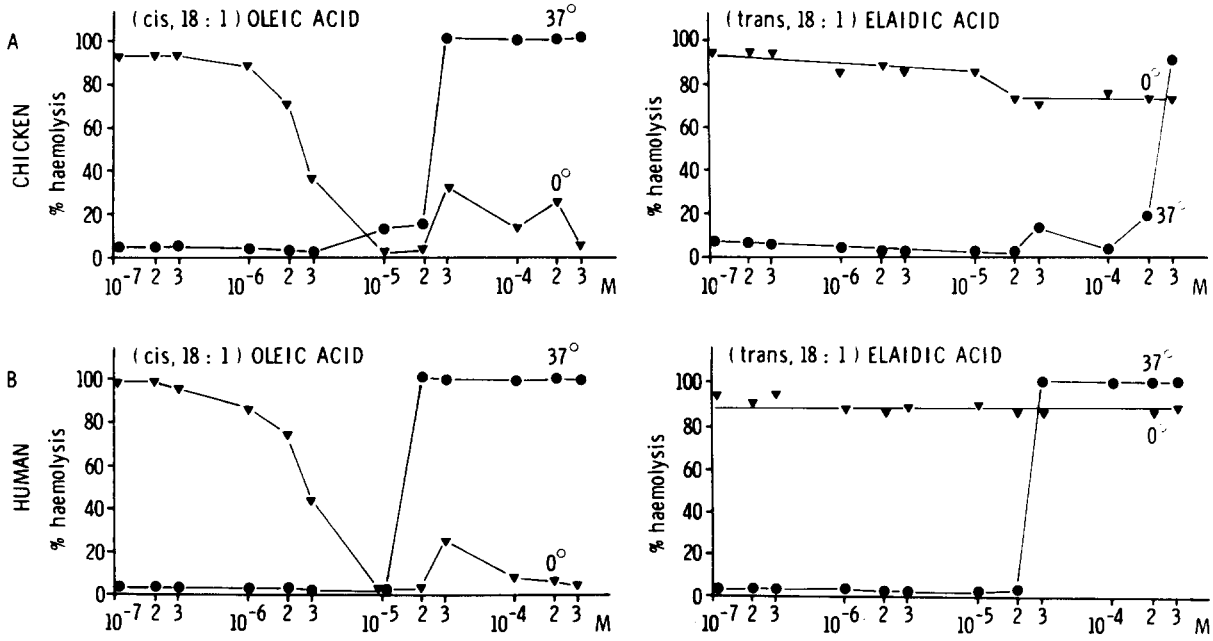

Fig. 2. Temperature-dependent effects of oleic acid (*cis*-18:1) and elaidic acid (*trans*-18:1) on chicken (A) and human (B) erythrocytes. These experiments are analogous to those described in Fig. 1 for sheep erythrocytes. Only two temperatures, 0°C and 37°C, are shown at the indicated concentrations. The degree of haemolysis at the lowest concentrations of fatty acids (10^{-7} M) is identical with the degree of haemolysis without addition of exogenous fatty acids at the respective temperatures. After equilibration at a certain temperature, first the methanolic solution of fatty acids and then 50 µl of the erythrocyte suspension were added to 5 ml of the hypotonic buffer solution. After 30 min of incubation, the samples were centrifuged (5 min at 2000 rpm) and the absorbance of the supernatant at 540 nm was determined. The reproducibility of haemolysis assays was within a range of ±5% haemolysis with the same freshly drawn blood samples on the same day. ▼, 0°C; ●, 37°C.

oleic acid below their melting points. It should be emphasized that elaidic acid stabilizes strongly at room temperature (23°C) and turns into a potent haemolytic agent at 37°C. Thus, the transition points between (a) complete lack of effects, (b) stabilization, and (c) haemolysis are all below the melting point of elaidic acid and are more likely correlated with transition points of the membrane. Similarly, oleic acid is not haemolytic, but strongly protects, below its melting point, at 0°C. Whether the suspension of free fatty acids in the aqueous haemolysis buffer can partition into the erythrocyte membrane does not seem to be a function of melting points of the fatty acids, but rather to be related to temperature-dependent properties of the membrane.

The question of binding of elaidic acid to the erythrocyte membrane was examined in the experiment depicted in Fig. 3. As shown in this figure, the indicated concentrations of elaidic acid were added to human erythrocytes at 0°C and subsequently incubated for 10 min at 0°C. After three washings with the isotonic buffer (buffer and centrifuge at 0°C), identical erythrocyte suspensions, representing treatments with different concentrations of elaidic acid, were tested as to their osmotic fragility at 0, 23 and 37°C with a hypotonic haemolysis buffer and compared with control erythrocytes which went through the same washing and centrifugation procedure at 0°C. As can be seen in Fig. 3, aliquots of the same red cells which exhibited no effect at 0°C showed a pronounced concentration-dependent protection at room temperature (23°C) and also haemolysis at 37°C (in those samples which were treated with the highest concentrations of elaidic acid at the beginning of the experiment). Thus, elaidic acid must be bound to the membrane in a way that it resists the subsequent washing procedure. The treatment at 0°C with different concentrations of elaidic acid led to a persistent change of the erythrocyte membrane, the quality of this change depending on the concentration of elaidic acid in the initial treatment before the washing of cells. This also is in agreement with the suggestion that the temperature-dependent transitions of the membrane are critical for the effects exhibited by free fatty acids which are bound to the erythrocyte membrane.

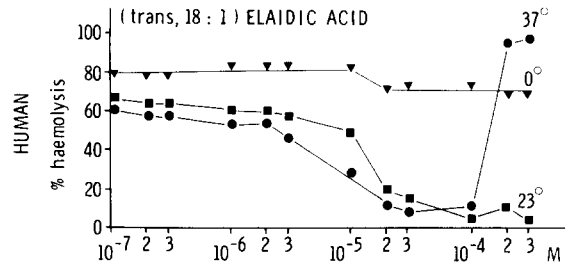

Fig. 3. Persistent protection of human erythrocytes by elaidic acid (*trans*-18 : 1) against hypotonic lysis after preincubation at 0°C. The indicated concentrations of elaidic acid were added to human erythrocytes at 0°C and subsequently incubated for 10 min at 0°C. After three washings and centrifugations of the treated red cells and the untreated controls at 0°C, the osmotic resistance of each sample was determined at three different temperatures. Aliquots of the same washed erythrocyte samples were incubated for 30 min at 0, 23 and 37°C. The isotonic buffer solution was diluted appropriately for each temperature to give comparable degrees of haemolysis for the controls at all three temperatures. Haemolysis at the lowest fatty acid concentrations is identical with the degree of haemolysis of the untreated controls. ▼, 0°C; ■, 23°C; ●, 37°C.

An important point regarding the mechanism of stabilization concerns the rapid kinetics of this phenomenon. The stabilization occurred instantly at 0°C, when first a methanolic solution of free fatty acids was admixed to the hypotonic buffer solution, and then the erythrocytes were added. Under these conditions, the presence of a *cis*-unsaturated C_{18} acid determined a change from almost 100% haemolysis to virtually no lysis.

In the light of these observations, we would like to postulate that the stabilization is a nonenzymatic process involving structural elements of the membrane which can distinguish between the molecular shape of the *cis*- and the *trans*-configurations of unsaturated C_{18} fatty acids at 0°C. At higher temperatures, however, this specificity is lost.

Discussion

The question of *cis*- and *trans*-configuration dependence was studied previously with other cells and also with liposomes [16,17]. Fatty acids have been shown to be taken up into the cell membrane and to cause major changes in the packing of lipid molecules by intercalation [16]. As a consequence of free fatty acid interaction with cells, a large

number of membrane-mediated cellular functions were reported to be altered [17–22], including surface receptor capping [17]. It was postulated that, due to a non-linear molecular shape, *cis*-unsaturated fatty acids partition into fluid domains, while linearly shaped *trans*-unsaturated and saturated fatty acids partition into gel-like domains [16].

The question arises of whether the present findings can be correlated with other studies on temperature-dependence of membrane parameters and of free fatty acid interactions with the cell membrane.

In a study of temperature effects, a lack of correlation was reported between anaesthetic action and perturbation of the bilayer structure, such as fluidity changes, phase separations and bilayer thickness [23]. Based on these experiments, the degenerate perturbation hypothesis was proposed, according to which, sets of protein-binding sites with different degrees of specificity were postulated. This hypothesis was substantiated by, for example, the stereoselective activity encountered with steroid anaesthetics [24].

Several distinct effects concerning the physical properties of the membrane were found with unsaturated free fatty acids, depending on the number of double bonds and the configuration [25]. Free fatty acids interact with the calmodulin-binding part of the membrane-bound Ca^{2+}-ATPase [26]. Other effects on the $(Na^+ + K^+)$-ATPase by long-chain fatty acids have been reported, but they do not seem to be specific for unsaturated fatty acids [22,27,28]. However, there is some degree of specificity in the case of bovine brain microsomal $(Na^+ + K^+)$-ATPase. Inhibition of this enzyme with unsaturated C_{18} fatty acids was reported to increase with the number of double bonds [28]. High K^+ or low temperature reduced the sensitivity of the enzyme to this inhibition, and fatty acids appeared to inhibit more effectively when the membrane was in a more fluid state [28]. For simplicity of interpretation, we used a buffer system which contained exclusively sodium and had no potassium as a cation. The present results obtained with this buffer system therefore suggest that the $(Na^+ + K^+)$-ATPase does not play a role in the stabilization of red cells against osmotic rupture by free fatty acids.

Inhibition of synaptosomal Na^+-dependent amino-acid uptake by free fatty acids was shown with pronounced specificity for *cis*-unsaturated fatty acids, while *trans*-unsaturated and saturated fatty acids showed negligible effects [21].

It remains to be shown whether the binding sites for free fatty acids, responsible for the phenomena investigated in this report, are identical with already known structures of the cell membrane, or whether they represent novel binding sites so far neglected. It also remains to be seen whether the behaviour of the erythrocyte membrane can be considered as a model for other cell types. The specificities of binding and changing of membrane properties would appear especially intriguing in examining proliferating or transformed cells [29].

Acknowledgements

We thank Professor Manfred Schweiger for his criticism of the manuscript. This research was supported by the Dr. Legerlotz Stiftung and also by Austrian Research Foundation, grant No. S 4104. The skilful technical assistance of Miss Katharina Stricker is appreciated.

References

1 Bodin IV, D.M., Birkenmeier, C.S. and Barker, J.E. (1984) Cell 37, 721–729
2 Bernstein, S.E. (1980) Lab. Animal Sci. 30, 197–205
3 Lux, S.E., Paese, B., Tomasselli, M.B., John, K.M. and Bernstein, S.E. (1979) in Normal and Abnormal Red Cell Membranes (Lux, S.E., Marchesi, V.T. and Fox, C.F., eds.), pp. 463–469, Alan R. Liss, New York
4 Kuypers, F., Van Linde-Sibenius-Trip, M., Roelofsen, B., Tanner, M.J.A., Anstee, D.J. and Op den Kamp, J.A.F. (1984) Biochem. J. 221, 931–934
5 Ridgwell, K., Tanner, M.J.A. and Anstee, D.J. (1983) Biochem. J. 213, 267–269
6 Seeman, P. (1966) Int. Rev. Neurobiol. 9, 145–221
7 Beutler, E. (1969) Pharmacol. Rev. 21, 73–103
8 Seeman, P. (1972) Pharmacol. Rev. 24, 583–665
9 Müller-Eberhard, H.J. (1975) Annu. Rev. Biochem. 44, 697–724
10 Raz, A. and Livne, A. (1973) Biochim. Biophys. Acta 311, 222–229
11 Csordas, A. and Schauenstein, K. (1984) Biochim. Biophys. Acta 769, 571–577
12 Piper, H.M., Sezer, O., Schwartz, P., Hütter, J.F. and Spieckermann, P.G. (1983) Biochim. Biophys. Acta 732, 193–203

13 Katz, A.M. and Messineo, F.C. (1981) Circ. Res. 48, 1–16
15 Kwant, W.O. and Van Steveninck, J. (1968) Biochem. Pharmacol. 17, 2215–2223
16 Klausner, R.D., Bhalla, D.K., Dragsten, P., Hoover, R.L. and Karnovsky, M.J. (1980) Proc. Natl. Acad. Sci. USA 77, 437–441
17 Klausner, R.D., Kleinfeld, A.M., Hoover, R.L. and Karnovsky, M.J. (1980) J. Biol. Chem. 255, 1286–1295
18 Hoak, J.C., Spector, A., Fry, G. and Warner, E. (1970) Nature 228, 1330–1332
19 Anderson, W.B. and Jaworski, C.J. (1977) Arch. Biochem. Biophys. 180, 374–383
20 Meade, C.J. and Mertin, J. (1978) Adv. Lipid. Res. 16, 127–165
21 Rhoads, D.E., Ockner, R.K., Peterson, N.A. and Raghupathy, E. (1983) Biochemistry 22, 1965–1970
22 Swann, A.C. (1984) Arch. Biochem. Biophys. 223, 354–361
23 Richards, C.D., Martin, K., Gregory, S., Keightley, C.A., Hesketh, T.R., Smith, G.A., Warren, G.B. and Metcalfe, J.C. (1978) Nature 276, 775–779
24 Richards, C.D. and Hesketh, T.R. (1975) Nature 256, 179–182
25 Stubbs, C.D. (1983) Essays Biochem. 19, 1–19
26 Wetzker, R., Klinger, R. and Funder, H. (1983) Biochim. Biophys. Acta 780, 196–200
27 Schmalzing, G. and Kutschera, P.G. (1982) Membrane Biol. 69, 65–76
28 Kato, A., Ando, K., Tamura, G. and Arima, K. (1971) Cancer Res. 31, 501–504