A SPECTROFLUORIMETRIC STUDY OF THE INTERACTION OF GLYCEROL MONO-OLEATE WITH HUMAN ERYTHROCYTE GHOSTS

A. KENNEDY and C. RICE-EVANS

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, 8 Hunter Street, London WC1N 1BP, UK

Received 27 July 1976

1. Introduction

It is now well established by the work of Lucy et al., that various unsaturated fatty acids and other chemical reagents (e.g. certain esters, polyols, dimethyl sulphoxide) will induce cell fusion for different types of cell under appropriate conditions [1–5]. Cell fusion may also be induced by other means including heat [2] and high pH with a high concentration of calcium ions [6]. It has been suggested that it may be necessary, in addition to other requirements, for lipids in natural membranes to be in a fluid condition for fusion to occur [4,5]. Kosower et al., have suggested that some increase in local fluidity favours cell fusion [7] from work involving membrane mobility agents which promote motion through cell membranes [8] by inducing local disorder and which actively promote the fusion of hen erythrocytes under similar conditions to those used by Lucy et al.

In the present communication evidence will be presented which indicates that treatment of human erythrocyte ghost membranes with the fusogenic lipid glycerol mono-oleate leads to an increase in the fluidity of the membrane lipids, whereas the chemically related nonfusogenic lipid glycerol monostearate has no effect.

2. Experimental

Erythrocyte membranes were prepared according to the method of Dodge et al. [9], from recently outdated blood bank blood. Each preparation was haemoglobin-free as judged by its absorbance at 414 nm. The ghost protein concentration was determined by the method of Lowry et al. [10] using crystalline bovine serum albumin (Sigma) as standard. The ghosts were finally suspended in phosphate buffer at pH 7.4.

The stock solutions of glycerol mono-oleate and glycerol monostearate (Sigma) were made in absolute ethanol and the appropriate volumes were added to the samples by injection. The magnesium salt of 1-anilino-8-naphthalene sulphonate (ANS) (Eastman) was used as the fluorescent probe without further purification. The freshly prepared stock solution was equilibrated at 25°C for two hours prior to each experiment. *N*-phenyl-1-naphthylamine (NPN) (Eastman) was recrystallised twice from ethanol and freshly prepared each day.

Fluorescence measurements were performed on a Perkin–Elmer Hitachi 204 spectrofluorimeter at 25°C. The extrinsic fluorescence of ANS and NPN were excited at 388 nm and 340 nm respectively and the emission intensities were observed at 460 nm and 410 nm respectively, except for experiments involving emission wavelength variation. The intrinsic fluorescence was excited at 282 nm and the emission observed at 330 nm. The membrane samples for the ANS experiments contained 200 μg ghost protein per ml and 20 μM ANS; for the NPN experiments samples contained 53 μg ghost protein per ml and 3 μM NPN, except for the binding experiments involving varying probe and membrane concentrations. Appropriate blanks were run on each occasion. The concentrations of glycerol mono-oleate used were calculated with reference to the amount used to

produce red cell fusion: 300 nmoles of fusogen per 3×10^8 cells [3]. The lipid:fusogen molar ratios were approximately the same for both sets of binding experiments.

3. Results

When ANS interacts with increasing concentrations of glycerol mono-oleate in buffer at pH 7.4, the fluorescence intensity is gradually increased until it reaches a plateau at approximately 300 μM glycerol mono-oleate, (fig.1A), and there is a shift in the wavelength of maximum emission from 525 nm to 470 nm. Thus the glycerol mono-oleate is in the form of micelles in aqueous solution.

In the presence of human erythrocyte ghosts, the ANS fluorescence is greatly increased and again blue-shifted compared with ANS in buffer. However, when ANS interacts with membrane ghosts perturbed by increasing concentrations of glycerol mono-oleate (fig.1A) the further progressive enhancement of fluorescence is accompanied by a red-shift of the emission maximum wavelength of magnitude 7 nm at a glycerol mono-oleate concentration of 336 μM. The influence of this perturbant to produce the red-shift in the ANS emission in the presence of membrane ghosts compared with a blue-shift in their absence provides evidence that the glycerol mono-oleate is interacting with the membrane as a monomer. The intrinsic tryptophan fluorescence intensity is unaffected by the presence of the fusogenic lipid.

The enhancement of the probe fluorescence intensity in the presence of glycerol mono-oleate may

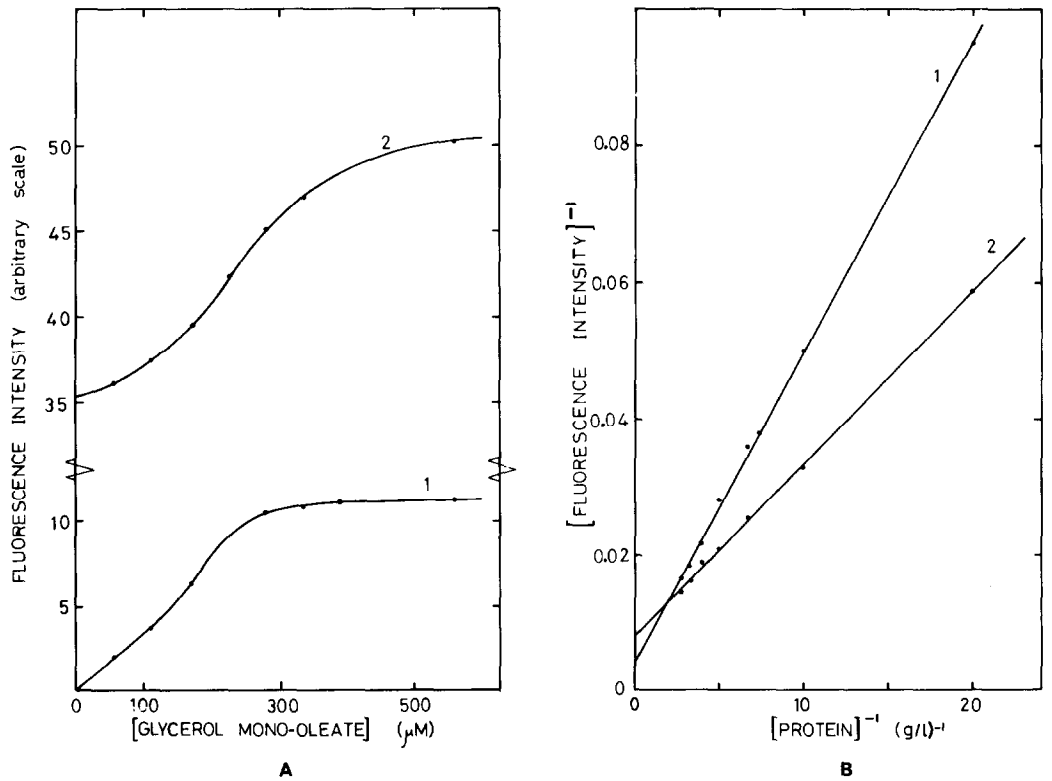

Fig.1(A). Effect of glycerol mono-oleate on the fluorescence of ANS (20 μM) in buffer and in the presence of erythrocyte membrane ghosts. 1. ANS in phosphate buffer, pH 7.4. 2. ANS with membrane ghosts (0.2 mg/ml protein) in pH 7.4 buffer. (B) Double reciprocal plots of ANS fluorescence (20 μM) as a function of membrane protein concentration. 1. In the absence of glycerol mono-oleate; 2. in the presence of 336 μM glycerol mono-oleate.

result from a change in the quantum yield of the bound dye, or from an increase in the number of dye molecules bound which could arise from the creation of new sites or from tighter binding [11]. In order to estimate the limiting ANS fluorescence enhancement when all the probe is bound to the membrane, the membrane protein concentration was varied at a fixed concentration of ANS and the double-reciprocal plot extrapolated to infinite protein concentration [12]. The quantum yield of ANS decreases on perturbation of the membrane by 336 μM glycerol mono-oleate (fig.1B).

To determine the number of binding sites for the ANS and the binding constants, the data can be analysed by a Scatchard plot [13] involving variation of the probe concentration at constant membrane concentration, using the values of the fluorescence of the ANS bound determined from the double-reciprocal plot. This approach requires the substantial assumptions that all the binding sites are identical in dye binding properties and that all the sites are independent. The results (fig.2) show that the influence of 336 μM glycerol mono-oleate is to increase the number of binding sites from 30 to 65 μmol/g and the affinity of the membrane for the probe, as shown by the lowering of the dissociation constant from 17 μM to 9 μM. The addition of the nonfusogen glycerol monostearate has no effect on the quantum yield, the fluorescence intensity or the wavelength of maximum emission of the probe bound to membrane ghosts.

The fluorescence of NPN in phosphate buffer at pH 7.4 is gradually increased and blue-shifted by increasing concentrations of glycerol mono-oleate (fig.3A). When NPN interacts with human erythrocyte ghosts the fluorescence is enhanced approximately 80-fold compared with the value for the probe in buffer and the emission maximum is blue-shifted by 58 nm. Perturbation of the membrane ghosts by 92.5 μM glycerol mono-oleate further increases the emission intensity of the probe (fig.3A) and the emission maximum is shifted by 10 nm to longer wavelengths. This red-shift is accompanied by a decrease in the quantum yield of the probe (fig.3B). A Scatchard analysis was performed on the data (fig.4). The affinity of the membrane for the probe is increased in the presence of 92.5 μM glycerol mono-oleate, the dissociation constant decreasing from 7.8 μM to 0.65 μM, and the number of probe molecules bound increases from 40 to 92 μmol/g. The presence of the fusogen has no significant effect on the conformational state of the membrane proteins as indicated by the unchanged intrinsic tryptophan fluorescence.

Comparable experiments involving the nonfusogen glycerol monostearate as membrane perturbant show no change in the quantum yield, fluorescence intensity or emission maximum wavelength of the probe.

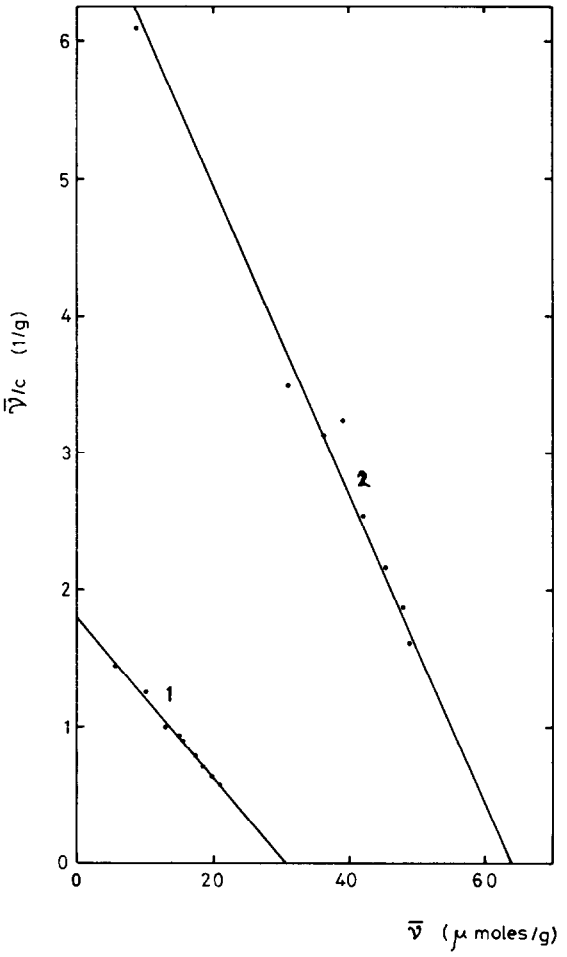

Fig.2. Scatchard plots for the binding of ANS (0–40 μM) to erythrocyte membranes (membrane protein concentration 0.2 mg/ml). 1. In the absence of glycerol mono-oleate; 2. in the presence of 336 μM glycerol mono-oleate.

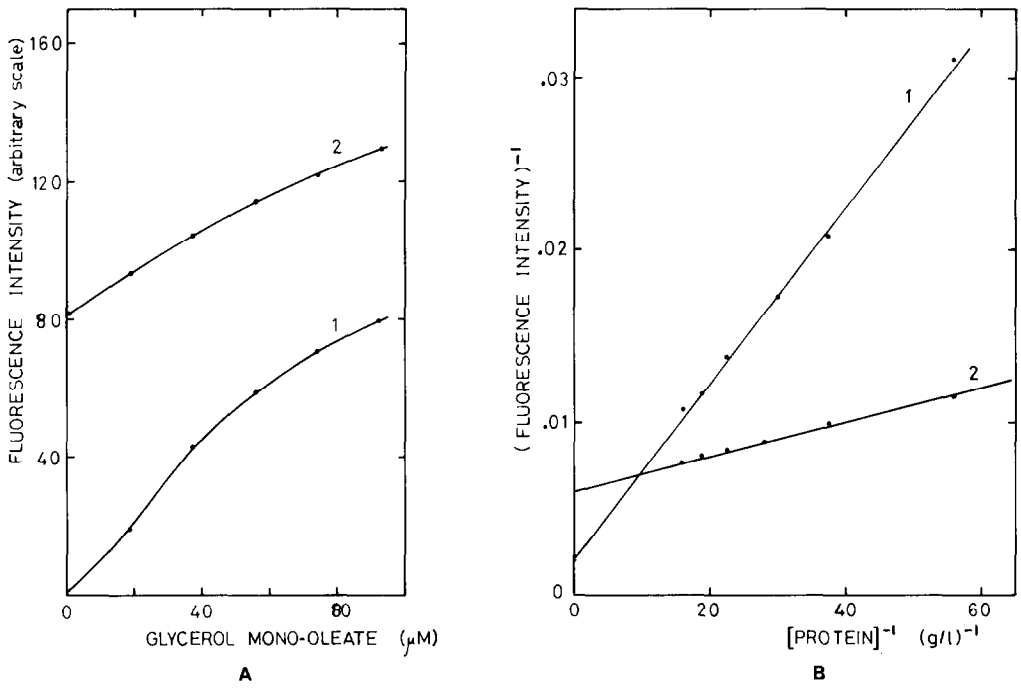

Fig.3(A). Effect of glycerol mono-oleate on the fluorescence of NPN (3 μM) in buffer and in the presence of erythrocyte membrane ghosts. 1. NPN in phosphate buffer, pH 7.4; 2. NPN with membrane ghost (0.053 mg/ml protein) in pH 7.4 buffer (B) Double reciprocal plots of NPN fluorescence (3 μM) as a function of membrane protein concentration. 1. In the absence of glycerol mono-oleate; 2. in the presence of 92.5 μM glycerol mono-oleate.

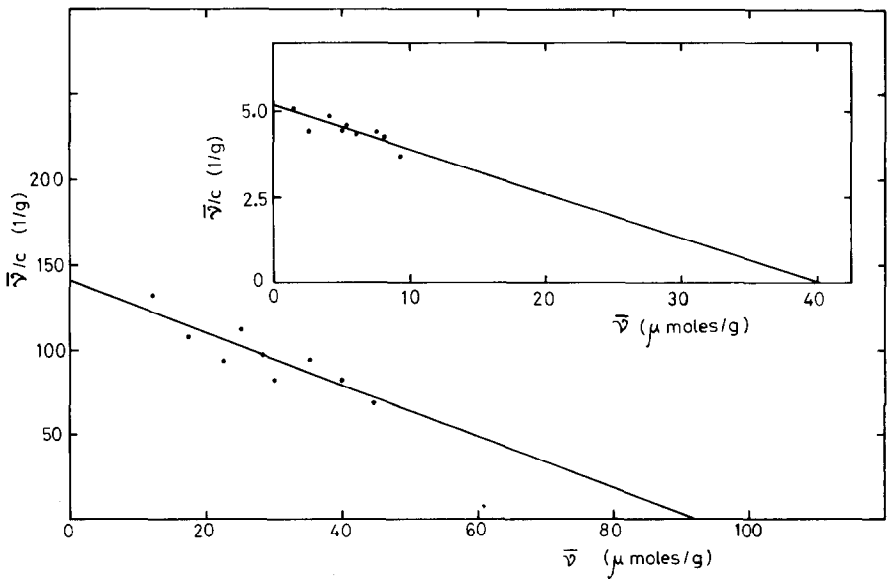

Fig.4. Scatchard plots for the binding of NPN (0–3 μM) to erythrocyte membranes (membrane protein concentration 0.053 mg/ml): in the presence of 92.5 μM glycerol mono-oleate, and, inset, in the absence of glycerol mono-oleate.

4. Discussion

There is good evidence that the negatively charged probe ANS binds to the membrane surface and orients with its sulphonic acid group in the aqueous phase [14,15]. The observation that ANS interacts with the erythrocyte membrane to produce an enhanced fluorescence and a blue-shift in the emission maximum compared with ANS in buffer is well documented and is consistent with the transfer of ANS to a more non-polar environment [16].

Changes in the packing of ANS between the polar headgroups and changes in the polar headgroup mobility express themselves through changes in the accessibility of water to the ANS and the reduction of the quantum yield [17]. An interpretation of the influence of the fusogen is that the decrease in quantum yield and the red shift are not simply a polarity effect but may be a result of the increased ability of the solvent molecules to rearrange during the lifetime of the excited state, that is, a decrease in the constraint around the probe molecules [11] producing a less ordered structure in the headgroup region. This is borne out by the observed increase in the affinity of the membrane for the probe, in the number of probe molecules bound and the enhancement of the fluorescence intensity.

The binding of ANS to membranes is strongly dependent on the surface charge of the membrane [18] and consequently may be used to indicate changes in surface potential [19]. Recent studies [20,21] have shown that the mean surface area per molecule and the mean surface potential per molecule of mixed phosphatidylcholine monolayers decreases as the content of glycerol mono-oleate in the monolayer increases due to a modification of the orientation of the polar groups of the choline-containing phospholipids. This is consistent with our observations on human erythrocyte ghosts of the increased binding of the amphiphilic probe at the membrane surface and the increased affinity of the membrane for the probe in the presence of glycerol mono-oleate.

The nonfusogen glycerol monostearate produces no effect on the quantum yield of ANS or its binding to erythrocyte membranes, but this may be accounted for by its possible inability to enter the membrane, in contrast to the fusogen. Furthermore it has been shown that this nonfusogen behaves ideally in mixed monolayers with phosphatidyl choline and has no effect on the membrane surface area and the surface potential [21].

NMR studies have shown that the fluorescent probe NPN penetrates deeply into the hydrocarbon region of the membrane [22]. The insertion of 92.5 μM glycerol mono-oleate into erythrocyte ghosts produces a decreased overlap between the emission and absorption spectra resulting in a lowered fluorescence quantum yield. However, fluorescence intensity is enhanced, the affinity of the membrane for the probe increases and more probe is bound. These observations support the suggestion that the glycerol mono-oleate is producing a less ordered structure in the membrane decreasing the constraint around the environment of the probe. Glycerol monostearate exerts no influence on the interaction of NPN with erythrocyte membranes.

We suggest that the insertion of glycerol mono-oleate into erythrocyte membrane ghosts increases the proportion of the hydrocarbon chains in the membrane that are in a relatively liquid state. Although no data have yet been published on the ability of glycerol mono-oleate to fuse ghosts, this work supports the suggestion that the role of the fusogenic lipid is at least partially a fluidising one.

Acknowledgements

We wish to thank Professor J. A. Lucy and Dr M. T. Flanagan for reading and commenting on the manuscript. We gratefully acknowledge personal support from the Medical Research Council to A. K.: intercalated B. Sc. course award.

References

[1] Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I. and Lucy, J. A. (1972) J. Cell Sci. 10, 769–787.
[2] Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I., Tampion, W., Verrinder, M. and Lucy, J. A. (1973) Nature 242, 215–217.
[3] Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1973) Biochem. J. 136, 147–155.
[4] Ahkong, Q. F., Fisher, D., Tampion, W. and Lucy, J. A. (1975) Nature 253, 194–195.
[5] Lucy, J. A. (1974) FEBS Lett. 40, S105–S111.

[6] Perez, H., Toister, Z., Laster, Y. and Loyter, A. (1974) J. Cell Biol. 63, 1–11.
[7] Kosower, Nechama, S., Kosower, Edward, M. and Wegman, Patricia (1975) Biochim. Biophys. Acta 401, 530–534.
[8] Kosower, E. M., Kosower, N. S., Faltin, Z., Diver, A., Saltoun, G. and Frensdorf, A. (1974) Biochim. Biophys. Acta 363, 261–265.
[9] Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[10] Lowry, O. H., Rosebrough, N. J., Parr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[11] Radda, G. K. (1971) in Current Topics in Bioenergetics (Rao Sanadi, D., ed), Vol. 4, pp. 81–126, Academic Press, New York and London.
[12] Brocklehurst, J. R., Freedman, R. B., Hancock, D. J. and Radda, G. K. (1970) Biochem. J. 116, 721–731.
[13] Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660–672.
[14] Lesslauer, W., Cain, J. and Blasie, J. K. (1971) Biochim. Biophys. Acta 241, 547–556.
[15] Radda, George, K. and Vanderkooi, Jane (1972) Biochim. Biophys. Acta 265, 509–549.
[16] Freedman, R. B. and Radda, G. K. (1969) FEBS Lett. 3, 150–152.
[17] Haynes, Duncan, H. and Staerk, Hubertus (1974) J. Membrane Biol. 17, 313–340.
[18] Träuble, Hermann and Overath, Peter (1973) Biochim. Biophys. Acta 307, 491–512.
[19] Flanagan, M. T. and Hesketh, T. R. (1973) Biochim. Biophys. Acta 298, 535–545.
[20] Maggio, B. and Lucy, J. A. (1975) Biochem. J. 149, 597–608.
[21] Maggio, Bruno and Lucy, Jack A. (1976) Biochem. J. 155, 353–364.
[22] Colley, C. M. and Metcalfe, J. C. (1972) FEBS Lett. 21, 241–246.