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INTERACTION OF TICLOPIDINE WITH THE ERYTHROCYTE MEMBRANE

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Abstract—The membrane effects of ticlopidine on the erythrocyte membrane were explored by the spin label method at the proteic and phospholipidic levels. This spectroscopic study was completed by polyacrylamide gel electrophoresis of proteins, measurement of the protection against haemolysis and observation of the erythrocyte shape changes induced by the drug. Two types of effects have been observed. At concentrations higher than 5×10^{-4} M, ticlopidine is a weak denaturating agent of the membrane proteins. At concentrations of pharmacological interest, the main effect of the drug is a protection against hypotonic haemolysis, and an increase in the fluidity of the membrane phospholipidic core. This last result could explain in part the interesting pharmacological effect of ticlopidine on various circulatory troubles.

Ticlopidine [5-(*O*-chlorobenzyl)-4,5,6,7-tetrahydrothieno-(3,2-c)-pyridine hydrochloride] is a platelet antiaggregant of low toxicity, in current clinical use in Europe. This highly hydrophobic molecule binds strongly and aspecifically on the erythrocyte membrane [1]. It was thus interesting to determine whether this drug induced physicochemical modifications of the erythrocyte membrane structure. For this aim we have used the spin label method and we have determined the osmotic resistance as well as the protein electrophoretic pattern of red blood cells incubated in the presence of increasing concentrations of ticlopidine.

MATERIALS AND METHODS

Pure ticlopidine was kindly supplied by Parcor (Toulouse).

Red blood cell samples. Blood was obtained from healthy young donors and collected on citrate–phosphate–dextrose. The red cells were washed free from plasma by centrifugation at 1000 *g* for 10 min in PBS (phosphate buffer saline) pH 8. Ghosts were prepared by the classical method of Dodge *et al.* [2]. Incubation in the presence of ticlopidine were performed for 10 min at 37°.

Spin labeling and recording of EPR spectra. The spin labels 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl and 2-(3-carboxypropyl)-2-tridecyl-4, 4-dimethyl-3-oxazolidinyloxyl [abbreviated as I (1,14) and I (12,3), respectively] were purchased from Syva (Palo Alto). The red blood cells were labeled by adding 1% of a 10^{-2} M stock solution of the label in DMSO to the red blood cell suspension.

Ghosts were labeled with MAL 5 (2,2′,5,5′-tetramethyl-4-maleimidopiperidinyl-*N*-oxyl) by incubation in a 10^{-4} M solution of the label in 5 mM phosphate buffer pH 8 for 12 hr at 4°. The unbound label was washed three times by centrifugation of the labeled ghosts in the same buffer.

Electron paramagnetic resonance spectra were recorded with an E3 or an E 109 Varian spectrometer equipped with a laboratory built temp. regulation device.

Osmotic resistance measurements. Osmotic resistance to haemolysis was measured in the following way: 500 μl of red blood cells preincubated in ticlopidine solution were diluted in 10 ml of 5 mM potassium phosphate buffer, pH 8. After 10 min at 4°, the suspensions were centrifuged at 45,000 *g* for 30 min. The hemoglobin concn in the supernatant was measured spectrophotometrically. The relative haemolysis was calculated by taking as 100% the haemolysis measured on red blood cell without ticlopidine

SDS polyacrylamide gel electrophoresis. Membrane proteins were separated by electrophoresis on 5–20% polyacrylamide gel plates in the presence of 0.1% sodium dodecyl sulfate according to Laëmmli [3]. After overnight coloration with Coomassie brilliant blue (2.5 g/l.) the gel was destained and then photographed. Densitometry of the dyed peptide bands was performed with a Vernon densitometer. The densitograms were digitized with a Hewlett–Packard HP 9874 A digitizer associated with a HP 9825 A desk computer and a HP 9872 A graphic plotter. The relative intensity of a given peptide band was defined as being the ratio of the area of this band to the total area of the densitogram.

Electron microscopy. For scanning electron micrography, erythrocytes were fixed in 1.5% glutaraldehyde in phosphate buffer and observed with an EM 300 Philips microscope.

RESULTS

Lipidic spin labeling of whole erythrocytes

I (12,3) *spin label.* Figure 1 shows the variation of the $2T_{//}$ parameter values measured in red blood cells incubated in the presence of 5×10^{-4} M ticlopidine as a function of temp. This parameter gives an indication on the degree of order of the phospho-

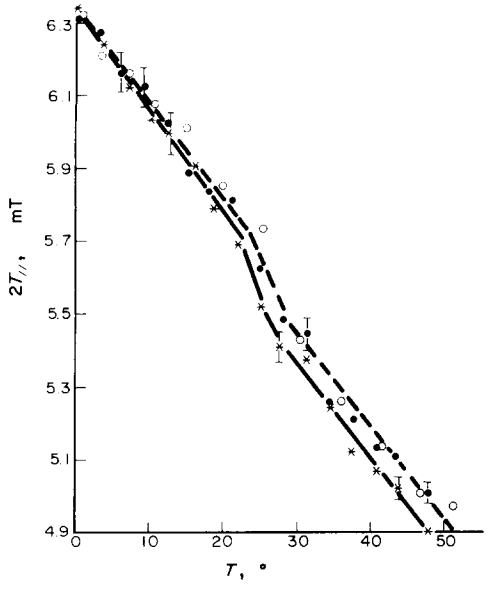

Fig. 1. Variation of 2 $T_{//}$ (mT) as a function of temp. when whole blood is incubated before washing the erythrocytes in PBS and labeling with 5 NS. (*)Incubation in 5×10^{-4} M ticlopidine for 60 min; (●) incubation in buffer; (○) without incubation.

lipidic fatty acid chains [4,5]. In this case ticlopidine was added to whole blood before washing in PBS and labeling with I (12.3). No variation in the 2 $T_{//}$ parameter is observed at a temp. below 20°. A diminution of 0.05–0.1 mT in 2 $T_{//}$ is measured at higher temps. This result is not observed with lower ticlopidine concns and when the incubation with the drug was performed on washed red blood cells.

I (1,14) *spin label.* The spectra obtained with this label have been interpreted by the classical formula [6] giving the apparent rotation frequency of the nitroxide group:

$$\nu = \left[6.5 \times 10^{-10} \times \Delta H_0 \left(\sqrt{\frac{h_0}{h_{+1}}} - 1\right)\right]^{-1},$$

were ΔH_0 is the line width of the central line and h_0 and h_{+1} are, respectively, the amplitude of the central and of the low field lines. Each sample of labeled red blood cells was studied between 0 and 45° and the spectra recorded at 3° intervals. The activation energy of rotation of the label was calculated from plots of the logarithm of the rotation frequency as a function of the reciprocal absolute temperature, by the Arrhenius equation:

$$\text{Ln } \nu = -\frac{E_a}{RT} + C.$$

The effect of ticlopidine has been studied for concns ranging from 5×10^{-6} to 5×10^{-4} M. For each concn 2–5 different red blood cell samples were used. A statistical analysis of variance was performed on all these results. The Arrhenius plots are linear in the whole range of temp. studied, and the activation energy values are not significantly modified by the drug (Table 1). However, the rotation frequency of the label was found to be significantly higher in the membrane of erythrocytes incubated in ticlopidine than in the control (Table 2), but no significant differences were observed between the different ticlopidine concentrations used.

Spin labeling of erythrocyte ghost proteins

The maleimide nitroxide spin label MAL 5 is covalently bound on the thiol groups of the membrane proteins. In the spectra one observes simultaneously

Table 1. Values of the activation energies of rotation of the 16 NS spin label in control and ticlopidine incubated erythrocytes

Ticlopidine concn (M)	0	5×10^{-6}	10^{-5}	5×10^{-5}	10^{-4}	5×10^{-4}
No. of experiments	5	2	4	4	3	3
Activation energies (kJ/mole)	14.36	13.50	12.88	13.22	14.95	13.33

No significant differences are observed.

Table 2. Values of the rotation frequency of the label ($\times 10^{-9}$) as a function of temp. for various ticlopidine concns

Ticlopidine concn (M)	Temp. (°)				
	4.15	14.5	26	34.3	43.1
0	1.98 ± 0.28	2.60 ± 0.23	3.15 ± 0.28	3.56 ± 0.32	4.33 ± 0.36
5×10^{-6}	2.46 ± 0.11 (0.001)	2.99 ± 0.27 (0.005)	3.8 ± 0.23 (0.005)	4.52 ± 0.34 (0.001)	4.89 ± 0.32 (0.005)
10^{-5}	2.38 ± 0.21 (0.001)	2.85 ± 0.23 (0.005)	3.45 ± 0.29 (0.025)	4.02 ± 0.36 (0.005)	4.37 ± 0.45 (0.02)
5×10^{-5}	2.47 ± 0.46 (0.005)	2.92 ± 0.25 (0.001)	—	4.17 ± 0.33 (0.001)	5 ± 0.47 (0.005)
10^{-4}	2.30 ± 0.15 (0.01)	2.95 ± 0.44 (0.01)	3.66 ± 0.62 (0.05)	4.43 ± 0.73 (0.005)	5.29 ± 0.84 (N.S.)
5×10^{-4}	2.34 ± 0.27 (0.01)	3.03 ± 0.35 (0.001)	3.54 ± 0.27 (0.02)	4.1 ± 0.45 (0.005)	5.18 ± 0.4 (0.02)

Numbers in parentheses indicate the level of confidence for the difference between assays and control.

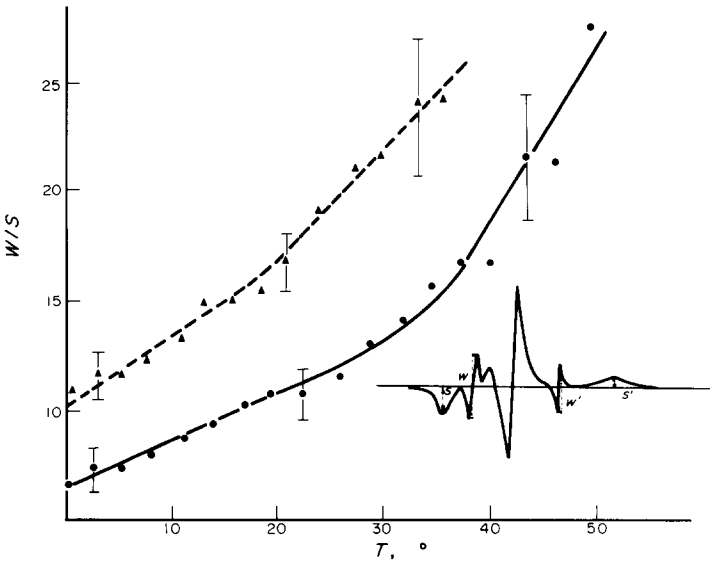

Fig. 2. Variation of the W/S parameter against temp. (●) Incubation of ghosts with 10^{-3} M ticlopidine; (▲) incubation of ghosts without ticlopidine.

lines which are due to mobile (lines W and W') and strongly immobilized labels (lines S and S') (Fig. 2). The ratio of the amplitude of lines W and S is an empirical, but widely used, means to quantify these spectra [7–9]. Figure 2 shows the effect obtained on the W/S ratio by the incubation of ghosts in 10^{-3} M ticlopidine. A decrease of approx. 60% is observed in the whole range of temp. studied. This effect is significant only at this high concn and is practically not reversed when the drug is washed off by centrifugation in PBS.

Effect of ticlopidine on the osmotic resistance

The compound strongly protects the red blood cells against haemolysis, since only 70% relative haemolysis is observed in hypotonic buffer in the presence of 2×10^{-5} M ticlopidine. This effect is biphasic as shown in Fig. 3. At high concns the drug induces haemolysis even in isotonic buffer.

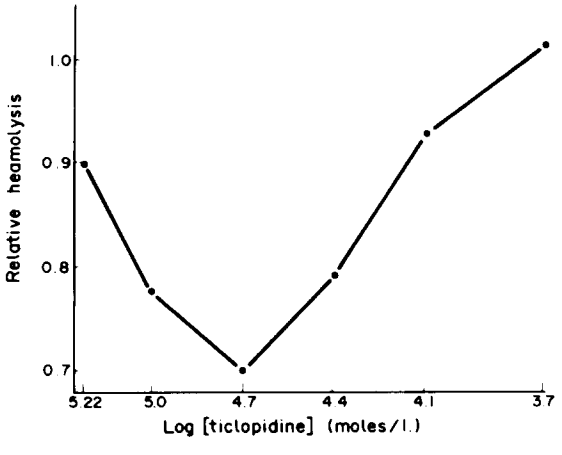

Fig. 3. Variation of the relative rate of haemolysis vs the concn of ticlopidine used for incubation.

Polyacrylamide gel electrophoresis of erythrocyte membrane proteins

Whole erythrocytes were incubated in PBS at 37° for 10 min in various concns of ticlopidine. After three washings, packed red blood cells (1 ml) were haemolysed and the ghosts centrifuged. The protein content was measured by the method of Lowry and aliquots of membranes corresponding to 150 μg protein were submitted to SDS polyacrylamide gel electrophoresis. The densitograms showed no effect of ticlopidine at concns lower than 5×10^{-4} M. At higher concns a perturbated electrophoretic pattern was observed.

This perturbation was also observed when ghosts were incubated in 10^{-3} M ticlopidine. Figure 4 shows that new peptide bands appear between bands 2 and 3, band 3 intensity is reduced, bands 4.1 and 4.2 seem to be displaced, and bands of low mol. wt are notably modified.

At lower drug concns the electrophoretic pattern is not significantly modified. However, although the intensity of all the bands is very reproducible, that of band 6 (glyceraldehyde-3-phosphate dehydrogenase) shows an important dispersion in the samples submitted to low concns of the drug compared with the control samples.

Shape of the erythrocytes

Examined by scanning electron microscopy, erythrocytes incubated for 15 min in 5×10^{-4} M ticlopidine virtually have the same shape as stomatocytes (Fig. 5). The same effect is observable with the light microscope on erythrocytes in suspension.

DISCUSSION

Our results show two types of effect of ticlopidine on the erythrocyte membrane. At high concns of the drug ($<5 \times 10^{-4}$ M), one observes haemolysis of the

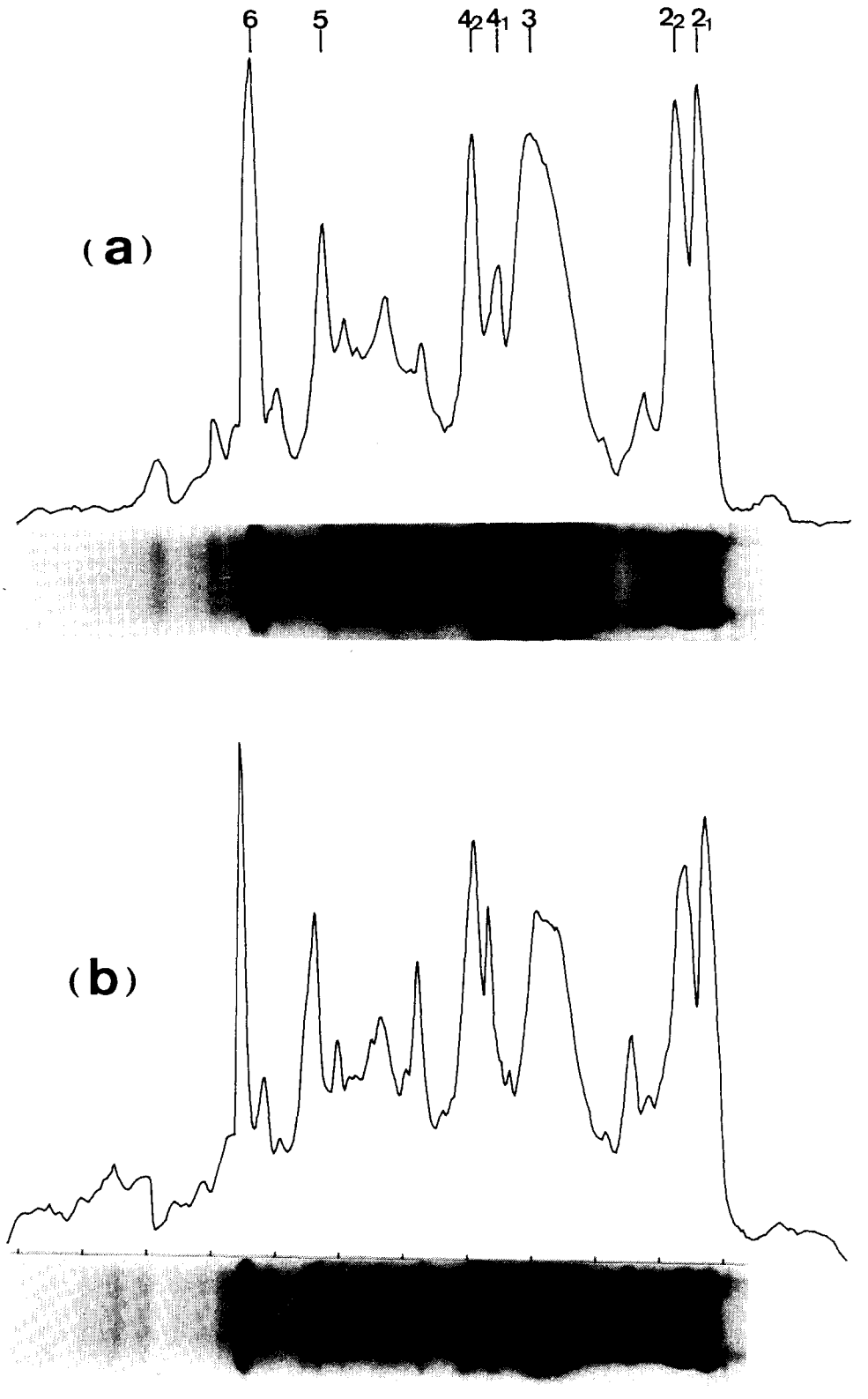

Fig. 4. Electrophoretic pattern of erythrocyte membrane proteins. (a) Control ghosts or ghosts incubated in 10^{-6} M ticlopidine; (b) ghosts incubated in 10^{-3} M of the drug.

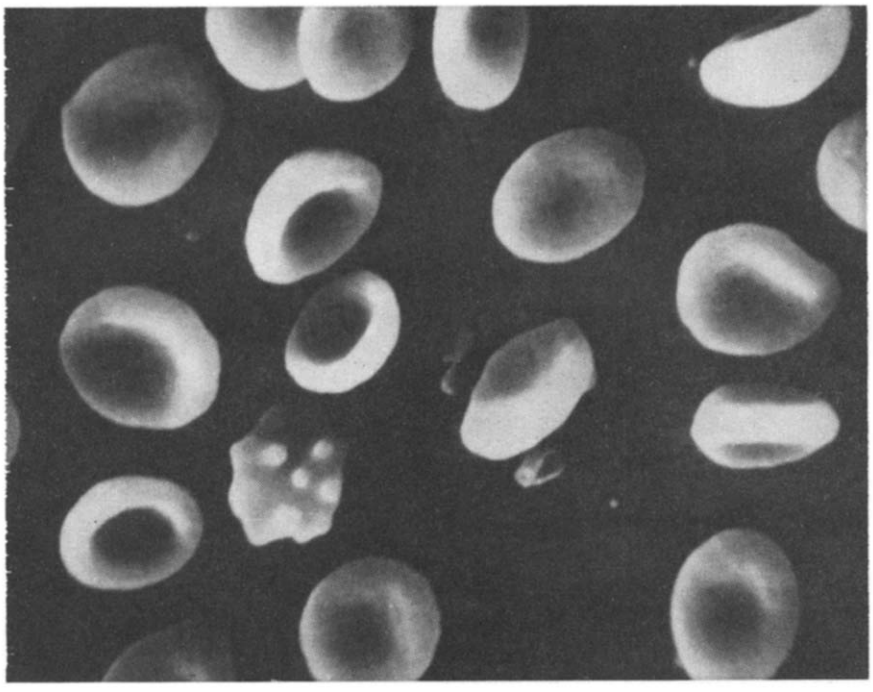

Fig. 5. Scanning electron micrography of erythrocytes incubated in 10^{-4} M ticlopidine (magnification, × 2500).

red blood cells and a denaturating effect on the membrane proteins as indicated by electrophoresis and irreversible modifications of the spectra of the spin labeled proteins. At low concns, the drug protects the erythrocytes against haemolysis, and increases the fluidity of the hydrophobic core of the phospholipidic bilayer.

The highest concns used in this study induces non-specific denaturating effects on the membrane proteins, similar to those observed with many hydrophobic compounds, such as phenothiazine derivatives [7] and detergents. This protein denaturation also leads to a partial disorganization of the phospholipidic bilayer as indicated by the decrease in the $2T_{//}$ parameter. The only interest of the results obtained at high ticlopidine concn is to indicate *a contrario* that this drug has no dramatic perturbating effects on the membrane in its normal pharmaceutical use, since the concn reached in the body fluids are never higher than 10^{-5} M (B. Lacaze, personal communication).

At pharmacologically useful concns the principal effects observed are the protection against haemolysis and the fluidification of the phospholipid bilayer. Non-specific interaction of ticlopidine with the erythrocyte membrane has been previously demonstrated with tritiated drugs by Sablayrolles *et al.* [1]. They showed a non-saturable and irreversible binding which was reversed neither by dilution nor by washing, and was not modified after denaturation of the membrane proteins by boiling. These results strongly suggest a preferential interaction of the drug with the phospholipids.

The stomatocytogenic effect of ticlopidine suggests a preferential interaction of the drug with the inner leaflet of the bilayer as proposed by Sheetz and Singer in their bilayer couple theory [10]. Recent results with chlorpromazine, which is also a cup former [10], argue in favour of this theory: fluidification of the membrane by the drug decreases the activity of adenylate cyclase by perturbing the coupling between the regulatory and enzymatic subunits of the enzyme complex, these subunits being located at the inner face of the membrane [11].

The mechanism of the protecting effects of many drugs against hypotonic haemolysis is unclear [12]. The biphasic curve generally observed could be due to the summation of opposite effects. At low concns these drugs most frequently induce an increase in membrane fluidity which decreases its stiffness. We have recently observed that reducing the bending stress of the membrane increases the phospholipidic fluidity [13]. At high concns the denaturing effects are predominant and the mechanical resistance of the membrane is impaired.

In conclusion, ticlopidine acts at low concns on the erythrocyte membrane by increasing the fluidity of the hydrophobic core of the phospholipidic bilayer. If the effect exists also on the platelet membrane it can, in part, explain the antiaggregating properties of the drug since platelet aggregation is strongly dependent on its membrane state [14,15]. Concerning the erythrocyte, this increase in fluidity could be beneficial from a rheological point of view, increasing the circulation in microcapillaries of both normal and pathological erythrocytes which could help in reducing the effects of sickle cell anaemia.

REFERENCES

1. M. Sablayrolles, H. Wajcman and D. Labie, *International Symposium on Abnormal Hemoglobins: Genetics Populations and Diseases*, p. 20. Jerusalem, 6–11 September 1981.

2. J. T. Dodge, C. Mitchell and D. J. Hanahan, *Archs. biochem. Biophys.* **100**, 119 (1963).
3. U. K. Laëmmli, *Nature, Lond.* **227**, 680 (1970).
4. L. J. Berliner, *Spin Labeling, Theory and Applications*, Vol I. Academic Press, New York (1976).
5. J. Viret and F. Leterrier, *Biochim. biophys. Acta* **436**, 811 (1976).
6. S. Henry and A. Keith, *Chem. Phys. Lipids* **7**, 245 (1971).
7. F. Leterrier, F. Rieger and J. F. Mariaud, *Biochem. Pharmacol.* **23**, 103 (1974).
8. J. L. Rigaud, C. M. Gary-Bobo and C. Taupin, *Biochim. biophys. Acta* **373**, 211 (1974).
9. J. Viret, D. Dormont, D. Molle, L. Court, F. Leterrier, F. Cathala, C. J. Gibbs, Jr. and D. C. Gajdusek, *Biochem. Biophys. Res. Commun.* **101**, 830 (1981).
10. M. P. Sheetz and S. J. Singer, *Proc. Natn. Acad. Sci. U.S.A.* **71**, 4457 (1974).
11. R. Salesse, J. Garnier and D. Daveloose, *Biochemistry* **21**, 1587 (1982).
12. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
13. M. Bitbol and F. Leterrier, *Biorheology* **19**, 482 (1982).
14. S. J. Shattil and R. A. Cooper, *Biochemistry* **15**, 4832 (1976).
15. R. W. Colman, J. Kuchibhotla, M. K. Jain and R. K. Murray, *Biochim. biophys. Acta* **467**, 273 (1977).