BINDING OF AMPHIPATHIC DRUGS AND PROBES TO BIOLOGICAL MEMBRANES

I. K. MOULES, E. K. ROONEY and A. G. LEE
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, S09 3TU, England

Received 30 November 1981

1. Introduction

It has been argued that small amphipathic molecules cannot bind to the lipid phase of biological membranes because of a 'large internal pressure' caused by the proteins in the membrane [1–3]. This idea, if generally true, would have a number of important consequences: (i) It would mean that many studies of membranes employing spectroscopic probes were incorrectly interpreted since typical electron spin resonance (ESR) and fluorescence probes are small amphipathic molecules which had been assumed to probe the lipid phase of the membrane [4]; (ii) Many drugs are small amphipathic molecules which have also been assumed to bind to the lipid phase of membranes; again this would be in error. The binding detected by centrifugal methods [5] had been attributed by Singer 'to some type of mixed micelle formation between the amphipath and some small amounts of components extracted by the amphipath from the membranes', the mixed micelles being loosely associated with the membrane.

Here we report ESR and fluorescence studies which suggest that amphipathic molecules can bind to the hydrophobic phase of typical biological membranes such as the sarcoplasmic reticulum from muscle, with no evidence for micelle formation. From ESR spectra observed for spin labelled fatty acids we argue that the fatty acids can bind normally to the membrane. We then use the fluorescence quenching properties of the spin-labelled fatty acids to argue that a fluorescent drug analogue, dansyl propranolol, can also bind to the membrane.

2. Materials and methods

Spin-labelled fatty acids were obtained from Synva and dansyl propranolol from Molecular Probes Inc. The hydrophobic tryptophan analogue *N*-palmitoyl–L-tryptophan–*n*-hexyl ester was prepared by esterification of tryptophan with thionyl chloride in hexanol followed by coupling to palmitic acid with *N,N'*-dicyclohexylcarbodiimide. Sarcoplasmic reticulum was obtained from rabbit muscle as in [6] and $(Ca^{2+} + Mg^{2+})$-ATPase was purified from it by the cholate procedure in [6]. The final lipid:protein molar ratio for the $(Ca^{2+} + Mg^{2+})$-ATPase was 30:1, whereas that in the original sarcoplasmic reticulum was 90:1, assuming M_r 115 000 for the ATPase. Lipid and protein concentrations were determined as in [6]. For convenience, concentrations of purified $(Ca^{2+} + Mg^{2+})$-ATPase are expressed in terms of the equivalent lipid concentration.

For ESR studies, spin label was dried from methanol onto the sides of flasks to which the membrane in buffer (10 mM sodium phosphate, 0.1 M NaCl, pH 7.2) was added. After incubation for 30 min, the samples (50 μl) were placed in melting point tubes. Spectra were recorded at 22°C on a Braker 200 D ESR spectrometer, digitized using a 12-bit Vector-Graphic A/D board and accumulated using a Z-80 based microcomputer system. Fluorescence spectra were recorded on a Perkin-Elmer MPF44A fluorimeter, and digitized using the same microcomputer system as for the ESR spectra. Dansyl propranolol and spin-labelled fatty acids were added to the membrane samples as concentrated solutions in methanol.

Enzyme activity was measured using a coupled enzyme system [6]. Free calcium concentrations were calculated using the binding constants in [7].

3. Results and discussion

Fig.1 compares the ESR spectra of 3 different fatty acid probes

Published by Elsevier Biomedical Press
0014593/82/0000–0000/$02.75

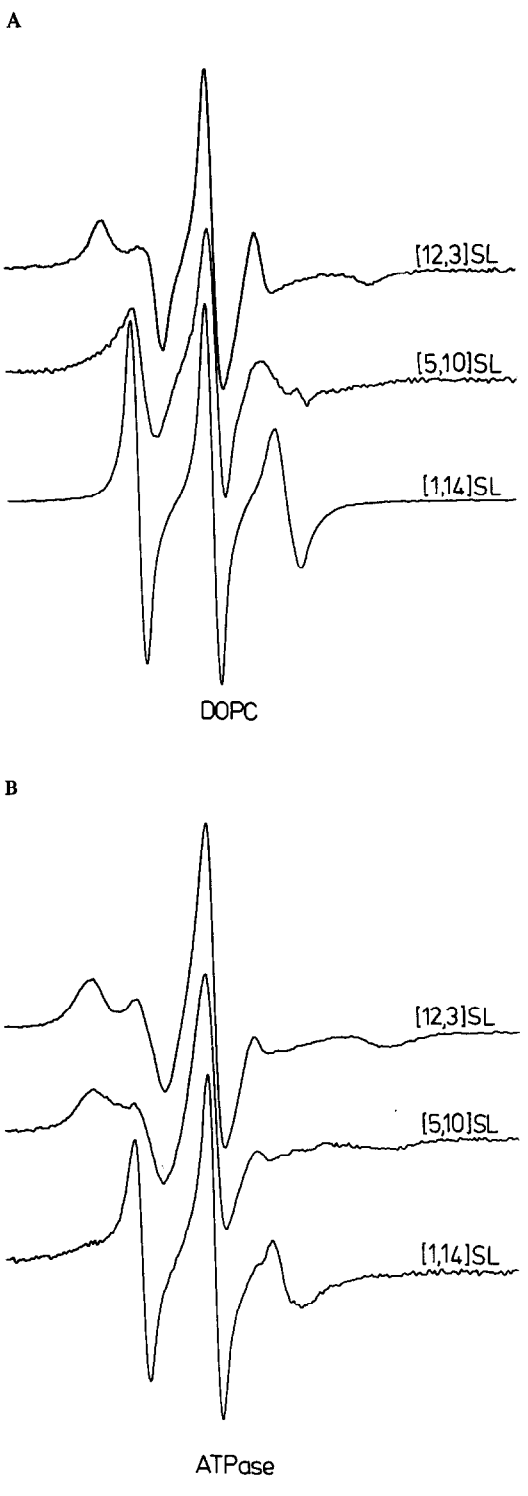
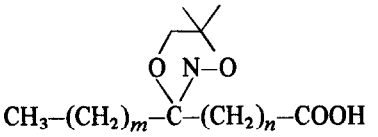


Fig.1. ESR spectra of spin-labelled fatty acids at lipid:probe molar ratios of 90:1 in (A) dioleoylphosphatidylcholine and (B) $(Ca^{2+} + Mg^{2+})$-ATPase.



incorporated into bilayers of dioleoyl phosphatidylcholine and into purified ATPase, at a 1:90 fatty acid:lipid molar ratio. Spectra of the [12,3] and [1,14] spin labels are very similar in the 2 systems, whereas the [5,10] spin label shows a greater immobilization in the ATPase system. The spectra in both dioleoyl phosphatidylcholine and in ATPase show increasing immobilization as the nitroxide group moves closer to the carboxyl group of the fatty acid, and an exactly similar result is seen in sarcoplasmic reticulum. Many observations of this kind have been made for other membranes using spin-labelled phospholipids and are attributed to the 'fluidity gradient' present in lipid bilayers with greater fluidity towards the centre of the bilayer [8]. These spectra then suggest that the fatty acid probes are in essentially similar environments in lipid bilayers and membranes. The greater immobilization seen in the presence of the ATPase is consistent with observations made with other probe techniques. For example, studies of the fluorescence depolarization of diphenyl hexatriene show a decreasing 'fluidity' with increasing molar ratio of protein to lipid, from dioleoyl phosphatidylcholine to sarcoplasmic reticulum to $(Ca^{2+} + Mg^{2+})$-ATPase (unpublished).

Micelle formation by spin-labelled fatty acids would be expected to give rise to spectra broadened by dipolar and exchange interactions between the nitroxide groups. Spectra such as those in fig.1b show no sign of any such broadening. However, broadening can be induced if the concentration of spin label within the membrane is increased. This is illustrated for dioleoyl phosphatidylcholine in fig.2a.

(i) These spectra demonstrate the partition of spin label between the lipid and aqueous phases. With decreasing amounts of lipid, there is an increase in the magnitude of the ESR spectrum corresponding to spin label free in the aqueous phase.

(ii) As the lipid concentration decreases, the spin label:lipid molar ratio within the membrane increases, leading to increased broadening of the spectra. Such broadened spectra can be readily simulated (unpublished) using the theoretical approach in [9].

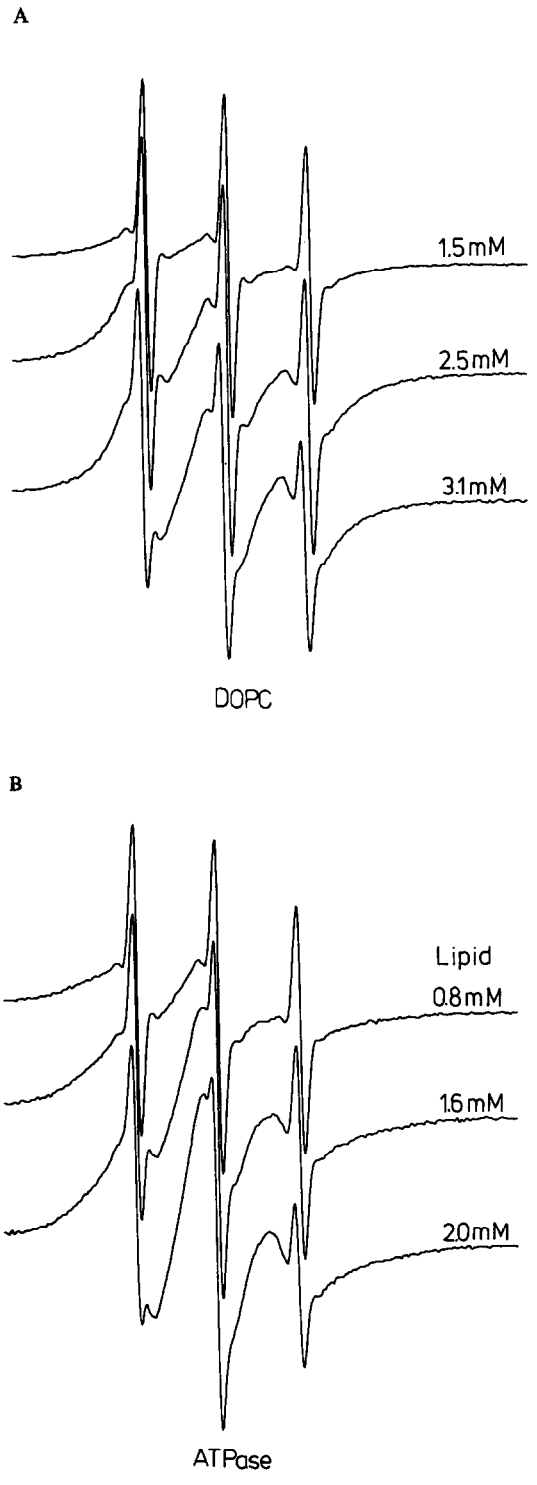


Fig.2. ESR spectra of [1,14] spin-labelled fatty acid (0.21 mM) in the presence of (A) dioleoylphosphatidylcholine and (B) $(Ca^{2+} + Mg^{2+})$-ATPase at the given lipid concentrations.

As shown in fig.2b, similar results are obtained with the $(Ca^{2+} + Mg^{2+})$-ATPase. Partitioning of spin label into this system is greater than for the simple lipid bilayer when calculated on the basis of lipid concentration, but this is probably rather misleading. Binding of spin label to the membrane is limited by the build up of charge on the membrane [10], and calculations of charge/unit area must take into account the area occupied by protein in the $(Ca^{2+} + Mg^{2+})$-ATPase.

It is clear that the observed changes in the ESR spectra as a function of spin label concentration are similar for dioleoyl phosphatidylcholine and for the $(Ca^{2+} + Mg^{2+})$-ATPase and are entirely consistent with lipid and spin-labelled fatty acids forming a mixed phase. If micelle formation had occurred, the ESR spectrum would have been expected to be a single, very broad line (as seen for micelles of spin-labelled fatty acid in water), the intensity of the line simply increasing with increasing concentration of spin label.

These ESR spectra are then entirely consistent with the spin labels partitioning into the lipid phase of the membrane. This is also suggested by the fluorescence quenching experiments shown in fig.3. Addition of liposomes of dioleoyl phosphatidylcholine to the hydrophobic tryptophan analogue *N*-palmitoyl–L-tryptophan–*n*-hexyl ester results in a large enhancement of fluorescence, consistent with partitioning into the lipid bilayer. Addition of spin-labelled fatty acids results in fluorescence quenching as shown in fig.3a. The quenching does not follow simple Stern-Volmer kinetics but is found to be dependent on lipid concentration, with the greatest quenching being observed for a given spin label concentration at the lowest lipid concentration. This is the expected result if it is not the free spin label concentration that is significant, but rather the concentration within the lipid bilayer. Quenching of the fluorescence of the $(Ca^{2+} + Mg^{2+})$-ATPase by spin-labelled fatty acid shows a similar dependence on membrane concentration (fig.3b), again consistent with partition of the fatty acid into the membrane. The majority of the tryptophan residues in the $(Ca^{2+} + Mg^{2+})$-ATPase are in hydrophobic regions of the sequence, presumably located within the membrane phase [11]. Fluorescence quenching by the hydrophilic quencher KI is much less marked than that observed with the spin labelled fatty acids.

For all the above reasons, therefore, we believe that spin labels do, indeed, probe the hydrophobic

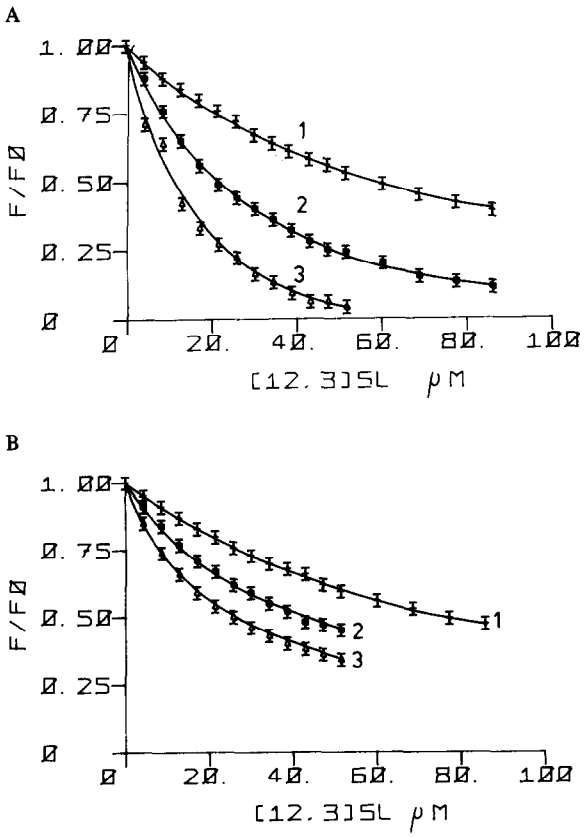


Fig.3.(A) Quenching of the fluorescence of *N*-palmitoyl–L-tryptophan–*n*-hexyl ester at a lipid:tryptophan molar ratio of 30:1 in dioleoylphosphatidylcholine at (1) 180, (2) 60 and (3) 30 μM, by [12,3] spin-labelled fatty acid. (B) Quenching of the tryptophan fluorescence of $(Ca^{2+} + Mg^{2+})$-ATPase at (1) 60, (2) 30 and (3) 15 μM lipid by [12,3] spin-labelled fatty acid.

regions of membranes and do not form micelles on the membrane surface. It then follows that it is possible to use the spin labels as markers for the hydrophobic phase of the membrane in fluorescence quenching experiments. We have explored this possibility with the fluorescent drug analogue dansyl propranolol.

Addition of dioleoyl phosphatidylcholine to dansyl propranolol results in a large enhancement of fluorescence that can be attributed to binding to the lipid bilayer (fig.4). The resulting spectrum can be well fitted to a sum of two skewed Gaussian peaks, one centred at 525 nm and characteristic of lipid bound probe and a small one centred at 565 nm characteristic of dansyl propranolol free in solution. Full details of these fitting procedures will be published

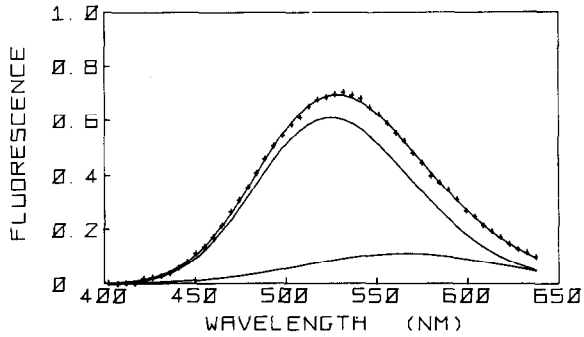


Fig.4. Fluorescence emission spectrum excited at 340 nm for 4 μM dansyl propranolol in the presence of dioleoylphosphatidylcholine + experimental data points. Solid lines, least-squares, best-fitted spectrum, and the 2 components of the fit.

elsewhere. Addition of $(Ca^{2+} + Mg^{2+})$-ATPase to dansyl propranolol also results in an enhancement of fluorescence, but the spectrum is somewhat blue-shifted compared to that in lipid alone. The blue shift is more marked at an excitation wavelength of 280 nm than at 340 nm. The former wavelength excites dansyl fluorescence via energy transfer from the protein tryptophan groups whereas the latter directly excites the dansyl fluorescence. As shown in fig.5, the spectrum can be well fitted to the sum of two skewed Gaussian peaks, centred at 489 nm and 525 nm (when fluorescence is excited at 280 nm, the contribution from dansyl propranolol in the aqueous phase is negligible). The latter is attributed to dansyl propranolol bound to lipid and the former to dansyl propranolol bound to protein sites within the membrane. Consistent with

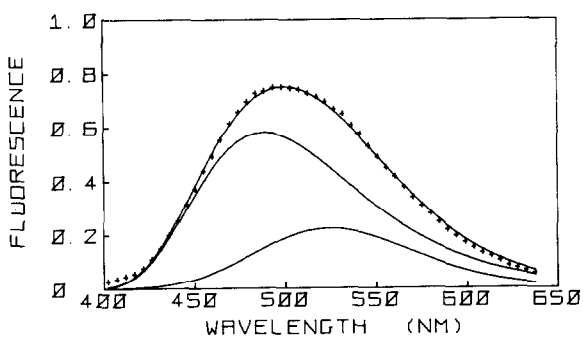


Fig.5. Fluorescence emission spectrum excited at 280 nm for 2.5 μM dansyl propranolol in the presence of 0.4 mg/ml $(Ca^{2+} + Mg^{2+})$-ATPase + experimental data points. Solid lines, least-squares best fitted spectrum, and the 2 components of the fit.

this interpretation we find that for sarcoplasmic reticulum the protein component of the spectrum is reduced in intensity relative to the lipid component when compared to purified (Ca^{2+} + Mg^{2+})-ATPase: the protein:lipid ratio in sarcoplasmic reticulum is lower than in purified ATPase. The component at 489 nm is also less marked in spectra excited at 340 nm, again consistent with the 489 nm component being due to protein-bound probe. We have found that the fluorescence emission spectrum depends both on the dansyl probe employed and on the membrane. Thus in fluorescence emission spectra for dansyl propranolol bound to membranes enriched in acetylcholine receptor from *Torpedo californica,* the lipid-bound component is much reduced compared to the protein component (unpublished) and with dansyl undecanoic acid, the separation between lipid and protein peaks in the spectrum when bound to (Ca^{2+} + Mg^{2+})-ATPase is greater than with dansyl propranolol (unpublished).

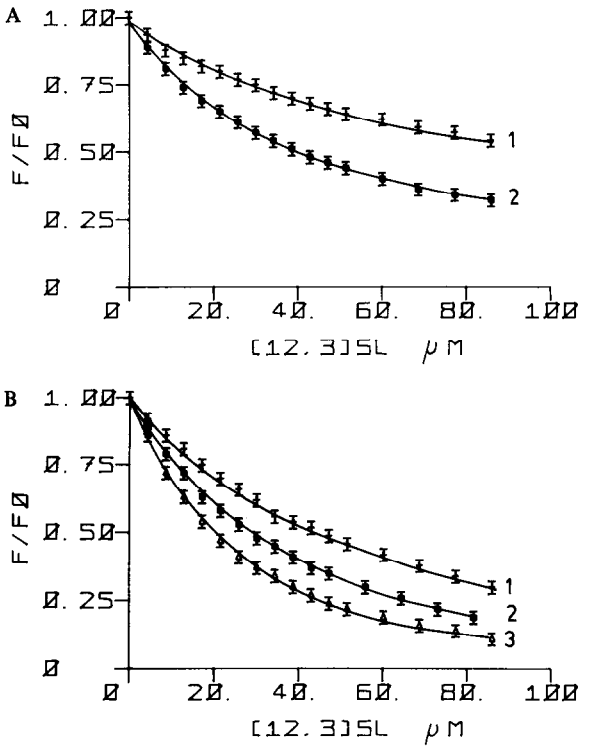


Fig.6. Fluorescence quenching of 2.5 μM dansyl propranolol by [12,3] spin-labelled fatty acid. Fluorescence was excited at 340 nm and measured at 530 nm: (A) in the presence of dioleoylphosphatidylcholine at (1) 180 and (2) 60 μM; (B) in the presence of (Ca^{2+} + Mg^{2+})-ATPase at (1) 60, (2) 30 and (3) 15 μM lipid.

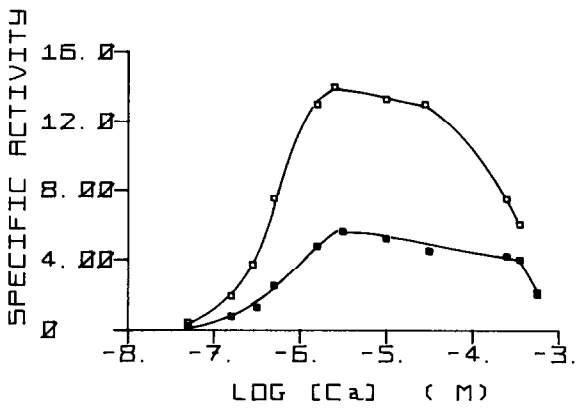


Fig.7. Effect of dansyl propranolol on the specific activity (IU) of (Ca^{2+} + Mg^{2+})-ATPase at 37°C as a function of [Ca^{2+}]: (□) zero drug; (■) 2 μM dansyl propranolol.

Fig.6 shows that spin labelled fatty acids cause a very similar quenching of dansyl propranolol in the presence of dioleoyl phosphatidylcholine and in the presence of (Ca^{2+} + Mg^{2+})-ATPase: directly comparable results are observed with sarcoplasmic reticulum. The dependence of quenching on lipid and membrane concentration is again consistent with quenching following from partition into the hydrophobic phase of the membrane. Both protein and lipid components of the spectra are quenched, suggesting that the spin labelled fatty acids have access to both the lipid and protein binding sites.

Finally, fig.7 shows the effect of dansyl propranolol on ATPase activity, as a function of [Ca^{2+}]. As we have found for a variety of other positively charged drugs, dansyl propranolol causes both a small reduction in the Ca^{2+} affinity of the ATPase and a reduction in the maximum activity. The effects are fully reversible.

4. Conclusion

We draw the following conclusions:

(1) The similarity of the ESR spectra, spectral broadening and spin-labelled fatty acid partitioning of simple lipid bilayers, sarcoplasmic reticulum and (Ca^{2+} + Mg^{2+})-ATPase, suggest that the spin labels are probing similar hydrophobic regions in both the lipid bilayers and the biological membranes.

(2) The quenching of protein tryptophan residues on the (Ca^{2+} + Mg^{2+})-ATPase by spin-labelled fatty acids are consistent with contact between the fatty acids and the tryptophans present in hydrophobic regions of the protein.

(3) The fluorescence spectrum of dansyl propranolol in the presence of membranes suggests that the drug analogue can bind to both lipid and protein sites within the membrane.

(4) The quenching of the fluorescence of dansyl propranolol in the presence of membranes by spin-labelled fatty acids is again consistent with dansyl propranolol binding to hydrophobic sites within the membrane.

None of the above observations can be readily interpreted as due to micelle formation on the surface of the membrane.

Acknowledgements

We thank the Science Research Council for a Studentship to I.K.M. and the Science Research Council and the Muscular Dystrophy Association of America for financial support. We thank Rosemary Diggens for the preparation of the tryptophan analogue and Jenny Schofield for preparation of membrane samples.

References

[1] Conrad, M. J. and Singer, S. J. (1979) Proc. Natl. Acad. Sci. USA 76, 5205–5206.
[2] Conrad, M. J. and Singer, S. J. (1981) Biochemistry 20, 808–818.
[3] Singer, S. J. (1981) Biochem. Soc. Trans. 9, 203–206.
[4] Lee, A. G. (1978) in: Receptors and Recognition (Cuatrecasas, P. and Greaves, M. F.) vol. 5A, Chapman and Hall, London.
[5] Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
[6] Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974) Proc. Natl. Acad. Sci. USA 71, 622–626.
[7] Vianna, A. L. (1975) Biochim. Biophys. Acta 410, 389–406.
[8] Berliner, L. J. ed (1976) Spin Labelling, Academic Press, New York.
[9] Sackmann, E. and Trauble, H. (1972) J. Am. Chem. Soc. 94, 4492–4498.
[10] Lee, A. G. (1978) Biochim. Biophys. Acta 514, 95–104.
[11] Allen, G., Trinnaman, B. J. and Green, N. M. (1980) Biochem. J. 187, 591–616.