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# A FLUORESCENCE POLARIZATION STUDY OF CALCIUM AND PHASE BEHAVIOUR IN SYNAPTOSOMAL LIPIDS

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**Steady-state fluorescence polarization of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene reported temperature-dependent lipid order in L-α-dimyristoylphosphatidylcholine, egg phosphatidylcholine and synaptosomal membranes. No change in lipid order was detected after depolarization of synaptosomes by veratridine (150 μM) even in the presence of 2 mM $CaCl_2$. However, $Ca^{2+}$ reduced the mobility of a second probe, dansylated dipalmitoylphosphatidylethanolamine, in dispersions of synaptosomal lipids. This effect, which was seen at a $Ca^{2+}$/total phospholipid ratio as low as 0.1, may represent an interaction between the cation and negatively-charged phospholipids. It is suggested that $Ca^{2+}$ promotes a phase separation in synaptosomal lipids which may be relevant to the process of neurotransmitter release.**

## Introduction

Although it is accepted that the lipids of mammalian cell membranes normally adopt a bilayer structure their large variety even in functionally 'simple' membranes is superfluous to such a requirement [1]. Also, in important details, many lipids are unsuited [2] to their putative roles as specific activators of membrane proteins. Moreover, given an invariant bilayer structure, it is difficult to envisage the mechanisms of such dramatic structural rearrangements as cell division and membrane fusion.

With such considerations in mind, we set out to investigate the disposition of synaptosomal membrane lipids during the process of neurotransmitter release and during membrane interaction with calcium, an event known to be central to evoked release [3,4]. We adopted the procedure of measuring steady-state polarization in fluorescently labelled membrane systems, and changes were sought against a background of varying temperature knowing that similar changes can be initiated by chemical events [1].

## Materials and Methods

### *Materials*

1,6-Diphenyl-1,3,5-hexatriene (DPH) and dansylated dipalmitoylphosphatidylethanolamine (dns-DPPE) were obtained from Molecular Probes Inc., Plano TX, U.S.A. Lyophilised L-α-dimyristoylphosphatidylcholine was supplied by the Sigma Chemical Co., Poole, Dorset, U.K. The other lipids were obtained from Lipid Products, Nutfield Nurseries, Nutfield, Surrey, U.K. and were of the best available grades. $^{45}CaCl_2$, specific activity 0.53 GBq per mg calcium, was purchased from Amersham International, Amersham, Bucks., U.K. Veratridine was obtained from Sigma and Sepharose 4B from Pharmacia, Uppsala, Sweden. All the other chemicals used were of at least reagent grade.

### *Preparation of synaptosomes, synaptosomal lipid extracts and proteoliposomes*

Synaptosomes were prepared from guinea-pig forebrain [5] and suspended in a modified

Krebs-Ringer medium (10 mM glucose/132 mM NaCl/5 mM KCl/1.2 mM $NaH_2PO_4$/1.2 mM $MgCl_2$/20 mM Tris-HCl/± 2 mM $CaCl_2$/± 2 mM EGTA, pH 7.4). Synaptosomal lipids were extracted into chloroform and methanol [6] with final partitioning by 0.9% NaCl to yield a chloroform solution containing 5–10 mg phospholipid per ml. Proteoliposomes (protein 10% w/w) were prepared by cholate dialysis of egg phosphatidylcholine (egg PC) labelled with diphenylhexatriene as described below and sodium cholate solubilized synaptic plasma membranes, all according to the general methods listed in Ref. 7, but omitting $CaCl_2$.

*Preparation of labelled samples*

Synaptosomes (10 mg protein per ml of medium) were labelled with diphenylhexatriene by adding the probe from a fresh 3 mM solution in tetrahydrofuran to a final concentration of 2 $\mu$M. Gentle stirring on ice was continued for 60 min to facilitate the incorporation of the probe into synaptosomal membranes. Lipids in solution in chloroform were mixed with diphenylhexatriene in tetrahydrofuran (2 nmol diphenylhexatriene per mg phospholipid) or dns-DPPE in chloroform/methanol (2 : 1, v/v) (final dns-DPPE concentration 1% w/w), dried by a stream of nitrogen and resuspended in medium (normally 140 mM NaCl/20 mM Tris-HCl/± 2 mM $CaCl_2$/pH 7.4, 1 ml per mg phospholipid). 1 ml aliquots of these suspensions were dispersed by probe sonication (MSE Scientific Instruments, Crawley, Sussex, U.K.) for $2 \times 5$ min bursts at 1.5 A, cooling the samples (except DMPC) in ice and water. In some experiments sonication was carried out under $N_2$ in $N_2$-saturated medium. When preparing proteoliposomes, egg PC with 1 mol% dns-DPPE was sonicated for 30 min under $N_2$ in a bath sonicator (Kerry Ultrasonics, Hitchen, Herts., U.K.) filled with ice and water.

*Fluorescence measurements*

An Aminco-Bowman spectrophotofluorimeter was equipped with polarizing filters (Kodak 'Polar Filter', Kodak Ltd., London, U.K.) conventionally orientated and fitted in the machine's moveable slit holders. With some further modifications up to four cuvettes could be accommodated simultaneously and heated or cooled, allowing appropriate controls to be included in each experiment.

Fluorescence intensities with vertical polarization and a vertical or horizontal analyser, $I_{\parallel}$ or $I_{\perp}$ respectively, yield

$$\text{Fluorescence polarization, } P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp}) \quad (1)$$

$$\text{Fluorescence anisotropy, } r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp}) \quad (2)$$

$$\text{Total fluorescence intensity, } F = I_{\parallel} + 2I_{\perp} \quad (3)$$

A grating correction factor [8] was applied to the fluorescence measurements and the depolarizing effect of sample scatter was almost entirely avoided by using horizontal cell slits [9] and by keeping sample absorbances at a level where successive dilutions showed that $P$ remained constant (usually an absorbance of about 0.1 at the excitation wavelengths used).

*Other measurements*

Protein was assayed by a modification of the method of Lowry et al. [10], further modified by the addition of 1% SDS to the copper-tartrate-carbonate reagent for estimations on lipid extracts. Bovine serum albumin was used for standards. Phospholipid phosphorous was estimated [11] after perchloric acid digestion and converted to phospholipid on the assumption that the phospholipid : phosphorous ratio is approximately 25 : 1 w/w. $^{45}Ca^{2+}$ association with lipid dispersions was measured by liquid scintillation spectrometry and the calcium content of lipid extracts was measured by atomic absorption spectrometry after drying and dispersion in double-distilled deionised water.

Lipid peroxidation was assessed by a version of the thiobarbituric acid colour reaction [12] expressing the results in absorbance units [13]. Calibration by measuring lipid oxygen consumption in an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) prior to the assay indicated that each unit per mg phospholipid corresponded to up to 2% (per)oxidation. (This figure is a maximum, as not all the $O_2$ consumed necessarily goes towards lipid peroxidation.)

Temperatures were measured with a Digitron No1754 probe thermometer (Digitron Instruments Ltd., Hertford, Herts., U.K.) and each reading is accurate to ±0.2%.

## Results

*Characterization of lipid preparations*

Following the application of 1 ml of sonicated synaptosomal lipid extract containing 1 mg of phospholipid (i.e. approx. 1 μmol) to a 1.5 × 30 cm Sepharose 4B column a single peak of ultraviolet absorbing material was obtained, at the void volume. When sonication was carried out in the presence of 2 mM $^{45}CaCl_2$ (specific activity 370 kBq per μmol $Ca^{2+}$) up to 0.2 μmol of calcium became associated with the lipid thus eluted. Transmission electron microscopy of negatively-stained [14] preparations on formvar-coated grids showed appearances consistent with multilamellar liposomes up to 1 μm in diameter. Proteoliposome preparations contained unilamellar liposomes 30–50 nm in diameter.

Lipid peroxidation during the period of sonication was measured in synaptosomal lipid extracts in media with or without added $CaCl_2$ and with or without $N_2$ purging and sonication under $N_2$. A mean of 3 units of peroxidation occurred per mg of phospholipid but there was no significant difference between samples with or without added $CaCl_2$ (paired $t$-test, $0.5 > p > 0.1$, $n = 3$) or indeed between samples prepared with or without $N_2$ (paired $t$-test, $0.5 > p > 0.1$, $n = 3$). Consequently samples were not sonicated under $N_2$ as a routine.

Hydrophobic proteins co-extracted with lipids from synaptosomes amounted to 27 ± 5 μg per mg phospholipid (mean ± S.D., $n = 4$) and the $Ca^{2+}$ content of the lipid extracts was 17 ± 2 nmol per mg phospholipid (mean ± S.D., $n = 4$).

*Diphenylhexatriene in synaptosomes and lipid dispersions*

The uncorrected excitation and emission maxima for diphenylhexatriene were 360 nm and 430 nm, respectively. Using the treatment developed by Jähnig [15] and Van Blitterswijk et al. [16] measurements of steady-state fluorescence anisotropy were divided into a fast decaying dynamic component, $r_f$, and an infinitely-slowly decaying static or structural component, $r_\infty$, the latter yielding an average lipid order parameter, $S_{DPH}$:

$$r = r_f + r_\infty \quad (4)$$

$$r_f = \gamma r_0 - \gamma r_s \quad (5)$$

$$r_\infty = (1+\gamma) r_s - \gamma r_0 \quad (6)$$

$$S_{DPH} = (r_\infty / r_0)^{1/2} \quad (7)$$

$r_0$ is the limiting fluorescence anisotropy (0.39 from Ref. 15) and $\gamma$ is the rotational correlation time of the probe ($\phi$) divided by its fluorescence lifetime ($\tau$). For diphenylhexatriene $\gamma$ is fairly constant at 0.125 [15].

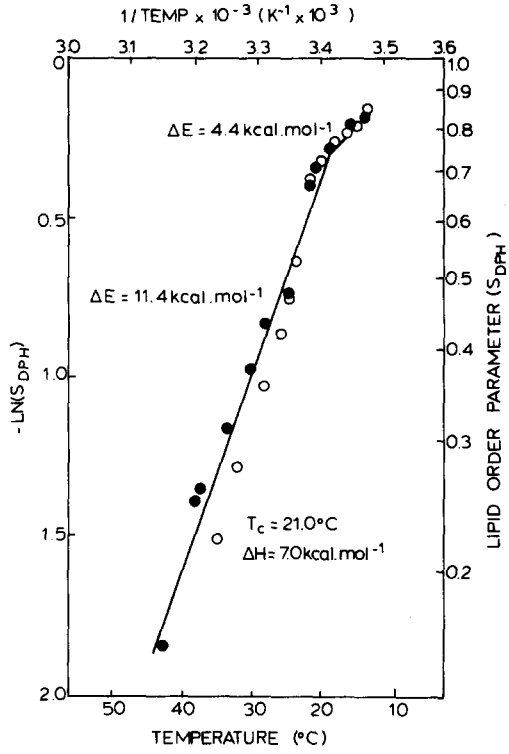


Fig. 1. Arrhenius plot of lipid order data for DMPC dispersions. Diphenylhexatriene-labelled DMPC was sonicated under $N_2$ and $S_{DPH}$ was determined from measurements of fluorescence anisotropy during heating (○) and cooling (●) scans as described in Materials and Methods. Assuming $S_{DPH} = A \cdot e^{-\Delta E/RT}$, $-\ln S_{DPH}$ is plotted against reciprocal temperature and activation energies ($\Delta E$) are determined from the slopes as indicated.

Fig. 1 is an Arrhenius plot of lipid order data obtained for the synthetic phospholipid DMPC labelled with diphenylhexatriene. The change in slope at 21.0°C is near the temperature of the main gel to liquid-crystalline phase transition as detected by scanning calorimetry (23°C or 23.7°C quoted in Ref. 1). The enthalpy change calculated from the difference in slopes of the plot above and below the break is 7.0 kcal · mol$^{-1}$. There is some evidence of an hysteresis effect between heating and cooling.

Egg PC was examined by heating scans only and showed no evidence of any discrete phase change, with $\Delta E = 4.2 \pm 0.7$ kcal · mol$^{-1}$ (mean ± S.D., $n = 3$) at 25°C. The calculated values of $r_f$, $r_\infty$ and $S_{DPH}$ at 25°C are compared in Table I to values calculated from data obtained at 45°C.

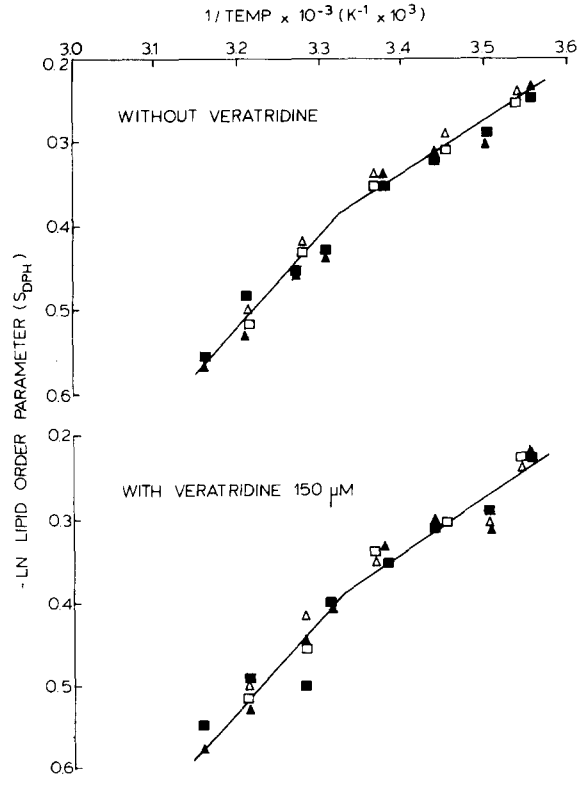


Fig. 2. Arrhenius plots of lipid order data for synaptosomes. Synaptosomes were labelled with diphenylehxatriene and temperature-dependent fluorescence anisotropy was measured as described in Materials and Methods during heating and cooling scans with 2 mM $CaCl_2$ (□ and ■, respectively) or with no added Ca but 2 mM EGTA (△ and ▲, respectively). The slopes were plotted after linear regression analysis of each group of observations above and below about 30°C. ($r > 0.93$ in each case).

TABLE I

FLUORESCENCE ANISOTROPY AND ORDER PARAMETERS FOR DIPHENYLHEXATRIENE IN EGG PHOSPHATIDYLCHOLINE

All dimensionless means ± S.D. from four experiments. $r_0$ = limiting fluorescence anisotropy (0.39). $r_f$ may vary from zero towards the limiting value 0.125 $r_0$ (i.e. 0.05) in an isotropic non-viscous medium. $r_\infty$ may vary from zero towards the limiting value $r_0$ in an anisotropic, fully-ordered membrane. $S_{DPH}$ may vary from zero towards a limiting value of unity in an anisotropic, fully ordered membrane.

| Temp. (°C) | 25 | 45 |
|---|---|---|
| Steady-state anisotropy $r$ | | |
| Dynamic component $r_f$ | 0.038 ± 0.001 | 0.040 ± 0.001 |
| Static component $r_\infty$ | 0.054 ± 0.003 | 0.033 ± 0.001 |
| Lipid order parameter $S_{DPH}$ | 0.372 ± 0.011 | 0.291 ± 0.007 |

Synaptosomes were labelled with diphenylhexatriene and typical scans are shown in Fig. 2. The main finding is the absence of any order change with veratridine-induced depolarization [17] even in the presence of 2 mM $CaCl_2$. At 25°C $\Delta E = 2.1 \pm 0.3$ kcal · mol$^{-1}$ (mean ± S.D., $n = 3$).

*dns-DPPE in lipid dispersions*

The uncorrected fluorescence excitation and emission maxima of dns-DPPE in lipid membranes were 360 nm and 430 nm, respectively. In contrast to diphenylhexatriene this probe is large and asymmetric, and to interpret fluorescence polarization measurements rotation about three major axes must be considered [18] to derive an average rate of rotation [19],

$$R = \left[ \frac{(P^{-1} - 1/3)}{(P_0^{-1} - 1/3)} - 1 \right] \tau^{-1} \qquad (8)$$

where $P_0$ is the limiting polarization of dns-DPPE (0.37, Ref. 19), and $\tau$ can be obtained for each measurement from a plot of total fluorescence intensity against temperature given that $\tau$ is 12.2 ns at 20°C [19]. Fluorescence lifetimes so calculated varied from 6.8 ns at 55°C to 15.2 ns at 6.5°C.

Fig. 3 is a typical plot of $R$ vs. temperature for dns-DPPE in synaptosomal lipid extract dispersions, the increase in probe mobility accelerating

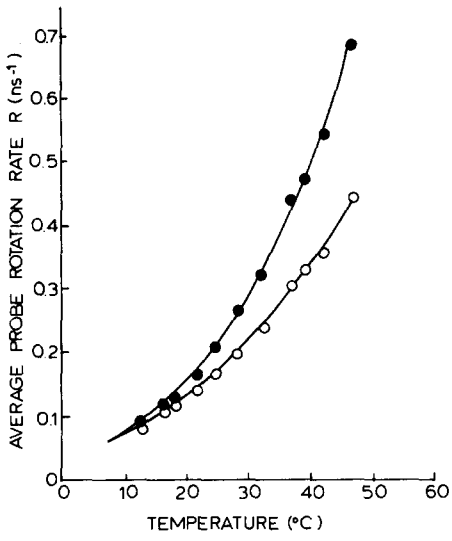


Fig. 3. Effect of $Ca^{2+}$ on dns-DPPE mobility in synaptosomal lipid dispersions. Synaptosomal lipid extracts were labelled with dns-DPPE either in the presence (○) or absence (●) of added $CaCl_2$ (2 mM) and $R$ was measured as described in Materials and Methods as the sample was heated.

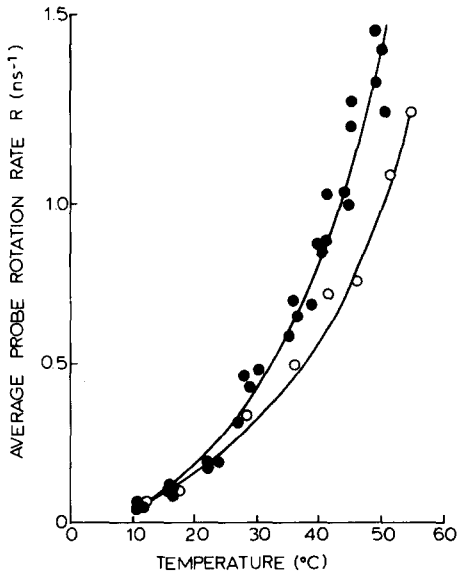


Fig. 4. Effect of $Ca^{2+}$ on dns-DPPE mobility in binary dispersions of egg PC and negatively-charged phospholipids. Egg PC dispersions were labelled with dns-DPPE as described in Materials and Methods and $R$ was measured as the samples were heated in the presence or absence of 2 mM $CaCl_2$ (●). Egg PC dispersions with 10 mol% phosphatidylserine were similarly labelled and R measured on heating with 2 mM $CaCl_2$ (○). Without $Ca^{2+}$ this mixture resembled egg PC alone. dns-DPPE-labelled binary mixtures of egg PC with 10 mol% of either phosphatidylethanolamine or sphingomyelin showed no $Ca^{2+}$-induced change in $R$ but with 10 mol% phosphatidylinositol 2 mM $CaCl_2$ reduced $R$ as in phosphatidylserine-containing preparations.

somewhat around 25°C. Adding $CaCl_2$ (2 mM in the figure) progressively decreases probe mobility as the temperature is raised. This effect was never seen in egg PC dispersions which were always studied simultaneously as controls with and without added calcium and was maximal by 1 mM $CaCl_2$ when up to 4 mM $CaCl_2$ was included. $CaCl_2$ was reduced to 100 $\mu$M, with 2 mM EGTA in control preparations, and the differences in $R$ at 38°C with and without $CaCl_2$ were still significant (mean difference 0.04 rotations per ns, paired $t$-test, $0.02 > p > 0.01$, $n = 3$).

Noting that synaptosomal membranes when compared to whole brain homogenate are relatively deficient in cholesterol but enriched in the major phospholipids [20] experiments were performed to establish whether any of the latter could contribute to the effect of calcium on probe mobility. Lipid dispersions of egg PC with 10 mol% of a second major phospholipid were labelled with dns-DPPE as described and the results of several experiments are summarised in Fig. 4. Of the binary lipid mixtures examined only those containing phosphatidylserine or phosphatidylinositol exhibited a reduction in probe mobility with calcium.

The possible involvement in this effect of hydrophobic proteins co-extracted from synaptosomes together with lipids was assessed using proteoliposomes formed from egg PC and solubilized synaptic plasma membranes. Although the proteoliposomes contained almost four times the protein of liposomes formed from synaptosomal lipid extracts only a barely-measurable calcium-induced reduction in probe mobility was seen.

## Discussion

DPH molecules in association with an ordered phospholipid bilayer appear to be situated centrally between the fatty acyl chains with their long axes normal to the plane of the membrane [21]. This is quite distinct from the behaviour of the probe in a medium which is isotropic, i.e. has no molecular order, so that $r$ of Equation 4 is zero

and re-arrangement of Equation 5 leads to the Perrin equation

$$r = r_0/(1+\tau/\phi) \quad (9)$$

allowing calculation of microviscosity from $\phi$ as in Ref. 22. The evaluation of membrane microviscosity (and its reciprocal fluidity) on the assumption of isotropy in biological membranes ignores the overwhelming contribution of membrane order to fluorescence anisotropy as discussed in Refs. 15 and 16 and illustrated in Table I. In addition, the values obtained show a marked dependence on the reference oil used for calibration [23].

Fig. 1 shows the thermotropic phase behaviour typical of a single lipid species. The break in the plot at 21.0°C is near the accepted main transition temperature $T_c$ [1] and although the enthalpy change is slightly high at 7.0 kcal · mol$^{-1}$ (cf. 6.3 or 6.6 from the last reference) both this value and $T_c$ are within the expected errors from our data [24]. Further values can be calculated from the results of Refs. 25 and 15, e.g. $\Delta H$ for DMPC calculated from the latter is 5.4 kcal · mol$^{-1}$. The lack of evidence of a sharp phase transition in egg PC dispersions reflects the varied lengths and saturations of the constituent acyl chains, and although the data for whole synaptosomes (Fig. 2) are fitted to two straight lines it would be expected that these membranes would also fail to show a sharp phase transition. It was noted that $\Delta E$ at 25°C decreased from 4.2 ± 0.7 kcal · mol$^{-1}$ for egg PC to 2.1 ± 0.3 kcal · mol$^{-1}$ for synaptosomal membranes (both means ± S.D., $n = 3$).

In synaptosomes there was no evidence that membrane lipid order (or indeed the dynamic component $r_f$ related to 'microviscosity') differed between depolarized and non-depolarized synaptosomes exposed to 2 mM $CaCl_2$ (Fig. 2). If calcium-dependent neurotransmitter release consists of depolarization-induced exocytosis of the contents of synaptic vesicles (see for example Ref. 26) possibly involving $Ca^{2+}$ in the transient fusion of vesicular and pre-terminal synaptic membranes [27], the lack of any change in membrane structure as particularly reflected in lipid order is unexpected. It remains possible that the probe may have become distributed within additional synaptosomal membrane systems [28] which could swamp this change.

The emission properties of dns-DPPE suggest that in phospholipid bilayer systems the molecule is orientated in much the same way as the unlabelled constituents [29]. Given that an average rotation rate can be calculated [19] its value should be sensitive to local lipid order and, in particular, to any phase change. Since calcium has been shown to promote phase transitions in negatively-charged phospholipids [2] in some cases in association with dramatic structural changes [30], the marked ability of $CaCl_2$ to reduce dns-DPPE mobility in synaptosomal lipids (Fig. 3) may primarily be due to an interaction of the cation with phosphatidylserine and phosphatidylinositol (Fig. 4) leading if not to a bilayer or hexagonal phase transition at least to important premelting or (more likely) prefreezing, lipid clustering, phenomena [1].

The effect of $CaCl_2$ on extracted lipids, maximal at 1 mM $CaCl_2$, was still apparent in experiments including only 100 nmol $CaCl_2$ per mg phospholipid, i.e. an approximate molar ratio of $Ca^{2+}$/total phospholipid of 0.1. Following extraction this ratio is already almost 0.02 (explaining the need for EGTA in controls for 100 $\mu$M $CaCl_2$) and in extracts exposed to $^{45}CaCl_2$ during sonication the ratio may reach 0.2, all the calcium being tightly bound. It may be concluded that certainly not more than equimolar quantities of $Ca^{2+}$ are needed for maximum effect, and probably appreciably less. This is relevant to the interaction of calcium with charged lipid membranes, as it seems likelier that the cation binds to specific charged groups rather than that it simply screens negative membrane charge [31]. Such an interpretation is relevant to the high specificity of $Ca^{2+}$ for neurotransmitter release. It should be emphasised that because of the electrical double-layer effect the concentration of divalent cations at a negatively-charged membrane surface will be considerably higher than in the bulk of the medium or in the cytosol.

In conclusion our data from the use of diphenylhexatriene show no evidence for large-scale structural order changes in the lipid membranes of depolarized cortical synaptosomes (which are necessarily unselected with regard to their neurotransmitter systems). Our results with dns-DPPE do suggest that $Ca^{2+}$ association may cause a phase separation in synaptosomal lipids involving acidic

phospholipid aggregation or transition to a gel-like phase. It is tempting to speculate that such a change (perhaps in association with certain membrane proteins) may underlie a channel-forming mechanism for neurotransmitter release.

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## References

1 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–281
2 Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 559, 399–420
3 Baker, P.F. (1972) Prog. Biophys. Mol. Biol. 24, 177–223
4 Miledi, R. (1973) Proc. R. Soc. B 183, 421–425
5 Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 79–87
6 Bligh, E.G. and Dyer, W.J. (1959) Canad. J. Biochem. 37, 911–917
7 King, R.G. and Marchbanks, R.M. (1982) Biochem. J. 204, 565–576
8 Chen, R.F. and Bowman, R.L. (1965) Science 147, 729–732
9 Teale, F.W.J. (1969) Photochem. Photobiol. 10, 363–374
10 Miller, G.L. (1959) Anal. Biochem. 31, 964
11 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
12 Hunter, F.E., Gebicki, J.M., Hoffstein, P.E. and Weinstein, J. (1963) J. Biol. Chem. 238, 828–835
13 Thiele, E.H. and Huff, J.W. (1960) Arch. Biochem. Biophys. 88, 203–207
14 Horne, R.W. and Whittaker, V.P. (1962) Z. Zellforsch. 58, 1–16
15 Jähnig, F. (1976) Proc. Natl. Acad. Sci. U.S.A. 76, 6361–6365
16 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer (1981) Biochim. Biophys. Acta 644, 323–332
17 Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 186, 21–33
18 Weber, G. (1971) J. Chem. Phys. 55, 2399–2407
19 Faucon, J.F. and Lussan, C. (1973) Biochim. Biophys. Acta 307, 459–466
20 Eichberg, J., Whittaker, V.P. and Dawson, R.M.C. (1964) Biochem. J. 92, 91–100
21 Engel, L.W. and Prendergast, F.G. (1981) Biochemistry 20, 7338–7445
22 Shinitzky, M. and Barenholtz, Y. (1974) J. Biol. Chem. 249, 2652–2657
23 Hare, F. and Lussan, C. (1977) Biochim. Biophys. Acta 467, 262–272
24 Klein, R.A. (1982) Q. Rev. Biophys. 15, 667–757
25 Andrich, M.P. and Vanderkooi, J.M. (1976) Biochemistry 15, 1257–1261
26 Kelly, R.B., Deutsch, J.W., Carlson, S.S. and Wagner, J.A. (1979) Annu. Rev. Neurosci. 2, 399–446
27 Katz, B. (1969) The Release of Neural Transmitter Substances, Liverppol University Press
28 Grunberger, D., Haimovitz, R. and Shinitzky, M. (1982) Biochim. Biophys. Acta 688, 764–774
29 Waggoner, A.S. and Stryer, L. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 579–589
30 Papahadjopoulos, D., Vail, M.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579–598
31 Ohki, S., Düzgüneş, N. and Leonards, K. (1982) Biochemistry 21, 2127–2133