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Evidence against protein-induced 'internal pressure' in biological membranes

Partition of 8-anilinonaphthalene-1-sulphonate into Triton X-100 micelles and submitochondrial particles

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It has recently been proposed that although small amphiphilic molecules partition into phospholipid vesicles this partition is reduced by a factor of 10^3–10^4-fold by the presence of proteins in biological membranes [Conrad & Singer (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5202–5206 and (1981) *Biochemistry* **20**, 808–818]. However, the affinity with which 8-anilinonaphthalene-1-sulphonate partitions into, or binds to, Triton X-100 micelles and submitochondrial particles is very similar and therefore does not support this proposal.

Liposomes and, to a lesser extent, detergent micelles are widely used as model systems of various physical features of biological membranes. The suitability of liposomes for such models has recently been questioned by Conrad & Singer (1979, 1981) on the basis that biological membranes have a high 'internal pressure' that is absent from artificial membranes composed only of phospholipids. They determined the partition coefficients of various amphilic molecules (decanol, 2,4-dinitrophenol, chlorpromazine and methochlorpromazine) into liposomes and a variety of biological membranes (erythrocyte ghosts, lymphoma cell ghosts and sarcoplasmic reticulum vesicles) using both the standard centrifugation technique and a newly developed technique, which they call hygroscopic desorption. The latter is essentially a filtration technique in which the aqueous suspending medium is removed by adsorption, through a suitable filter and without a pressure gradient. The partition coefficient of chlorpromazine and methochlorpromazine into the biological membranes is approx. 10^4 times higher when measured by the centrifugation technique (1000) than when measured by the hygroscopic desorption technique (0.1). In contrast the partition coefficient (1500) of these two compounds into phospholipid vesicles is similar when measured either by the centrifugation technique or by hygroscopic desorption. Conrad & Singer (1979, 1981) conclude that the partition coefficients derived from the centrifugation technique are in error and that the presence of proteins in the membrane alters the lipid phase to such an extent as to reduce partition by a factor of about 10^4. Such a reduction would seem to necessitate an extremely high degree of interaction between membrane proteins and lipids. This has not been observed by using other techniques [e.g. e.s.r., n.m.r. and differential scanning calorimetry (Chapman *et al.*, 1982)].

These conclusions are entirely dependent on the difference measured by Conrad & Singer (1979, 1981) between the partition of chlorpromazine and methochlorpromazine into biological membranes and into liposomes being real and not an artefact of the hygroscopic desorption technique. The simplicity of this technique would seem to guarantee its faultlessness. But this is also true of the centrifugation technique, which has, for example, been used extensively to measure the partition of anaesthetics into membranes. This has shown that the partition coefficient of anaesthetics into synaptosomes is the same as that into erythrocytes, and further, for neutral anaesthetics this partition coefficient is one-fifth of the corresponding water/octanol partition coefficient (Roth & Seeman, 1972). Further, for a given anaesthetic there is a correlation between its anaesthetic concentration and its partition coefficient into phosphatidylcholine liposomes and erythrocytes (Janoff *et al.*, 1981), and another between its anaesthetic concentration and the concentration that leads to a given degree of protection of erythrocytes against osmotic haemolysis (Roth & Seeman, 1972). These data are internally consistent and would seem to be in direct contradiction to the results of Conrad & Singer (1979, 1981).

In the present paper the partition coefficients of 8-anilinonaphthalene-1-sulphonate into Triton X-

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100 micelles and into submitochondrial particles have been determined. This determination is based on the fluorescence signals arising from the equilibrium binding of the fluorochrome *in situ* and is therefore not subject to artefacts that may arise in the separation of bound from free fluorochrome.

Triton X-100 micelles have been used in preference to liposomes made from extracted submitochondrial particle lipids, because of the relatively slow permeation of 8-anilinonaphthalene-1-sulphonate into liposomes (Fig. 3 of Gains & Dawson, 1975) and the consequent uncertainty as to whether equilibrium between the inside and outside aqueous phases has been reached.

Materials and methods

The preparation of submitochondrial particles (Beyer, 1967; Hansen & Smith, 1964) from ox heart mitochondria (Blair, 1967; Smith, 1967) has been described previously (Gains & Dawson, 1976). Protein was determined with the biuret reagent (Gornall *et al.*, 1949) using bovine serum albumin as the standard protein. The submitochondrial suspensions were clarified before the protein determination with 0.2% Triton X-100.

Fluorescence was measured in a front-face laboratory built fluorimeter (Gains & Dawson, 1975, 1976) at 30°C using an excitation wavelength of 380nm and an emission wavelength of 480nm.

8-Anilinonaphthalene-1-sulphonate was from Eastman Kodak and Triton X-100 was from BDH. Other reagents were of analytical grade.

Theory

For the partition of a fluorochrome (F) from an aqueous phase (w) into a hydrophobic phase (h) the partition coefficient is described by:

$$K_p = [F]_h/[F]_w \quad (1)$$

If the concentration of F is expressed as its concentration in the total volume (V_b, where $V_b = V_w + V_h$), then:

$$[F]_{wb} = [F]_h \cdot \frac{V_w}{V_b K_p} \quad (2)$$

and

$$[F]_{hb} = [F]_w \cdot K_p \cdot \frac{V_h}{V_b} \quad (3)$$

The total fluorochrome concentration, $[F]_t$, is derived by adding eqns. (2) and (3). If eqn. (1) is also used to eliminate $[F]_w$ from the sum this gives:

$$[F]_t = [F]_h \left(\frac{V_w}{V_b K_p} + \frac{V_h}{V_b}\right) \quad (4)$$

Multiplying through by V_h/V_b and rearranging gives:

$$[F]_{hb} = [F]_t \left(\frac{V_h K_p}{V_h K_p + V_w}\right)$$

Assuming that the relationship between fluorescence (f) and fluorochrome concentration is approximately hyperbolic (Gains & Dawson, 1979*a*,*b*) gives:

$$f = [F]_{hb} K_f \left(\frac{K_c}{K_c + [F]_t}\right)$$

$$= [F]_t K_f \left(\frac{V_h K_p}{V_h K_p + V_w}\right)\left(\frac{K_c}{K_c + [F]_t}\right)$$

where K_f is a constant with units of fluorescence (in arbitrary units) per unit concentration and K_c is the total fluorochrome concentration at half the maximal value (assuming a hyperbolic relationship). Rearrangement gives:

$$f = \frac{K_c K_f V_h K_p}{V_h K_p + V_w} - \frac{f K_c}{[F]_t} \quad (5)$$

A plot of f against $f/[F]_t$ should therefore be linear and the intercept on the $f/[F]_t$ axis, when f and $[F]_t$ are zero, is given by:

$$\left(\frac{f}{[F]_t}\right)_{f=0} = \frac{K_f V_h K_p}{V_h K_p + V_w} \quad (6)$$

Rearrangement gives:

$$\left(\frac{f}{[F]_t}\right)_{f=0} = K_f - \left(\frac{f}{[F]_t}\right)_{f=0} \frac{V_w}{V_h K_p} \quad (7)$$

Therefore a plot of these intercept values against the same intercept values multiplied by the appropriate values of V_w/V_h should give a straight line with an intercept on the $(f/[F]_t)_{f=0}$ axis equal to K_f and with an intercept on the $(f/[F]_t)_{f=0} \cdot (V_w/V_h)$ axis of K_f/K_p.

Under the conditions where the terms f and $[F]_t$ of eqn. (5) are extrapolated to zero the distinction between binding and partition is lost. In the case of a saturable site(s) and starting from the mass action equation it can be shown that the term K_p in eqns. (5), (6) and (7) is equivalent to:

$$\sum_{i=1}^{n} d_i v_i/(K_{D_i} MW_i)$$

where d is the density of the ligator and v its volume fraction in the membrane, and MW is its molecular weight in g.

Results

Fig. 1(*a*) is a plot of fluorescence against fluorescence × [fluorochrome]$^{-1}$ for 8-anilino-

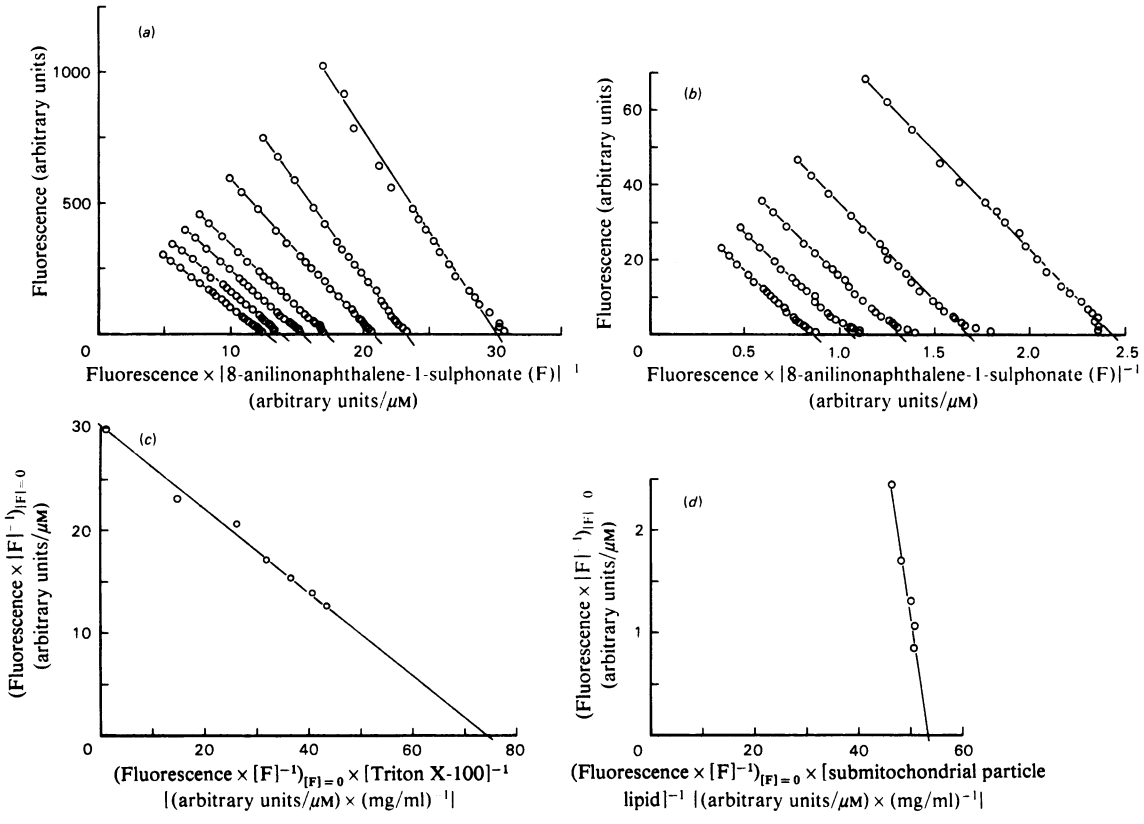

Fig. 1. *8-Anilinonaphthalene-1-sulphonate binding (a) to Triton X-100 micelles and (b) to submitochondrial particles, and (c and d) determination of the partition coefficient from the intercept values on the abscissa of plots (a) and (b), respectively*

In (*a*) the Triton X-100 concentrations were, from left to right, 0.47, 0.52, 0.60, 0.73, 1.0, 1.8 and 50.0 g · l^{-1}. In (*b*) the submitochondrial particle concentrations were, from left to right, 0.053, 0.066, 0.083, 0.111 and 0.166 g of protein · l^{-1}. In both the 8-anilinonaphthalene-1-sulphonate concentration range was from 0.5 to 60 μM. In (*c*) the intercept values from the abscissa of (*a*) are replotted; the Triton X-100 concentrations that appear as the denominator of the abscissa scale are the values given above less the critical micellar concentration of 0.14 g · l^{-1} (De Vendittis *et al.*, 1981). In (*d*) the values from the abscissa of (*b*) are replotted; the lipid concentrations that appear as the denominator of the abscissa scale assume a submitochondrial particle lipid-to-protein ratio of 1:3.1 (Parsons & Yaro, 1967). In (*a*) the buffer contained 250 mM-sucrose/5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH (pH 7.55); in (*b*) the buffer contained 250 mM-sucrose/5 mM-Hepes/20 mM-Tris/HCl (pH 7.55) and rotenone at 2 μg · ml^{-1}.

napthalene-1-sulphonate dissolved in various concentrations of Triton X-100. The straightness of the plots indicates that, over the fluorochrome concentration used, fluorescence bears an approximately hyperbolic relationship to fluorochrome concentration. This hyperbolic relationship could arise from one or a combination of the following: firstly, the binding of the fluorochrome to a saturable site on the Triton X-100 micelles; secondly, a concentration-dependent decrease in the partition coefficient caused by the concentration dependent increase in fluorochrome binding and, therefore, increase in the surface charge of the micelle; or thirdly, to the hyperbolic relationship that exists between fluorescence and fluorochrome concentration (Gains & Dawson, 1979*a*,*b*). At the highest Triton X-100 concentration, the right-hand curve of Fig. 1(*a*), there is a five-fold excess of Triton X-100 micelles over the highest fluorochrome concentration [60 μM, assuming a critical micellar concentration of 0.14 g · l^{-1} (De Vendittis *et al.*, 1981) and a micellar weight of 90000 (Kushner & Hubbard, 1954)]. At the lowest Triton X-100 concentration, the left-hand curve of Fig. 1(*a*), the hyperbolic relationship is apparent before the total fluorochrome concentration reaches the micellar concentration (3 μM,

sixth datum). Further, if it is assumed for the plot at the lowest Triton X-100 concentration that, as the intercept value on the abscissa is less than half that of the maximal value in Fig. 1(*a*), only half of the 8-anilinonaphthalene-1-sulphonate ions are bound, then the average number of 8-anilinonaphthalene-1-sulphonate ions bound per micelle is less than one up to the ninth datum (6 μM). It would seem therefore that the primary cause of the approximately hyperbolic relationship between fluorescence and fluorochrome concentration is that derivable from the Beer–Lambert equation (Gains & Dawson, 1979*a*). It should be noted however that if this were the only contributing factor the plots in Fig. 1(*a*) would be parallel.

Fig. 1(*b*) is a plot of fluorescence against fluorescence × [fluorochrome]$^{-1}$ for 8-anilinonaphthalene-1-sulphonate binding to submitochondrial particles. It is similar to Fig. 1(*a*) except that the fluorescence values are smaller. This arises from the necessity of using low concentrations of submitochondrial particles in order to avoid artefacts arising from light scattering.

Figs. 1(*c*) and 1(*d*) are plots of the intercept values derived from the abscissa of Figs. 1(*a*) and 1(*b*), respectively, against the same intercept values divided by the appropriate concentration of Triton X-100 or submitochondrial particle lipid. As these concentrations are given in mg · ml^{-1} the partition coefficient between 1g of Triton X-100 or membrane lipid and 1ml of buffer will be the reciprocal of the slope ×1000. The partition coefficients found from these plots are 2550 for Triton X-100 micelles and 3000 for submitochondrial particle lipids.

Discussion

The concept of partition of amphiphilic molecules into biological membranes can only be a limited one. Unlimited partition will give rise to a change in the composition and therefore to a change in the intrinsic properties of the membrane. For charged amphiphiles, the change in surface charge that occurs on binding will decrease the affinity and therefore lead to a partition coefficient that is apparently dependent on the concentration of the bound amphiphile. In both instances it could appear as if the partitioning molecule were binding to a saturable site. The graphical analysis in Fig. 1 was chosen as it allows the partition coefficient to be calculated from values that have been extrapolated to zero amphiphile concentration. However, although this value for K_p is independent of the amphiphile concentration the conditions used are such that partition cannot be distinguished from binding. In the case of binding the value derived for K_p is:

$$\sum_{i=1}^{n} d_i v_i/(K_{D_i} MW_i)$$

(see above under Theory). In the case of fluorochromes and where $\varepsilon_{c.d.} < 0.5$ the analysis used in Fig. 1 obviates the necessity of correcting the data for any nonlinearity between fluorescence and fluorochrome concentration (Gains & Dawson, 1979*a*).

The data in Fig. 1 show that it is possible to determine partition coefficients for 8-anilinonaphthalene-1-sulphonate binding to membranes and micelles without having to separate bound from free fluorochrome. This is technically possible at relatively low membrane and fluorochrome concentrations, and should be applicable to any fluorochrome provided that the signal from the bound is much higher than from the free fluorochrome. The partition coefficients of 8-anilinonaphthalene-1-sulphonate into Triton X-100 micelles (2550) and submitochondrial particles (3000) are similar. These values are calculated on a weight-to-volume basis (e.g. between 1g of membrane lipid and 1ml of buffer). However, precisely how comparable these partition coefficients are is uncertain. Partition will depend, among other things, on the total surface area of the hydrophobic–hydrophilic interface; on a weight basis this is not likely to be the same for Triton X-100 micelles and submitochondrial particle lipids.

If Conrad & Singer (1979, 1981) are correct the partition coefficient measured for 8-anilinonaphthalene-1-sulphonate between submitochondrial particles and buffer does not represent the partition coefficient of the membrane, but of micellar structures associated with the membrane. However, Conrad & Singer (1981) propose that amphiphilic molecules form micelles that are nucleated by some component derived from the membrane. They explain the difference between the centrifugation data and the hygroscopic desorption data by proposing that the micelles are loosely associated with the membrane and are co-precipitated with the membranes by centrifugation whereas the same micelles are stripped from the membranes throughout the process of hygroscopic desorption. They rule out the possibility that the micelles are only stripped from the membrane as this starts to become less hydrated during the final stage of hygroscopic desorption. This is based on the observation that in hygroscopic desorption amphiphilic molecules are lost, more or less, linearly with time (see Fig. 2 of Conrad & Singer, 1979). From this it should follow that the association of the proposed micelles with the membrane is very weak and that they would be lost, not only during hygroscopic desorption, but also during normal filtration. However, there are three grounds that render the above proposals unlikely. In

the experiments made by Conrad & Singer (Table 1 of Conrad & Singer, 1979) with intact erythrocytes and B and T lymphoma cells, the binding of chlorpromazine, 2,4-dinitrophenol and decanol was not detected by hygroscopic desorption. If it is assumed that these molecules penetrate the cell membrane, as was shown for chlorpromazine, it is necessary to explain either how the micelles associated with the cytoplasmic side of the membrane are lost while the cytoplasm is retained during hygroscopic desorption, or propose why the micelles only exist on the extracellular surface of the membrane. The second ground is that after filtration of an 8-anilinonaphthalene-1-sulphonate/rat liver mitochondria suspension the filtrate is not fluorescent (data not shown). This indicates either that there are no micelles present in the filtrate or that the fluorescence of the micelle-associated 8-anilinonaphthalene-1-sulphonate is quenched. Further, solubilization (in 0.2% Triton X-100) of the rat liver mitochondria retained on the filter shows that 8-anilinonaphthalene-1-sulphonate remains associated with the mitochondria. Therefore, the fluorescence of the unfiltered suspension arises from membrane-associated, and not micelle-associated, 8-anilinonaphthalene-1-sulphonate. The third ground is one of availability of material. Conrad & Singer (1979, 1981) do not speculate as to what the micelle-forming, membrane-derived component might be, nor did they attempt to identify it in the filtrates. However, for the sake of the argument, if this component is lipid and if 10% of the total lipid is in the form of membrane-associated micelles, then it would mean that the partition coefficient of 8-anilinonaphthalene-1-sulphonate for these micelles would have to be 30000, an order of magnitude higher than into Triton X-100 micelles. Such a high value does not seem likely. On the other hand, if the partition coefficient is smaller, then the fraction of the membrane lipid that is in the form of the proposed membrane-associated micelles must be proportionally larger. This would leave less lipid as an integral membrane component.

Further arguments against the proposals of Conrad & Singer (1979, 1981) can be derived from e.s.r. data. If the presence of proteins in biological membranes reduced the amount of doxyl fatty acid to one-thousandth of that which can be bound to liposomes made from their extracted lipids, then the e.s.r. signal would be too small to detect. The abundance of spectra in the literature for doxyl fatty acids bound to biological membranes contradicts this. As Moules *et al.* (1982) have pointed out, if doxyl fatty acid probes do not partition into biological membranes but instead form membrane-associated micelles, then the resulting spectra should show line broadening arising from spin–spin exchange between the doxyl groups. This is not found and, further, the spectra of doxyl fatty acid probes in both biological membranes and in liposomes made from their lipids are qualitatively similar (for example, see Esser & Lanyi, 1973; Fretten *et al.*, 1980; Hauser *et al.*, 1982). Perhaps in accord with the proposals of Conrad & Singer (1979, 1981), the order parameter derived from the spectrum of these probes is, at a given temperature, higher in the biological membranes than in the corresponding liposomes [this has been found for *Halobacterium cutirubrum* (Esser & Lanyi, 1973), chromaffin granule membranes (Fretten *et al.*, 1980) and small intestine brush border vesicle membranes (Hauser *et al.*, 1982)]. However, there is no established relationship between this spectral order parameter and either the 'internal pressure' of membranes or the partition coefficient of small amphiphilic molecules into membranes. Some slight support for the proposals of Conrad & Singer (1979, 1981) comes from the partition of TEMPO (2,2,6,6-tetramethylpiperidinooxyl) into small intestine brush border vesicles and into liposomes made from their extracted lipids (Hauser *et al.*, 1982). Although a true partition coefficient cannot be derived from these data, they show that at 37°C partition into the liposomes is about 20% higher and that this increases to about 50% as the temperature is decreased towards 0°C.

The data in the present paper do not eliminate the likelihood that a direct comparison of the partition of amphiphilic molecules into biological membranes with that into liposomes prepared from their extracted lipids would show that, based on the lipid concentration, the proteins reduced the partition coefficient. However, they do indicate that this reduction, if any, is extremely small in comparison to the 10^3–10^4-fold reduction proposed by Conrad & Singer (1979, 1981).

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