Membrane solubility of chlorpromazine

Hygroscopic desorption and centrifugation methods yield comparable results

Margarita LUXNAT, Hans-Jürgen MÜLLER and Hans-Joachim GALLA

Fachgebiet Biochemie, Institut für Organische Chemie und Biochemie, Technische Hochschule Darmstadt, Petersenstrasse 22, D-6100 Darmstadt, Federal Republic of Germany

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Binding of the positively charged drug chlorpromazine to artificial and erythrocyte bilayer membranes was investigated by the filtration method called hygroscopic desorption [Conrad & Singer (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5202–5206] and by the conventional centrifugation method. Only minor differences in the partition coefficients were observed using the two methods. Our finding is not consistent with the observation of Conrad & Singer that amphipaths are completely excluded from biological membranes. However, the partition coefficient is dependent on membrane composition, which means dependent on the physical properties of a membrane.

The study of the interaction of small amphipaths with membranes is of considerable interest in biology and physiology. Artificial bilayer membranes are in widespread use as models for biological membranes to investigate drug solubility by the determination of their partition coefficient. A common method is to centrifuge the equilibrated membranes and to analyse the pellet and the supernatant (Seeman & Kwant, 1969).

Recently a new method, hygroscopic desorption filtration (Conrad & Singer, 1979), was developed and led to surprising results. Partition coefficients of different amphipaths in artificial lipid bilayers were found to be identical by both methods. In biological membranes, however, almost no solubility was found with the filtration method whereas the centrifugation method yielded comparable results for artificial and natural membranes. These data were interpreted by the assumption of an 'internal pressure' in biological membranes that excludes the amphipaths. The use of artificial membranes as models for biological membranes became doubtful.

The surprising results were confirmed in a further paper (Conrad & Singer, 1981). The high partition coefficients obtained with the centrifugation method were explained by the adsorption of micelles formed by the amphipaths on the bilayer surface, which sedimented with the cells. Using the hygroscopic desorption technique, these micelles are thought to be stripped off the cell surface and to pass through the filter, thus leading

to the true partition coefficient, which was found to be almost zero.

However, Forrest *et al.* (1984) could not detect any micelles in their n.m.r. study. Furthermore, Pjura *et al.* (1984) investigated the partition of fatty acids between pure lipid and plasma membranes by the hygroscopic desorption technique and found essentially no difference. The same was true for chlorpromazine.

We used both the centrifugation and the hygroscopic desorption techniques to measure the partition coefficients of radioactively labelled chlorpromazine in pure phosphatidylcholine, mixed phosphatidylcholine/cholesterol and erythrocyte membranes as well as in whole erythrocytes. Our results oppose those of Conrad & Singer (1979) and underline the view that artificial bilayer membranes are still useful as models for biological membranes.

Materials and methods

Materials

Dimyristoylphosphatidylcholine was obtained from Fluka (Neu-Ulm, Germany), checked by t.l.c. and used without further purification. [³H]-Chlorpromazine with a specific radioactivity of 18.5 Ci/mmol was from New England Nuclear.

Large unilamellar vesicles

There were prepared by the reversed phase evaporation technique according to Szoka &

Papahadjopoulos (1978). The lipid or lipid/cholesterol (17mg) was dissolved in 1ml of chloroform/buffer (2:1, v/v). The buffer solution was 140 mM-NaCl/10 mM-Tris/HCl, pH 7.5. After a short ultrasonication the solvent was evaporated under vacuum. The remaining aqueous lipid dispersion was diluted with 10ml of buffer, centrifuged at 12500g and the pellet was washed twice. The last sediment was dispersed in 10ml of buffer solution. The final lipid concentration was 0.5–1.0mg/ml for pure dimyristoylphosphatidylcholine and 1.2–1.5mg/ml for cholesterol-containing vesicles. Phosphate determination was performed by the method of Chen *et al.* (1956). [^3H]Chlorpromazine was added to the membrane suspension at an activity of 1 $\mu\text{Ci/ml}$, which corresponds to a concentration of $5.4 \times 10^{-8}\text{ M}$. The vesicles were incubated for 30 min at 37°C. Aliquots were used for the centrifugation and the hygroscopic desorption.

Centrifugation was performed at 12500g. The pellet was dissolved in 10ml of Ready-Solv MP (Beckman) and the radioactivity was measured with a Tri-Carb scintillation counter. The hygroscopic desorption was performed as described by Conrad & Singer (1979). Nucleopore top filters (2.5cm diameter, 5 μm thickness, 0.2 μm pore diameter) were used to retain the vesicles. Small volumes (0.4ml) of lipid dispersion were layered on top of the filter. After soaking, the dry filter was either dissolved in 5ml of Filter Count (LSC-Cocktail; Packard Instruments) or was used for phosphate determination. [^3H]Inulin was used to correct for buffer remaining on the filter.

Erythrocytes

Erythrocytes were prepared from human blood by washing with 0.9% NaCl solution at 4°C. Erythrocyte ghosts were prepared according to Dodge *et al.* (1963) and 10 vol. of buffer were added to 1 vol. of the pellet. After addition of [^3H]chlorpromazine at $5.4 \times 10^{-8}\text{ M}$ the cells were incubated at 37°C for 30 min. Top filters of 0.4 μm pore diameter were used for the filtration technique. The membrane volume was calculated from the phosphate analysis by using the known erythrocyte composition and a density of 1.1g/ml for cell membranes.

Results

Hygroscopic desorption filtration

The filtration was performed exactly as described by Conrad & Singer (1979), but with severe experimental difficulties. Top filters with pore diameters of 0.08 μm or less could not be used. Even in the presence of poly(vinylpyrrolidone) these filters showed negligible flow rates without

application of vacuum. Even with the pore diameters of 0.2 μm used for filtration of lipid vesicles and 0.4 μm used for erythrocyte membrane filtration, typical filtration times were between 30 and 90 min, which is extremely longer than reported by Conrad & Singer (1979, 1981). Preconditioning treatment with poly(vinylpyrrolidone) had no remarkable effect on filtration time. The application of vacuum (Maher & Singer, 1984) cannot be recommended due to a dispersion of liposomes and cells during the filtration procedure. In contrast, filtration through nucleopore filters of a given pore size may be used to form vesicles of a given size (Olson *et al.*, 1979). Thus hygroscopic desorption under vacuum may lead to erroneous results.

Partition coefficients in artificial and in erythrocyte membranes

Large unilamellar vesicles made from dimyristoylphosphatidylcholine alone and dimyristoylphosphatidylcholine/cholesterol mixtures were investigated. The loss of lipid in the filtrate during hygroscopic desorption or in the supernatant during centrifugation was less than 1%. The partition coefficients are given in Table 1. Each value is the average of at least three determinations. Even with a relatively high s.d., a decrease in the partition coefficient with increasing cholesterol content could easily be determined. Upon centrifugation of erythrocytes or erythrocyte ghosts we obtained a loss of material of 5% or 10–15%, respectively. During filtration the loss of membrane content was 10% for ghosts, whereas intact erythrocytes could be filtered without loss. Even taking into account the large s.d., our value for the partition coefficient of chlorpromazine between erythrocyte membranes and water is between 300 and 500, and is clearly in contrast to the values reported by Conrad & Singer (1979, 1981) which were substantially less than 1.0 in all cases. Our values for a biological membrane are still of the order of the partition coefficients found in artificial bilayer membranes containing large amounts of cholesterol. They by no means approach zero.

Discussion

We tried to reproduce the hygroscopic desorption filtration developed by Conrad & Singer (1979). From earlier studies on erythrocyte membranes (Galla & Luisetti, 1979) with spectroscopic probes we know that these probes incorporate well into biological membranes. The papers of Singer's group clearly demonstrate that this should not be possible and they claim that artificial bilayer membranes should not be used as

Table 1. Partition coefficients (k_p) in dimyristoylphosphatidylcholine (DMPC), DMPC/cholesterol and erythrocyte membranes determined by the centrifugation and the hygroscopic filtration techniques

The decrease of k_p with increasing cholesterol content is explained by the rigidifying effect of cholesterol characterized by the corresponding decrease in the pressure-induced shift of the phase transition temperature dT_i/dP (dipalmitoylphosphatidylcholine was used instead of DMPC).

System	Loss of material (%) during:		Partition coefficient (k_p) determined by:		dT_i/dP^* (K/bar)
	Filtration	Centrifugation	Filtration	Centrifugation	
DMPC	2	2	1650 ± 50	1800 ± 20	0.029
DMPC/5 mol% cholesterol	1	<1	1200 ± 50	1400 ± 50	0.024
DMPC/10 mol% cholesterol	1	<1	900 ± 50	1000 ± 40	0.023
DMPC/20 mol% cholesterol	<1	<1	700 ± 100	900 ± 30	0.020
Erythrocyte ghosts	10	10–15	500 ± 100	700 ± 100	–
Erythrocytes	0	5	300 ± 100	390 ± 100	–

* From Müller & Galla, 1983.

models for biological membranes (Conrad & Singer, 1979). Therefore we determined the partition coefficients of chlorpromazine in artificial pure phospholipid and cholesterol-containing membranes and in a biological membrane with astonishing results.

Performance of the filtration method was difficult. Filters with pore diameter of less than $0.1 \mu\text{m}$ had extremely low flow rates independent of preconditioning treatment. Application of vacuum, which should be avoided, had no considerable acceleration effect at this small pore diameter. Thus the filtration method could not be used to separate small vesicles. Moreover, one of the goals of the hygroscopic desorption method as described was the possibility to filter without vacuum only by sucking the aqueous phase into a hygroscopic bottom filter in order to keep the membranes intact. This fails at small pore diameters and takes a long time (up to 1 h) with larger pore diameters. In a recent paper Maher & Singer (1984) tried to overcome these difficulties by modifying the filtration technique. They now used a sintered glass filter as medium filter and applied vacuum. However, under these conditions the hygroscopic desorption filtration is changed into a normal filtration which might disperse the membranes.

Despite these difficulties, we finally applied the hygroscopic desorption method and we determined partition coefficients between erythrocyte membranes and water which are comparable with the values found in artificial bilayer membranes containing high amounts of cholesterol. Thus in our experiments the k_p value of about 500 in erythrocyte ghosts may well be explained already

by the high cholesterol content of erythrocyte membranes. A complete exclusion of amphipath has never been observed. The centrifugation method, however, yields slightly higher values compared with the hygroscopic desorption method, which may be interpreted by adsorption of the drug to the bilayer surface. This may be specially true for the negatively charged erythrocyte membranes. Preliminary experiments using glycophorin-containing vesicles showed such an adsorption of chlorpromazine, which can be stripped off during hygroscopic desorption (M. Luxnat & H.-J. Galla, unpublished results). However, the assumption of an almost complete adsorption of mixed chlorpromazine micelles to membrane surfaces cannot be supported by our experiments.

We interpret the reduced partition coefficients found in cholesterol-containing membranes, including erythrocyte membranes, by a strengthened interaction within the lipid bilayer. Cholesterol is known to condense fluid bilayer membranes (Marsh & Smith, 1973). More recently Müller & Galla (1983) reported a reduced pressure sensitivity of the lipid phase transition temperature in the presence of cholesterol. The values dT_i/dP included in Table 1 underline the condensation effect of cholesterol. This physical parameter characterizing the membrane rigidity may be an easy but plausible explanation for the reduced solubility of amphipaths. This effect is assumed to be even larger in natural bilayer membranes containing integral proteins in addition to high amounts of cholesterol.

Our results are contradictory to the results given by Singer and coworkers, but consistent with the

finding of Pjura *et al.* (1984) who also found substantially no difference between partition coefficients determined by centrifugation or hygroscopic desorption. However, their partition coefficients vary between 10^2 and 10^5 , which makes a comparison impossible. In our experiments we used a very low chlorpromazine concentration of 5.4×10^{-8} M. Pjura *et al.* (1984) used 4.3×10^{-6} M and Conrad & Singer (1981) used a 6×10^{-5} M solution. It should be noted that these concentrations are all far below the critical micelle concentration of 4×10^{-3} M. A better agreement of our data is found with values given by Gaffney *et al.* (1983). They measured partition coefficients of spin-labelled fatty acids by e.s.r. spectroscopy. Their value of $k_p = 279$ for ghost membranes and $k_p = 1449$ for dimyristoylphosphatidylcholine vesicles are comparable in their tendency with our results using chlorpromazine.

We conclude that the rigidifying effect of cholesterol is responsible for the decrease in the k_p value for erythrocyte membranes. The hygroscopic desorption and centrifugation techniques yield comparable results. We have no reason to postulate the formation and adsorption of micelles on a significant scale. N.m.r. studies (Forrest *et al.*, 1984) were also not able to show micelle formation in the presence of amphipaths. The influence of protein on the solubility of amphipaths now requires investigation.

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