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Anion transport across varying lipid membranes – the effect of lipophilicity†

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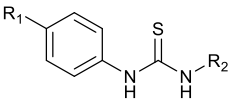
The anion transport properties of a range of alkyl-substituted phenylthioureas were tested in vesicles of different lipid composition. Although changes in the bilayer affected the rate of transport for all compounds in the series, the 'ideal' log P for peak activity did not change depending on the composition of the bilayers tested.

The development of small-molecule transmembrane anion carriers has attracted significant attention recently, as these compounds have potential as future therapeutics for treatment of conditions such as cystic fibrosis in additional to being useful tools for the study of transport processes across cell membranes.[1, 2] Transport studies are often carried out using large unilamellar vesicles (LUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).[1] Biological membranes, however, are significantly more complex, containing a wide range of phospholipids, sterols, proteins and other molecules.[3, 4] Little work has been done to directly compare the performance of synthetic anion transporters in different lipid environments.

The lipophilicity of an anion carrier is known to affect its transport activity[5, 6] and calculated log P (clogP) values (the n-octanol/water partition coefficient) have been shown to be key in determining the transport activity of simple thioureas.[7] Additionally, Quesada *et al.* have shown that for the tambjamine class of transporters, there exists an optimum log P for transport.[8]

It is known that energy barriers exist for solutes crossing lipid bilayers that vary in magnitude depending on lipid composition. In particular, computational studies have shown that the magnitude of these barriers affect the partitioning of solutes into the head or tail regions of the bilayer depend on the hydrophobic nature of the solute[9]. We wished to investigate whether similar effects could affect the ideal log P for anion receptors in different lipid bilayers therefore we investigated how the transport activity of a series of simple phenylthiourea molecules varied between bilayers formed of different lipids.



#	R_1	R_2	clog P	#	R_1	R_2	clog P
1	H	Et	2.19	9	Pe	Pe	5.94
2	Me	Et	2.68	10	Pe	Hx	6.40
3	Et	Et	3.14	11	Hx	Hx	6.85
4	Et	Pr	3.66	12	Hx	Hp	7.31
5	Pr	Pr	4.12	13	Hp	Hp	7.77
6	Pr	Bu	4.57	14	Hp	Oc	8.22
7	Bu	Bu	5.03	15	Oc	Oc	8.68
8	Bu	Pe	5.49				

Fig. 1 General structure and calculated log P values for the thiourea series.

A range of thioureas (**Fig. 1**) was synthesised with an increasing length of alkyl substituent, such that the set of compounds spanned the full range of log P values from 2 to 8.5. Thioureas were chosen as they are simple yet highly effective anion transporters.[7, 10] The length of the alkyl chains was increased in a symmetrical manner, in accordance with the recently described principle of lipophilic balance.[10] The compounds were synthesised from the appropriate isothiocyanates and anilines and full synthetic details are given in the ESI†.

The association constants for anion binding were determined for compounds **1**, **7** & **15** by ^{1}H NMR titration in DMSO-d_6/0.5% H_2O using the WINEQNMR2 program[11]. The results are shown in Table 1.

Lipid vesicles were prepared by previously reported methods[12-14].

Table 1 Anion binding constants for **1**, **7** & **15** and various TBA salts in DMSO-d_6/H_2O (0.5 %) at 298 K. Values calculated using WINEQNMR2 assuming a 1:1 binding mode. Errors ≤ 10 %. See ESI for details†.

Guest \ Host	**1**	**7**	**15**
Cl^-	14	17	16
NO_3^-	<10	<10	<10
$H_2PO_4^-$	180	220	160
SO_4^-	200	220	250

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†Electronic Supplementary Information (ESI) available: Experimental details of thiourea synthesis, transport studies and NMR titration data. See DOI: 10.1039/c000000x/

Chloride efflux from the vesicles was measured using a chloride ion-selective electrode. After 5 minutes, the vesicles were lysed to calibrate the readings to 100% efflux. Transport activity was quantified by a linear approximation, as described previously by Quesada *et al.*[8]

Initial transport rates were plotted against clog P values obtained from the ALOGP[15] method (via the VCC Lab web applet[16, 17], see **Fig. 1**). This model was chosen over a selection of other computational methods as it gave the best correlation with retention times obtained by RP-UHPLC (see ESI for details†).[18]

For POPC bilayers, the peak transport rate was observed for clog P = 5-6 (**Fig. 3**). The existence of the optimum log P range has been rationalised as being due to a balance between the receptor being lipophilic enough to cross the tail region of the membrane, without being so lipophilic as for delivery to the bilayer to be an issue[19]. It might be expected that if the effect were purely a partitioning effect then the optimum log P would be the same for the same bilayer composition. This result suggests otherwise as it is different from that found in studies of other sets of compounds[8], and presumably the effect is more complex and the optimum log P range is specific to transporter class.

The experiments were also conducted in vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (**Fig. 2**), both having the same tail groups as POPC. PG lipids have a glycerol head-group in the place of the choline moiety in PC lipids and have an overall negative charge in the head-group region. Across the whole series, transport was slower across POPG bilayers (**Fig. 3**), possibly as the formation of the negatively charged chloride complex was disfavoured. The peak in activity with respect to log P remained unchanged.

POPE has a similar structure to POPC but lacks the three methyl groups appended to the nitrogen atom (**Fig. 2**). Vesicles of a 3:1 POPE:POPC ratio were used as POPE will not form vesicles on its own (due to the higher transition temperature for this lipid and the likelihood of forming a combination of gel and liquid crystalline phases at the concentrations used in these experiments[20]). In contrast to POPG, the transport rate across the mixed lipid bilayer was significantly faster than that across the pure POPC bilayer for the whole series (**Fig. 3**), where it might be expected that increased head-group – head-group interactions for PE would increase the energy barrier in the polar region of the membrane[9]. Instead, the transporter molecules may be relieving some potential energy introduced by inclusion of the non-bilayer-forming lipid in the membrane[3]. Again, the peak in activity did not appear to change with respect to log P, although **6** & **10** also matched the rate of **7**, **8** & **9**, suggesting that a different rate-determining step in transport may be operating in this system.

To investigate the effect of changing the lipid tail group, the screening was repeated using a fully-saturated PC lipid DPPC (**Fig. 2**). These experiments were carried out at higher temperature due to the higher phase transition temperature of DPPC. The rate across the series was not significantly different from the POPC control (**Fig. 4**). This would suggest that the rate-limiting step for transport is not the diffusion across the tail region of the bilayer.

Experiments were also conducted using POPC vesicles doped with cholesterol. The condensing and ordering effects of cholesterol (Chol) on the membrane are well described,[21] hence cholesterol assays have previously been used as evidence for a mobile carrier mechanism in anion transport[5, 22] (as slower diffusion across a more

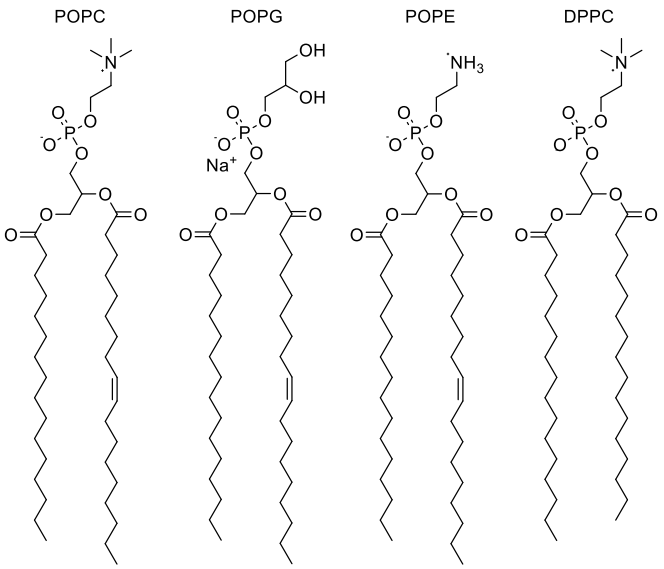

Fig. 2 Structures of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and dipalmitoyl-sn-glycero-3-phosphocholine (DPPC).

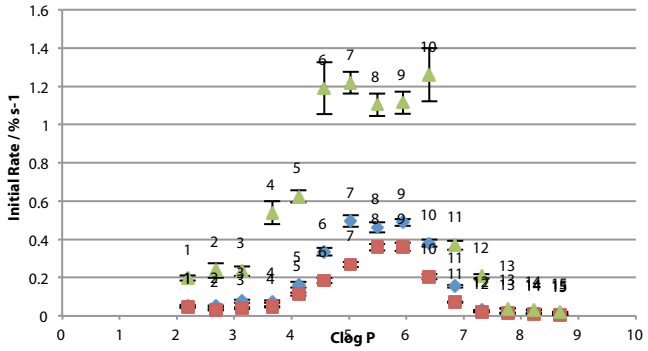

Fig. 3 Plot of initial rate of chloride efflux from various lipid vesicles for **1** – **15**, plotted against clog P of the transporter molecule. Blue diamonds: 100 % POPC; red squares: 100 % POPG; green triangles: 3:1 POPE:POPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

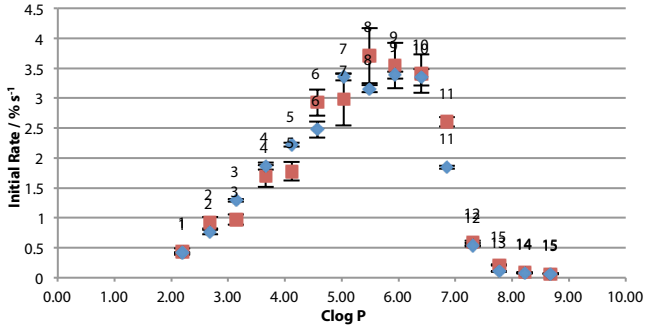

Fig. 4 Plot of initial rate of chloride efflux from DPPC vesicles for **1** – **15** at 55°C, plotted against clog P of the transporter molecule. Blue diamonds: 100 % POPC; red squares: 100 % DPPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

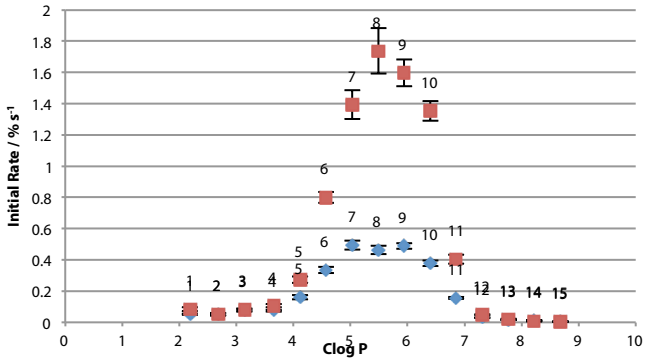

Fig. 5 Plot of initial rate of chloride efflux from 7:3 POPC:Chol vesicles for **1** – **15** plotted against clog P of the transporter molecule. Blue diamonds: 100 % POPC; red squares: 7:3 POPC:Chol. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

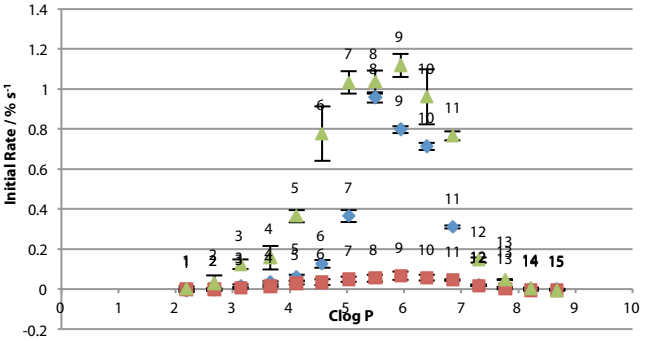

Fig. 6 Plot of initial rate of chloride efflux from various lipid vesicles for **1** – **15**, in the presence of external $NaSO_4$ after initiation with $NaNO_3$. Rate plotted against clog P of the transporter molecule. Blue diamonds: 100 % POPC; red squares: 100 % POPG; green triangles: 3:1 POPE:POPC. Each point represents the average of 3 trials. Error bars represent the standard error on the linear regression.

rigid membrane is evidence that supports a mobile carrier over a channel mechanism).

For this series of thioureas, transport rate increased ubiquitously in 7:3 POPC:Chol vesicles (**Fig. 5**). Crucially, the ideal lipophilicity range remained constant. This provides further evidence that diffusion across the tail region is not the rate-determining step, and in addition to other previous results[19, 23] that cholesterol assays are not sufficient evidence of a mobile carrier assay mechanism.

Finally, to explore any effect of the delivery of the compounds into the membrane, experiments in POPC/POPG/POPE:POPC 3:1 were repeated in the presence of external $NaSO_4$. The SO_4^{2-} ion has a high energy of solvation[24] and is generally considered not able to be transported through lipid bilayers mediated by small molecule transporters although there are compounds that have been shown to be capable of transporting this anion[6, 25]. After 2 minutes allowing the transporter to equilibrate with the SO_4^{2-} suspended vesicles, transport was initiated with the addition of a pulse of $NaNO_3$.

In the three systems, the relative peak in optimal log P was retained in the presence of sulfate (**Fig. 6**). In the POPG system, transport rates were dramatically reduced for all compounds. We propose that the sulfate may bind to the glycerol moieties in this lipid, restricting the ability of chloride ions or the transporters to locate in the head region to form a complex for transport. Alternatively sulfate could be forming complexes with the transporters at the interface so reducing the concentration of free transporter available for chloride transport.

A degree of Cl^- transport was observed in the 3:1 POPE:POPC system before the nitrate spike addition. In further assays, we found no direct evidence of sulfate transport or vesicle leakage. A slight dependence of chloride transport on the present cation was found however, indicating that MCl co-transport may make a minor contribution to the overall efflux of chloride. Slight de-acidification of the vesicle interior was also seen for the most active compounds in an HPTS pH assay, indicating a small amount of possible HCl transport. We suspect these two mechanisms may explain this effect in this system. This data is presented in full in the ESI†.

For the other systems, the compounds in the middle of the log P range appear to transport faster in the sulfate system. We attribute this to the longer equilibration time allowing majority of transporters to be ideally located within the membrane when transport is initiated, giving a boost to the initial rate. Conversely, those at the hydrophobic and hydrophilic extremes of the series transport more slowly. The more polar compounds may be involved in competitive binding to SO_4^{2-} at the interface, whilst the hydrophobic compounds are likely further confined to the tail region due to the strengthening of the hydrophobic effect by the sulphate ion.

Conclusions

We have shown that in the different bilayer systems tested, the ideal range of log P for maximum transport rate by this series of compounds does not change. We note that the ideal log P range appears to be a property of the transporter class and not of the membrane.

Optimising log P is still an important factor for maximising transport rate for a given compound series, although it is important to note that other design factors must determine the absolute rate in different lipid systems. We have shown that different lipid environments have a significant influence on transporter activity and it will be important in further work to identify what the limiting steps are for different receptor classes. This will aid more targeted molecular design for natural target membranes and help ensure that future model bilayers accurately represent the target cellular environment to avoid potentially misleading results.

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