Interactions of Small Molecules with Phospholipid Bilayers

BINDING TO EGG PHOSPHATIDYLCHOLINE OF SOME ORGANIC ANIONS (BROMOSULPHOPHTHALEIN, OESTRONE SULPHATE, HAEM AND BILIRUBIN) THAT BIND TO LIGANDIN AND AMINOAZO-DYE-BINDING PROTEIN A

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1. To assess the possible involvement of ligandin and aminoazo-dye-binding protein A in intracellular transport it is necessary to know how their ligands, most of which are molecules with hydrophobic moieties, interact with cellular membranes. To obtain such information we examined the interactions of bromosulphophthalein, oestrone sulphate, haem and bilirubin with aqueous dispersions of egg phosphatidylcholine and egg phosphatidylcholine/cholesterol (1:1, molar ratio) by equilibrium dialysis and spectrophotometry. 2. In all four cases, saturation effects were observed. Values of $\bar{v}_{\max}$. ($\bar{v}$ = mol of compound bound/mol of lipid phosphorus) at 25°C were: for bromosulphophthalein, ~0.1; for oestrone sulphate, ~0.25; for haem, ~0.25 (all at pH 7.4); and for bilirubin 0.1–0.2 (at pH 8.2). 3. Limiting values of $\bar{v}/c$ (c = unbound concentration) as $\bar{v} \rightarrow 0$ at 25°C and pH 7.4 are: for bromosulphophthalein, 6.25×10^4 litre·mol⁻¹; for oestrone sulphate, 7.8×10^2 litre·mol⁻¹; for haem, 4.5×10^5 litre·mol⁻¹; and for bilirubin, $\sim 1.2 \times 10^4$ litre·mol⁻¹. For haem the result depends on the assumption that only the monomeric form binds to the lipid. 4. The binding of each compound was decreased by cholesterol; bromosulphophthalein and oestrone sulphate were affected more than haem and bilirubin. 5. Bromosulphophthalein at saturating concentration decreased the limiting values of $\bar{v}/c$ of the other three compounds by approximately one order of magnitude. 6. By assuming that the interactions with egg phosphatidylcholine resemble those with the phospholipid components of mammalian intracellular membranes the binding data for phosphatidylcholine, together with data for binding to the intracellular proteins ligandin and aminoazo-dye-binding protein A, enable the subcellular distributions of the four compounds to be estimated. For the rat hepatocyte up to 92, 51, 98 and 47% of the total bromosulphophthalein, oestrone sulphate, haem and bilirubin respectively may be membrane-bound.

The intracellular transport of small molecules with hydrophobic moieties is most likely to be accomplished by binding to water-soluble carrier proteins, such as ligandin and aminoazo-dye-binding protein A, and by lateral diffusion in the lipid bilayers of membranes. To attempt to quantify the diffusional fluxes it is necessary to know the concentration gradients and diffusion coefficients for the aqueous and membrane phases of the cell. Values for diffusion coefficients in aqueous solution of small molecules and proteins are readily available, and recently there have been a number of estimates of the lateral-diffusion coefficients, in both pure lipid bilayers and in biological membranes, of a number of small molecules (for a review see e.g. Edidin, 1974). The relative amounts of diffusate in the aqueous and membrane

phases depend on the binding affinities of the membranes and of the carrier proteins. For ligandin and protein A such information is available (Kamisaka *et al.*, 1975; Ketley *et al.*, 1975; Ketterer *et al.*, 1975, 1976; Tipping *et al.*, 1976a,b,c, 1978), and so the assessment of the importance of these two proteins in intracellular transport awaits measurements of the partition coefficients of their ligands between aqueous solution and lipid bilayers.

In the preceding paper (Tipping *et al.*, 1979) we reported equilibrium-dialysis and spectrophotometric studies of the binding of four uncharged ligands of ligandin and protein A to dispersions of lipids (egg phosphatidylcholine and egg phosphatidylcholine/cholesterol). Here we report experiments with the same techniques, but with compounds that are ionized at neutral pH: bromosulphophthalein, oestrone sulphate, haem and bilirubin (Fig. 1). Each of these binds to both ligandin and protein A at a

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Fig. 1. Formulae of the compounds studied

Ionization states are shown as the form predominating in aqueous solution (i.e. unbound to phosphatidylcholine) under the conditions of the experiments. In bromosulphophthalein, the phenolic hydroxy groups are slightly ionized at pH 7.4 and almost fully ionized at pH 9.0. In the monomeric form of haem (which is shown) either $X = H_2O$ and $Y = OH^-$ or X and $Y = H_2O$; in the dimeric form two molecules are linked via an $Fe-O-Fe$ bridge, the other co-ordination groups being H_2O and H_2O or H_2O and OH^- (Brown *et al.*, 1970). In bilirubin, the monomeric non-colloidal

form is probably the dianion. The lactam form is shown $\left(\begin{smallmatrix} O \\ \parallel \\ C-N \\ | \\ H \end{smallmatrix} \right)$, but it is not certain whether this or the lactim $\left(\begin{smallmatrix} HO \\ \backslash \\ C=N \end{smallmatrix} \right)$ predominates (cf. Bonnett *et al.*, 1976).

single primary site with association constants in the range 10^5 – 10^8 litre·mol $^{-1}$ (Ketterer *et al.*, 1976; Tipping *et al.*, 1976a,b,c).

Experimental

Materials

The following compounds were obtained from the Sigma Chemical Co., Poole, Dorset, U.K.: bilirubin, bromosulphophthalein, cholesterol, L- α -glycerophosphocholine, haem, oestrone sulphate, phosphocholine, L- α -phosphatidyl-L-serine and sodium dodecyl

sulphate. Egg phosphatidylcholine was obtained from Lipid Products, South Nutfield, Surrey, U.K., and [6,7- 3H]oestrone sulphate (sp. radioactivity 1100 Ci/mol) was from The Radiochemical Centre, Amersham, Bucks., U.K.

Phospholipid dispersions

Unilamellar liposomes of phosphatidylcholine and phosphatidylserine were prepared by the method of Brunner *et al.* (1976) (cf. Tipping *et al.*, 1979). Multilamellar liposomes of phosphatidylcholine alone or mixed with equimolar amounts of cholesterol were

prepared by hand-shaking followed by brief sonication to make the dispersions suitable for spectrophotometry.

Equilibrium dialysis

Experiments with oestrone sulphate were carried out as described by Tipping *et al.* (1979). Experiments with bromosulphophthalein utilized bags made of Visking tubing (6mm diam.) containing 1 ml of lipid dispersion that were placed in 2 ml of bromosulphophthalein solution in small test tubes. After sealing the tubes they were shaken gently in a water bath at the desired temperature. Final bromosulphophthalein concentrations were determined by rendering samples of the solutions outside the bags alkaline and reading the absorbance at 580nm. Control experiments indicated that equilibrium was attained within 24h at all concentrations of bromosulphophthalein used.

Spectrophotometry

A Pye-Unicam SP. 1800 spectrophotometer was used. Titrations of lipids with haem and bilirubin were carried out essentially as described for the binding of these two compounds by ligandin and protein A (Tipping *et al.*, 1976b,c). Titrations with bromosulphophthalein were carried out by preparing a series of suspensions of phosphatidylcholine at pH7.4 containing various amounts of bromosulphophthalein. After equilibration at 25°C overnight their absorbances at 580nm were determined.

Expression of binding results

Our results are expressed throughout in terms of the parameters $\bar{v}$ (mol of compound bound/mol of lipid P) and c , the unbound concentration of compound (cf. Tipping *et al.*, 1979). In the present work, we also make use of the parameters $\bar{v}_{\max}$, (the maximum, or saturation, value of $\bar{v}$) and the limit as $\bar{v} \rightarrow 0$ of $\bar{v}/c$ (i.e. a parameter reflecting the maximum ratio of bound to free ligand).

Results and Discussion

Bromosulphophthalein

Fig. 2 shows the results of equilibrium-dialysis experiments with bromosulphophthalein and liposomes composed of either egg phosphatidylcholine or egg phosphatidylcholine/cholesterol (molar ratio, 1:1), plotted by the method of Scatchard (1949). Saturation was observed, the $\bar{v}_{\max}$ being approx. 0.1, i.e. liposome phospholipids can accommodate one bromosulphophthalein molecule for every ten of themselves. At pH7.4 the extrapolation of the plot for phosphatidylcholine to $\bar{v}=0$ gives a limiting value of $\bar{v}/c$ of approx. 6.25×10^4 litre $\cdot$ mol $^{-1}$. Because of the large amounts of intracellular lipid and binding proteins (see below) very little if any of the small molecules considered in the present work is likely to

Fig. 2. The binding of bromosulphophthalein to egg phosphatidylcholine liposomes

Open symbols are the results of equilibrium-dialysis measurements at 25°C: O, phosphatidylcholine only, pH7.4; Δ, phosphatidylcholine/cholesterol (1:1, molar ratio), pH7.4; □, phosphatidylcholine only, pH9.0. The results shown are for multilamellar liposomes, but in the case of the experiments involving phosphatidylcholine alone essentially identical data were obtained with unilamellar liposomes. Filled circles (●) are from spectrophotometric measurements at 25°C, pH7.4; c = concentration of unbound bromosulphophthalein. The concentration range of lipid was 0.05–0.5 mM.

be unbound under physiological conditions and so such limiting values of $\bar{v}/c$ are probably most relevant to the situations *in vivo* that interest us. Bromosulphophthalein is seen to bind less well to phosphatidylcholine/cholesterol. Its binding to phosphatidylcholine alone is pH-dependent, being weaker at pH9.0 than at pH7.4. It is also temperature-dependent. Fig. 2 refers to experiments at 25°C. However, at 4°C the limiting value of $\bar{v}/c$ as $\bar{v} \rightarrow 0$ is somewhat higher at 1.1×10^5 litre $\cdot$ mol $^{-1}$, although the $\bar{v}_{\max}$ is unchanged. All these results are summarized in Table 1.

At pH7 aqueous solutions of bromosulphophthalein are purple because of ionization of the phenolic hydroxy groups. However, if sufficient egg phosphatidylcholine is added to such a solution it becomes colourless, indicating that the species of bromosulphophthalein with its phenol groups unionized (i.e. the dianion as opposed to the tri- or

Table 1. *Parameters for the binding of bromosulphophthalein, oestrone sulphate, haem and bilirubin by egg phosphatidylcholine liposomes*

Values of $\text{Lim}(\bar{v}/c)$ and $\bar{v}_{\max}$ are obtained from the ordinate and abscissa intercepts of Scatchard plots (Figs. 2, 3, 7 and 9) and are expressed as litre $\cdot$ mol $^{-1}$ and mol of ligand bound/mol of lipid P respectively. In cases where linear regression was not applied (i.e. bromosulphophthalein, oestrone sulphate and bilirubin) the intercepts were estimated by eye. Abbreviation: N.D., not determined. For the phosphatidylcholine/cholesterol results $\bar{v}$ has been expressed in terms of phospholipid only and also in terms of total liposome component (i.e. phospholipid+cholesterol). Results for bromosulphophthalein, oestrone sulphate and haem refer to pH 7.4, and for bilirubin to pH 8.2.

	Binding parameters							
	Phosphatidylcholine/cholesterol at 25°C							
	Phosphatidylcholine only at 25°C		$\bar{v}$ in terms of phospholipid		$\bar{v}$ in terms of phospholipid+cholesterol		Phosphatidylcholine only at 4°C	
	$\text{Lim}(\bar{v}/c)$ $\bar{v} \rightarrow 0$	$\bar{v}_{\max}$	$\text{Lim}(\bar{v}/c)$ $\bar{v} \rightarrow 0$	$\bar{v}_{\max}$	$\text{Lim}(\bar{v}/c)$ $\bar{v} \rightarrow 0$	$\bar{v}_{\max}$	$\text{Lim}(\bar{v}/c)$ $\bar{v} \rightarrow 0$	$\bar{v}_{\max}$
Bromosulphophthalein	6.25×10^4	0.1	1.65×10^4	N.D.	8.25×10^3	N.D.	1.1×10^5	0.1
Oestrone sulphate	7.8×10^2	0.3	1.1×10^2	N.D.	55	N.D.	1.2×10^3	0.33
Haem	4.5×10^5	0.27	3.5×10^5	0.22	1.75×10^5	0.11	N.D.	N.D.
Bilirubin	4×10^3	0.1–0.2	2.5×10^3	0.1–0.2	1.25×10^3	0.05–0.1	N.D.	N.D.

tetra-anion) is preferentially bound. On the assumption that only this latter form of bromosulphophthalein binds, c , the unbound concentration of bromosulphophthalein, in phosphatidylcholine/bromosulphophthalein mixtures containing significant amounts of both bound and free bromosulphophthalein can be determined simply by measuring the amount of purple colour, i.e. A_{580} , and binding isotherms can be constructed. The results of such an experiment at pH 7.4 are shown as filled circles in Fig. 2. The good agreement between these results and those from equilibrium-dialysis experiments confirms the assumption that the un-ionized form is preferentially bound. This is also in accord with the weaker binding at pH 9.0 compared with that at pH 7.4. However, if the dianion were the only species able to bind it would be expected that the Scatchard plot at pH 9.0 would be the pH 7.4 plot reduced by a factor of anti-log (9.0–7.4), i.e. 40. Since the reduction factor obtained experimentally is only about 10 (Fig. 2) it appears that some binding of the tri- and tetra-anions can occur.

In view of the low pK of the sulphonate group each bromosulphophthalein molecule that enters the lipid bilayer is accompanied by two negative charges, and repulsion between these charges must be a crucial factor in determining the value of $\bar{v}_{\max}$. The most likely orientation for bromosulphophthalein in the bilayer has the hydrophobic tetrabromobenzene ring immersed in the hydrocarbon chain region, whereas the phenolic and sulphonate groups are in the head-group region, exposed to water. Because bromosulphophthalein is roughly 'Y-shaped' (the tail being the tetrabromobenzene ring) its binding to phosphatidylcholine is presumably accompanied by some

local distortion of the bilayer structure. This may be a second factor limiting the value of $\bar{v}_{\max}$, and also provides an explanation for the decrease in binding affinity brought about by cholesterol, since the well-known condensing effect of the sterol (for a review see Demel & de Kruijff, 1976) would decrease the potential local distortion.

Oestrone sulphate

Equilibrium-dialysis measurements of the binding of oestrone sulphate to egg phosphatidylcholine liposomes gave the Scatchard plots shown in Fig. 3. From these plots limiting values of $\bar{v}/c$ as $\bar{v} \rightarrow 0$, and those of $\bar{v}_{\max}$ were obtained (Table 1). As with bromosulphophthalein the limiting value of $\bar{v}/c$ at 4°C is higher than that at 25°C, and the presence of cholesterol in the liposomes causes a substantial decrease in binding. Bromosulphophthalein itself is extremely effective in decreasing the attractiveness of the liposomes for oestrone sulphate. As shown above $\bar{v}_{\max}$ for bromosulphophthalein is 0.1, but even this degree of binding causes the limiting value of $\bar{v}/c$ for oestrone sulphate to decrease approx. 15-fold (Fig. 3).

There can be little doubt that the sulphate group of oestrone sulphate is located in the head-group region of the bilayers. However, the position of the 17-oxo group is less obvious. The parent steroid oestrone (with a 3-phenol group) binds to egg phosphatidylcholine liposomes with a $\bar{v}/c$ value (constant in the range $\bar{v} = 10^{-5}$ – 2×10^{-3}) of 3.1×10^3 litre $\cdot$ mol $^{-1}$ at 25°C, but to phosphatidylcholine/cholesterol liposomes with $\bar{v}/c = 7 \times 10^2$ litre $\cdot$ mol $^{-1}$ at 25°C ($\bar{v}$ in terms of phospholipid) (Tipping *et al.*, 1979). This effect of cholesterol can be interpreted to mean that

Fig. 3. The binding of oestrone sulphate to egg phosphatidylcholine liposomes

Open symbols are the results of equilibrium-dialysis experiments at 25°C: ○, phosphatidylcholine only; △, phosphatidylcholine/cholesterol (1:1, molar ratio); □, liposomes of phosphatidylcholine only + 1 mM-bromosulphophthalein. Filled circles refer to phosphatidylcholine only at 4°C. The results shown are for multilamellar liposomes, but in the case of the experiments involving phosphatidylcholine alone essentially identical data were obtained with unilamellar liposomes. The concentration range of lipid was 1–3 mM.

oestrone is orientated in the cholesterol-free bilayer with its hydrocarbon nucleus parallel to the bilayer plane and with the 3-phenol and 17-oxo groups able to form hydrogen-bonds with water or head-group components (Tipping *et al.*, 1979). The same interpretation may also be applied to oestrone sulphate, i.e. its 17-oxo group may be located in the head-group region as well as the 3-sulphate group. Such an orientation would be expected to generate considerable local distortion in the bilayer so that the condensing effect of cholesterol could have a profound effect on the binding of oestrone sulphate. In phosphatidylcholine/cholesterol bilayers oestrone sulphate could be parallel to the bilayer plane, in which case the low values of $\bar{v}/c$ would suggest a high degree of local distortion, or perpendicular to it, when the low values of $\bar{v}/c$ could reflect the energetically unfavourable location of the 17-oxo group in the hydrocarbon region.

The decreased binding of oestrone sulphate to egg phosphatidylcholine liposomes when bromosulphophthalein is present at saturating concentrations is pro-

bably due to charge–charge repulsion between the sulphonate groups of bromosulphophthalein and the sulphate group of oestrone sulphate. In accord with this bromosulphophthalein has little effect on the binding of oestrone, testosterone and 4-dimethylaminoazobenzene (all of which are uncharged), which suggests that bilayer perturbation brought about by bromosulphophthalein is much less significant than the increased charge that accompanies its binding.

Haem

Haem forms dimers in neutral aqueous solution (Brown *et al.*, 1970) and this complicates binding studies. The dimerization is reflected by concentration-dependent spectra in the Soret region. At low concentrations, where monomer predominates, the spectrum is sharp and intense, whereas at higher concentrations the dimer gives rise to broader, less intense bands. By following the variation in absorption coefficient with haem concentration at various pH values, Brown *et al.* (1970) showed that the aggregation process conforms to the equilibrium:

where M represents monomer and D represents dimer. At pH 7.0 and I 0.16 (potassium phosphate/KCl buffer) we have found previously (Tipping *et al.*, 1976c) that $[D]/[M]^2 = 10^7$ litre·mol⁻¹, a value somewhat smaller than that of 4×10^7 litre·mol⁻¹ at pH 6.98 and I 0.10 (sodium phosphate/potassium phosphate buffer) reported by Brown *et al.* (1970). In the present studies we have used NaCl/Tris buffer, pH 7.4 (I 0.15), and have based our value of $[D]/[M]^2$ under these conditions on our previous result. At this slightly higher pH $[D]/[M]^2$ is calculated to be $10^7 \times (10^{-7.0}/10^{-7.4}) = 2.5 \times 10^7$ litre·mol⁻¹, if it is assumed that the H^+ activity coefficient is the same at pH 7.4 and I 0.15 as at pH 7.0 and I 0.16.

Fig. 4 shows spectra of 5×10^{-6} M-haem in buffer, 1 mM-phosphatidylcholine, 1 mM-phosphatidylserine and in 0.1 M-sodium dodecyl sulphate (cf. Simplico, 1972). Although the transfer of haem from buffer to any one of these amphiphiles is accompanied by qualitatively similar spectral changes, it is clear that the nature of the head group is an important determinant of the bound spectrum. Thus, the spectrum of haem bound to sodium dodecyl sulphate resembles quite closely that of monomeric haem bound to proteins such as serum albumin (Beaven *et al.*, 1974), ligandin and aminoazo-dye-binding protein A (Tipping *et al.*, 1976c). Haem bound to phosphatidylserine and to phosphatidylcholine gives spectra with lower $\epsilon_{\max}$ values (7.4×10^4 and 5.65×10^4 litre·mol⁻¹·cm⁻¹ respectively) than that for dodecyl sulphate-bound haem (9.2×10^4 litre·mol⁻¹·cm⁻¹), and therefore it is not completely certain that these spectra represent monomeric haem. However, in the case of phosphatidylcholine the observed spectrum of

Fig. 4. Spectra of haem in phosphatidylcholine (—), phosphatidylserine (— · — ·), sodium dodecyl sulphate (---) and buffer (····).

The concentration of haem was $5\mu\text{M}$. Measurements were made in 1 cm cells at pH 7.4.

$5 \times 10^{-6}\text{M}$ -haem does not vary with concentration of lipid over the range $2 \times 10^{-4}\text{M}$ to 10^{-2}M and so it is unlikely that there is any aggregation of haem in the bound state.

Because of the dependence of haem spectra on the nature of the amphiphile head-group discussed above we investigated the possibility of interactions between haem and the head-group components of phosphatidylcholine and as shown in Fig. 5 both phosphocholine and L- α -glycerophosphocholine bring about substantial changes in the spectrum of haem. Choline also induced a measurable spectral change. The interactions are reversible as shown by the fact that on the addition of sodium dodecyl sulphate the spectra are the same as those in the presence of the detergent (Fig. 4), but in the absence of the head-group components. From these observations it is highly likely that the binding of haem to phosphatidylcholine involves interactions with the phospholipid head-groups. However, the fact that the spectrum of haem in phosphatidylcholine is different again from those in phosphocholine or glycerophosphocholine suggests that the hydrocarbon chains of the lipid are also important in the binding process and so it seems

Fig. 5. Effects of components of the phosphatidylcholine head-group on haem spectra

For each spectrum the concentration of haem was $5\mu\text{M}$: ····, Tris buffer, pH 7.4; —, 1 mM-L- α -glycerophosphocholine; ---, 10^{-2}M -phosphocholine.

likely that the dimethyl-divinyl 'end' of the haem molecule is immersed in the hydrocarbon region, whereas the iron atom and the hydrophilic propionic acid side chains are in the head-group region. Detailed studies of the free head-group-haem interactions would probably give more precise indications of the position of haem in the bilayer.

Haem in aqueous solution does not dialyse and is unstable, and for these reasons rapid spectrophotometric methods are the most appropriate for studying its binding characteristics (cf. Beaven *et al.*, 1974; Tipping *et al.*, 1976c). Accordingly the difference in spectrum between haem bound to phosphatidylcholine and free aqueous haem was exploited to obtain quantitative measurements of binding. Titrations were carried out at various concentrations of phosphatidylcholine and the difference in A_{411} between haem+lipid and haem alone was monitored. The choice of 411 nm is dictated by the dimerization of haem (see above): at this wavelength both monomers and dimers of the unbound tetrapyrrole absorb equally, which means that the measured absorbance differences result only from binding to lipid, i.e. they do not depend on the state of aggregation of the unbound haem (Tipping *et al.*, 1976c).

Fig. 6 shows the results of titrations of phosphatidylcholine with haem. These were carried out by adding small volumes of concentrated haem solutions to suspensions of the lipid and reading the absorbance difference against solutions of haem of the same final

Fig. 6. Difference-spectrophotometric titrations of egg phosphatidylcholine with haem

The difference in A_{411} between haem in phosphatidylcholine and equimolar haem in buffer was measured. Phosphatidylcholine concentrations were: 1 mM, $\circ$; 0.211 mM, $\bullet$; 0.15 mM, $\square$; 63.3 μ M, $\blacksquare$; 21.1 μ M, $\triangle$.

concentration, in buffer alone. The spectral changes were complete within the time required to mix the solutions and to start recording the absorbance differences (10–20 s) and very similar results were obtained whether unilamellar or multilamellar liposomes were used. Taken together these observations suggest that haem crosses the bilayers fairly easily, since although the rapid development of the absorbance differences might be explained by a complete lack of penetration of the liposomes, i.e. binding only to the exterior monolayer is possible, this would mean that multilamellar liposomes would bind much less haem than unilamellar ones, which was not found.

The titration results were analysed as follows. By extrapolating a plot of the reciprocal of the initial slope against the reciprocal of the phosphatidylcholine concentration to $[\text{phosphatidylcholine}]^{-1} = \text{zero}$, a limiting value of $\Delta A/[\text{haem}]$ of 1.56×10^4 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ was obtained. This value is the difference in absorption coefficient at 411 nm ($\Delta \epsilon_{411}$) between haem in the completely bound state, at $\bar{\nu} \rightarrow 0$, and unbound haem, and can be used to calcu-

late values of $\bar{\nu}$ and c for each titration point from the equations:

$$\bar{\nu} = \frac{[\text{haem}]_{\text{bound}}}{[\text{phosphatidylcholine}]} = \frac{\Delta A_{411}/(1.56 \times 10^4)}{[\text{phosphatidylcholine}]} \quad (2)$$

$$c = [\text{haem}]_{\text{total}} - [\Delta A_{411}/(1.56 \times 10^4)] \quad (3)$$

This approach is based on the assumption that the value of $\Delta \epsilon_{411}$ is independent of $\bar{\nu}$, i.e. that the difference spectrum is the same no matter how many haem molecules are present in the bilayer. The likelihood of the assumption being correct decreases as $\bar{\nu}$ increases, since at high occupancies a degree of haem–haem interaction might occur and give rise to new difference spectra. However, for reasons given above for bromosulphophthalein, the part of the binding isotherm that interests us is the region of low $\bar{\nu}$ and so the possibility of aggregation of bound haem is not of prime concern. Values of $\bar{\nu}$ and c obtained from eqns. (2) and (3) are plotted by the method of Scatchard (1949) in Fig. 7(a). The curvature of the plot makes extrapolation to $\bar{\nu} = 0$ impossible and so the limiting value of $\bar{\nu}/c$ cannot be obtained. Recalling, however, that the unbound haem is composed of monomers and dimers, whereas, at least at low values of $\bar{\nu}$, the bound form is probably monomeric, it is useful to replot the data of Fig. 7(a) in terms of $\bar{\nu}$ and the unbound concentration of monomer, $[M]$. Values of $[M]$ can be obtained from c by application of eqn. (1) and the plot shown in Fig. 7(b) is thus obtained. Bearing in mind the number of numerical manipulations required to reach this stage, the plot in Fig. 7(b) is a reasonable approximation to a straight line ($r = 0.89$) and extrapolations to the ordinate and abscissa respectively give a limiting value of $\bar{\nu}/[M]$ of 4.5×10^5 litre $\cdot$ mol $^{-1}$ and $\bar{\nu}_{\text{max.}} = 0.27$. Since the lower is the concentration of unbound haem the more of it is monomeric, the limiting value of $\bar{\nu}/c$ is also 4.5×10^5 litre $\cdot$ mol $^{-1}$. A similar analysis for liposomes composed of equimolar egg phosphatidylcholine and cholesterol (for which $\Delta \epsilon_{411} = 2.1 \times 10^4$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$) gave a limiting value of $\bar{\nu}/[M]$ of 3.5×10^5 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ and $\bar{\nu}_{\text{max.}} = 0.22$ ($\bar{\nu}$ in terms of phospholipid only). Although we acknowledge that these results for haem are subject to greater errors than those for bromosulphophthalein and oestrone sulphate, they are probably of the correct order and it is clear that the combination of hydrophobic and head-group interactions discussed above produces a high binding affinity.

In the presence of 1 mM-bromosulphophthalein, haem binds less readily to egg phosphatidylcholine (Figs. 7a and 7b). Since the pK of the propionic acid groups of haem is about 4.5 (Falk, 1964) it is likely that in the form of haem bound to phosphatidyl-

Fig. 7. The binding of haem by egg phosphatidylcholine (a) Scatchard plot of $\bar{v}/c$ against $\bar{v}$, where c is the total unbound concentration (monomers+dimers). (b) Scatchard plot of $\bar{v}/[M]$ against $\bar{v}$, where $[M]$ is the concentration of unbound monomeric haem; the line is fitted by linear regression ($r = 0.89$) and gives $\bar{v}_{\max} = 0.27$. In both Figures the symbols refer to the phosphatidylcholine concentrations shown in Fig. 6; in addition the closed triangles ($\blacktriangle$) refer to results in the presence of 1 mM-bromosulphophthalein. See the text for details.

choline they are ionized, and so the decreased binding brought about by bromosulphophthalein could

be due to charge-charge repulsion, as proposed for oestrone sulphate (see above).

Bilirubin

As shown by Mustafa & King (1970) the spectrum of bilirubin in lipid is red-shifted from its spectrum in aqueous solution. The same kind of shift is also observed on its binding to proteins such as serum albumin (Blauer & King, 1970), ligandin and aminoazo-dye-binding protein A (Tipping *et al.*, 1976b), and signifies that the bound bilirubin is in a hydrophobic environment. Bilirubin is like haem in that studies of its binding characteristics are restricted by its failure to dialyse and its instability, and so the spectral changes offer the most convenient means of measuring binding. Difference-spectrophotometric titrations of egg phosphatidylcholine were carried out at pH 8.2 over a range of lipid concentrations and the results are shown in Fig. 8. The spectral differences developed within the time required to mix the solutions and start recording, and were the same whether multilamellar or unilamellar liposomes were used. As with haem (see above) these observations suggest that bilirubin passes easily across the bilayers.

By the same procedure as used for haem (see above) we obtained a value for $\Delta\epsilon_{480}$ of 1.4×10^4 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ from a plot of the reciprocal of the initial slope

Fig. 8. Difference-spectrophotometric titrations of egg phosphatidylcholine with bilirubin

The difference in A_{480} between bilirubin in phosphatidylcholine and equimolar bilirubin in buffer was measured. Phosphatidylcholine concentrations were: 2.8 mM, $\circ$; 0.95 mM, $\bullet$; 0.285 mM, $\square$; 58 μ M, $\blacksquare$.

against $[\text{phosphatidylcholine}]^{-1}$ and this was used to calculate values of $\bar{v}$ and c . These are presented as a Scatchard plot in Fig. 9. The scatter of the points (which reflects the instability of monomeric bilirubin and its tendency to adsorb to glass cuvettes) makes extrapolation to either axis uncertain. It seems reasonable to assign a value of $4 \times 10^3 \text{ litre} \cdot \text{mol}^{-1}$ for the limiting value of $\bar{v}/c$; for $\bar{v}_{\text{max}}$, we can only conclude that it is at least 0.1, but probably less than 0.2. Egg phosphatidylcholine/cholesterol (1:1, molar ratio) gives a limiting value of $\bar{v}/c$ ($\bar{v}$ in terms of phospholipid only) of $2.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1}$ and again $0.1 \leq \bar{v} \leq 0.2$.

We chose to carry out most of our titrations of lipid with bilirubin at pH 8.2 because at lower pH values the pigment is very difficult to work with, the rate and extent of colloid formation increasing greatly as the pH is lowered (Brodersen & Theilgaard, 1969). However, we have also made some measurements at pH values lower than 8.2 (see Fig. 10) that show that the affinity of egg phosphatidylcholine for bilirubin increases substantially as the pH is lowered, paralleling the acid/base titration curve of bilirubin (Overbeek *et al.*, 1955) and its tendency to form colloids. We conclude that un-ionized bilirubin is preferentially bound by the lipid. If it is assumed that $\Delta\epsilon_{480}$ and $\bar{v}_{\text{max}}$, do not vary with pH then it can be

Fig. 9. The binding of bilirubin by egg phosphatidylcholine. The symbols refer to the phosphatidylcholine concentration of Fig. 8, i.e. 0.95 mM (●), 0.285 mM (□) and 58 μM (■).

Fig. 10. Egg phosphatidylcholine-bilirubin binding as a function of pH

The concentrations of phosphatidylcholine (10^{-4} M) and bilirubin (10^{-5} M) were kept constant. The buffers were 0.1 M-NaCl/0.01 M- Na_2HPO_4 /0.01 M-Tris adjusted to the appropriate pH with HCl. The values of $\bar{v}$ were calculated by assuming that $\Delta\epsilon_{480}$ ($1.4 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at pH 8.2) is independent of pH.

calculated that the limiting value of $\bar{v}/c$ at pH 7.4 is approx. 3 times that at pH 8.2, i.e. about $1.2 \times 10^4 \text{ litre} \cdot \text{mol}^{-1}$.

In the crystal form bilirubin is extensively intramolecularly hydrogen-bonded (Bonnett *et al.*, 1976). The propionyl carboxy groups are un-ionized and form three hydrogen-bonds each. The B-ring carboxy group forms two hydrogen-bonds with the pyrrole nitrogen atom of the D ring and one hydrogen-bond with the oxygen atom of the D ring, the C-ring carboxy group forms two hydrogen-bonds with the pyrrole nitrogen atom of the A ring and one hydrogen-bond with the oxygen atom of the A ring. These bonds result in the molecule as a whole having a ridge-tile structure so that the A and B rings are in a plane at right angles to the C and D rings. It is likely that bilirubin is in this conformation when dissolved in organic solvents such as chloroform (Bonnett *et al.*, 1976), and possibly also when in the colloidal state and when bound to lipids. This being so it is not obvious how bilirubin in phosphatidylcholine bi-

layers might be oriented. Cholesterol has a significant, but not especially great, effect on binding, which suggests that the bilirubin-lipid arrangement is to some extent flexible. Bromosulphophthalein is more effective than cholesterol at decreasing binding; at concentrations of bromosulphophthalein and phosphatidylcholine of 0.5 mM and 0.3 mM respectively the limiting value of $\bar{v}/c$ is approximately one tenth of that in the absence of bromosulphophthalein. The mechanism of this effect is unclear because, as discussed above, the bound bilirubin does not appear to be ionized and so bromosulphophthalein could not decrease its binding by direct charge-charge repulsion as proposed for bromosulphophthalein itself, oestrone sulphate and haem. Possibly the intramolecular hydrogen-bonding of bilirubin is disturbed by the presence in the bilayer of bromosulphophthalein in itself, or by the increase in negative charge which bromosulphophthalein confers upon the bilayer.

Our results for bilirubin binding to egg phosphatidylcholine are markedly different from those of Nagaoka & Cowger (1978) for the binding of bilirubin to sphingomyelin and phosphatidylserine (both from cow brain), bovine heart phosphatidylglycerol and synthetic dipalmitoylphosphatidylethanolamine and dipalmitoylphosphatidylcholine. These authors monitored the quenching by bilirubin of 1,3-diphenylhexa-1,3,5-triene incorporated into liposomes and derived expressions relating the extent of quenching to the binding parameters of bilirubin. From their results they concluded that only small numbers of bilirubin molecules could bind (corresponding to $\bar{v}_{\max}$ values of 0.002–0.004 for all lipids studied except phosphatidylglycerol, which had a value of 0.014), but that they did so with high apparent association constants (1.6×10^5 – 3.7×10^6 litre·mol⁻¹). That such values for $\bar{v}_{\max}$ cannot be reconciled with the absorbance

differences observed by ourselves and by Mustafa & King (1970) is shown by the following considerations. Taking an average value of 0.003 for $\bar{v}_{\max}$, the maximum concentration of bound bilirubin at a lipid concentration of 58 μ M would be $0.003 \times 58 \times 10^{-6} = 0.174 \mu$ M. In our titration of this concentration of egg phosphatidylcholine with bilirubin (cf. Fig. 8) the highest absorbance difference we measured was 0.06 (although this was by no means a saturation value) and so if the maximum bound bilirubin concentration is 0.174 μ M then the difference in absorption coefficient between the bound and free forms of the tetrapyrrole is at least $0.06/(0.174 \times 10^{-6}) = 3.45 \times 10^5$ litre·mol⁻¹·cm⁻¹. This value is too high: the peak (440 nm) absorption coefficient of bilirubin in aqueous solution is only 5.2×10^4 litre·mol⁻¹·cm⁻¹ (Blauer & King, 1970). Mustafa & King (1970) working with lipids extracted from mitochondria found that in pH 7.5 buffer at a lipid concentration of 15 μ g/ml (approx. 20 μ M) and with a total bilirubin concentration of 13.2 μ M, the bilirubin spectrum was quite different from that of the same concentration of bilirubin in the absence of lipid. At 490 nm the absorbance difference between the two solutions was approx. 0.2, which if there are only 0.003 'binding sites' per lipid molecule, corresponds to a difference absorption coefficient of $0.2/(20 \times 10^{-6} \times 0.003) = 3.3 \times 10^6$ litre·mol⁻¹·cm⁻¹. Moreover, Mustafa & King (1970) showed that at about 440 nm the absorbance difference was of opposite sign, i.e. the bound bilirubin absorbed less than the free form. For this to occur the absolute bound spectrum would need to have a negative absorption coefficient at this wavelength, which is impossible. It seems therefore that the theoretical basis for extracting binding parameters from the results of experiments of the kind performed by Nagaoka & Cowger (1978) may need reappraising.

Table 2. *Calculated subcellular distributions of bromosulphophthalein, oestrone sulphate, haem and bilirubin*
The results are calculated from eqn. (4) for free aqueous concentrations of 0.001 μ M and 0.1 μ M. Values for K_L and K_A refer to pH 7.0 and are from Tipping *et al.* (1976a,b,c) and from Ketterer *et al.* (1976). Data refer to 4°C for bromosulphophthalein and oestrone sulphate and to 25°C for haem and bilirubin. Numbers in parentheses are percentages of the total in each case.

	Ligand concn. (μ M)									
	Unbound aqueous		Bound to ligandin		Bound to protein A		Bound to phospholipid		Total	
Free aqueous concentration (μ M) ...	0.001	0.1	0.001	0.1	0.001	0.1	0.001	0.1	0.001	0.1
Bromosulphophthalein	0.001	0.1	1.09	52.4	0.076	7.06	8.80	688	99.6	748
	(0)	(0)	(10.9)	(7.0)	(0.8)	(0.9)	(88.3)	(92.0)		
Oestrone sulphate	0.001	0.1	0.066	6.2	0.030	2.9	0.096	9.6	0.193	18.8
	(0.5)	(0.5)	(34.2)	(32.9)	(15.5)	(15.5)	(49.7)	(51.1)		
Haem	0.001	0.1	0.99	50.0	0.40	28.6	36.0	3040	37.4	3120
	(0)	(0)	(2)	(1.6)	(1.1)	(0.9)	(96.3)	(97.5)		
Bilirubin	0.001	0.1	0.695	41.2	1.96	66.7	0.96	960	3.62	204
	(0)	(0.05)	(19.2)	(20.2)	(54.2)	(32.7)	(26.5)	(47.1)		

The subcellular distributions of bromosulphophthalein, oestrone sulphate, haem and bilirubin

As discussed in the preceding paper (Tipping *et al.*, 1979) the combination of data for binding to proteins and to phospholipids allows the subcellular distribution of a given molecule to be estimated. This is accomplished by application of the following equation (eqn. 3 of Tipping *et al.*, 1979):

$$c_{\text{total}} = c \left\{ 1 + \frac{10^{-4}K_L}{1 + cK_L} + \frac{10^{-4}K_A}{1 + cK_A} + 0.08 (\bar{v}/c)_c \right\} \quad (4)$$

where K_L and K_A are the association constants for ligandin and protein A respectively and for lipid binding $(\bar{v}/c)_c$ is the value of $\bar{v}/c$ corresponding to the particular value of c . The factors 10^{-4} and 0.08 arise from the hepatocyte concentrations of ligandin and protein A (both approx. 10^{-4}M) and of phospholipid (0.08M). By using eqn. (4), the results reported in the present study together with results for the binding by ligandin and protein A of bromosulphophthalein and oestrone sulphate (Tipping *et al.*, 1976a; Ketterer *et al.*, 1976), bilirubin (Tipping *et al.*, 1976b) and haem (Tipping *et al.*, 1976c) give the subcellular distributions shown in Table 2. It should be pointed out that for bilirubin we have estimated values of $\bar{v}/c$ for pH 7.4, basing our estimates on the results at pH 8.2 together with the pH-dependent plot in Fig. 10. In the preceding paper (Tipping *et al.*, 1979), where a number of uncharged compounds (4-dimethylaminoazobenzene, 2-acetylaminofluorene, oestrone and testosterone) were considered we chose to set $c = 10^{-7}\text{M}$, and this concentration was sufficiently low so that saturation effects on neither protein- nor lipid-binding were significant. Since the compounds studied in the present paper bind more strongly to proteins in all cases and to lipids also in the cases of bromosulphophthalein and haem, a free aqueous concentration of 10^{-7}M gives rise to significant degrees of saturation. We have therefore performed the calculations for a value of c of 10^{-9}M as well as 10^{-7}M . Because some of the cellular phospholipid may not be available to bind small molecules (cf. Tipping *et al.*, 1979) we have calculated the percentages of the compounds that would be bound to lipid at a lipid concentration of 0.04M (Table 2 uses a value of 0.08M). They are, at $c = 10^{-9}\text{M}$, 79.0% for bromosulphophthalein, 33.1% for oestrone sulphate, 92.8% for haem and 15.3% for bilirubin.

As with the uncharged compounds studied in the preceding paper (Tipping *et al.*, 1979) these calculations suggest that membrane lipids are important subcellular binders of ligands of ligandin and protein

A and this binding seems likely to have a major influence on their intracellular transport, even though diffusion coefficients in lipid bilayers are 1–2 orders of magnitude lower than those for small molecules or proteins in aqueous solution (see e.g. Edidin, 1974; Vanderkooi & Callis, 1974).

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