Biochem. J. (1979) **180**, 319–326
Printed in Great Britain

Interactions of Small Molecules with Phospholipid Bilayers

BINDING TO EGG PHOSPHATIDYLCHOLINE OF SOME UNCHARGED MOLECULES (2-ACETYLAMINOFLUORENE, 4-DIMETHYLAMINOAZOBENZENE, OESTRONE AND TESTOSTERONE) THAT BIND TO LIGANDIN AND AMINOAZO-DYE-BINDING PROTEIN A

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(*Received* 18 *October* 1978)

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 have examined the interactions of 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone with aqueous dispersions of egg phosphatidylcholine and egg phosphatidylcholine/cholesterol (1:1, molar ratio) by equilibrium dialysis and spectrophotometry. 2. At 25°C and pH7.4, the partition coefficients for binding to phosphatidylcholine [expressed as (mol of ligand bound/mol of phosphatidylcholine)/unbound ligand concentration] were: for 2-acetylaminofluorene, 5.0×10^3 litre·mol^{-1}; for 4-dimethylaminoazobenzene, 2.1×10^4 litre·mol^{-1}; for oestrone, 3.1×10^3 litre·mol^{-1}; and for testosterone, 4.2×10^2 litre·mol^{-1}. In the ranges studied these values were independent of concentration. The results for the two steroids confirm those of Heap, Symons & Watkins [(1970) *Biochim. Biophys. Acta* **218**, 482–495]. 3. The introduction of cholesterol into the lipid bilayers caused large decreases in the partition coefficients of oestrone and testosterone, but had relatively little effect on the binding of 2-acetylaminofluorene and 4-dimethylaminoazobenzene. 4. By assuming that the interactions with egg phosphatidylcholine bilayers resemble those with the phospholipid components of mammalian intracellular membranes the phosphatidylcholine partition coefficients, 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 98, 99, 89 and 58% of the total 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone 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 of small molecules and proteins in aqueous solution 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 (see e.g. Edidin, 1974, for a review). The relative amounts of diffusing compound 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.*, 1976*a*,*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 present paper we report equilibrium-dialysis and spectrophotometric measurements of the binding of some uncharged ligands of ligandin and protein A to dispersions of lipids. We have used egg phosphatidylcholine and egg phosphatidylcholine/cholesterol (1:1, molar ratio) as models for cellular membranes. The ligands studied, 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone (Fig. 1), bind to both ligandin and protein A with association constants of approx. 10^5

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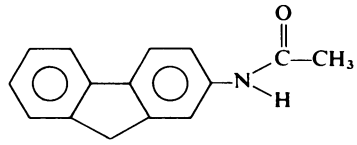
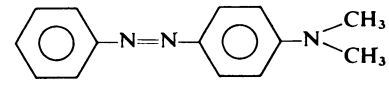


2-Acetylaminofluorene



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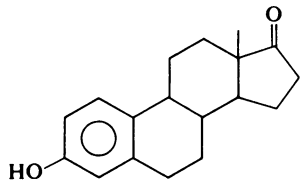
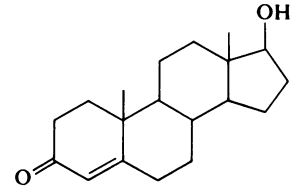


Oestrone



Testosterone

Fig. 1. *The structures of the ligands studied in the present work*

litre·mol^{-1} (Ketterer *et al.*, 1976; Tipping *et al.*, 1976*a*; the present work).

Experimental

Materials

Cholesterol, oestrone and testosterone were purchased from the Sigma Chemical Co., Poole, Dorset, U.K., 4-dimethylaminoazobenzene was from Koch–Light Laboratories, Colnbrook, Bucks., U.K., egg phosphatidylcholine was from Lipid Products, South Nutfield, Surrey, U.K., and 2-acetylaminofluorene was from the Aldrich Chemical Co., Gillingham, Dorset, U.K.

2-Acetylamino[9-^{14}C]fluorene (sp. radioactivity 30 Ci/mol) was purchased from NEN Chemicals G.m.b.H., Frankfurt, Germany, [*carboxy*-^{14}C]-cholic acid (sp. radioactivity 59.5 Ci/mol), [6,7-^{3}H]-oestrone (sp. radioactivity 46000 Ci/mol) and [1,2-^{3}H]testosterone (sp. radioactivity 42000 Ci/mol) were from The Radiochemical Centre, Amersham, Bucks., U.K. 4-Dimethylaminoazobenzene, generally tritiated in the unsubstituted benzene ring (sp. radioactivity 50 Ci/mol), was a gift from Dr. John Roberts, Chester Beatty Research Institute, Chalfont St. Giles, Bucks., U.K.

All materials were used as supplied except for 4-dimethylaminoazobenzene, which was purified before use by passage through alumina in benzene.

Phospholipid dispersions

Two methods were used to prepare dispersions of phospholipids, the hand-shaking procedure of Bangham (1968), which gives multilamellar liposomes, and the technique of Brunner *et al.* (1976), which involves the quantitative removal of cholate from phospholipid/cholate mixed micelles by passage through Sephadex G-50, and gives unilamellar liposomes. In the latter case egg phosphatidylcholine (50–200mg) was mixed with 2–4ml of 5% sodium cholate and the resulting clear dispersion applied to a column (2.5cm × 50cm) of Sephadex G-50 equilibrated with 0.1M-NaCl/0.05M-Tris/HCl buffer, pH 7.4. The material emerging at the free volume contained less than 0.05% of the original cholate, as determined by using a trace of [^{14}C]cholate, and was faintly opaque. The final molar ratio of cholate/phospholipid was ≤ 0.005. Examination of the liposomes prepared by this technique, by electron microscopy after negative staining with phosphotungstate (Bangham & Horne, 1964), showed that the lipid was in the form of unilamellar vesicles, in agreement with Brunner *et al.* (1976). Equimolar phosphatidylcholine/cholesterol dispersions were prepared by the hand-shaking method. Phospholipid concentrations were determined by the method of Rouser *et al.* (1966), as modified by Ketterer *et al.* (1973).

Preparation of proteins

Ligandin and aminoazo-dye-binding protein A were prepared as described by Tipping *et al.* (1976*a*) and Ketterer *et al.* (1976) respectively. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Equilibrium dialysis

Experiments were carried out as described previously (Tipping *et al.*, 1976*a*), in 0.1M-NaCl/0.05M-Tris/HCl buffer, pH7.4, at phospholipid concentrations of 0.1–1.0mM. Control experiments with buffer only indicated that for all four compounds studied equilibrium was attained within 24h. Adsorp-

tion to the apparatus occurred to a small degree (up to 10% of the total) with 2-acetylaminofluorene, oestrone and testosterone, and to a greater extent (up to 80%) with 4-dimethylaminoazobenzene. These adsorption processes should not invalidate our results, however, since measurements of radioactivity were made on samples from both sides of the membrane, i.e. the total amounts of radioactivity in solution were determined. Moreover, measurements made 24h apart on the same solutions showed that the adsorption processes were complete within the time for equilibration across the membrane to occur. However, the high degree of adsorption of 4-dimethylaminoazobenzene meant that the results obtained with this compound were less reproducible than those with the others (see the Results and Discussion section).

Radioactivity was determined by using a Packard 3385 liquid-scintillation spectrometer, with scintillation fluid consisting of 10g of 2,5-diphenyloxazole, 500ml of Triton X-100 and 2 litre of toluene.

Spectrophotometry

Measurements were made with a Pye–Unicam SP. 1800 spectrophotometer. Titrations of egg phosphatidylcholine with 4-dimethylaminoazobenzene were carried out by the addition of small volumes (0–0.1 ml) of stock solutions of 4-dimethylaminoazobenzene in methanol to 10ml of phosphatidylcholine dispersion in a 4cm cuvette. In all cases the spectral changes that developed were complete within the time of mixing (10–20s).

Expression of binding results

Our results are expressed throughout in terms of the parameters $\bar{\nu}$ (mol of compound bound/mole of lipid P) and c (the unbound concentration of compound). Since there is no reason to believe that the structure of the lipid bilayers varies with concentration in the range used in our experiments (0.01–3mM), $\bar{\nu}$ and c are intensive quantities, i.e. they do not depend on the concentration of lipid.

An alternative and sometimes more convenient means of expression involves the conversion of $\bar{\nu}$ values to concentrations in the 'two-dimensional liquid' of the bilayer. Taking the density of phospholipid bilayers as 1.01g/ml (Huang, 1969) and the molecular weight of an average phospholipid as 750, then $\bar{\nu}/c$ is converted into the quantity (concentration in the membrane)/(concentration in water) by multiplying by $1.01 \times 10^3/750$ ($= 1.35 \text{mol} \cdot \text{litre}^{-1}$).

Results and Discussion

4-Dimethylaminoazobenzene

In the wavelength range 350–500nm the spectrum of 4-dimethylaminoazobenzene is sensitive to the dielectric constant of the medium (Fig. 2). When bound to egg phosphatidylcholine the shape of its spectrum corresponds to that which would result from an environment somewhat more polar than methanol, which suggests that 4-dimethylaminoazobenzene positions itself at or near the hydrocarbon/head-group interface of the bilayer.

The difference in spectrum between aqueous and lipid-associated 4-dimethylaminoazobenzene was used to monitor the extent of binding. The quantity $\Delta A_{410} - \Delta A_{480}$ (i.e. the peak minus the trough of the difference spectrum when the absorbance of 4-dimethylaminoazobenzene + phosphatidylcholine is read against that for 4-dimethylaminoazobenzene in buffer) was measured as a function of 4-dimethylaminoazobenzene and phosphatidylcholine concentrations (Fig. 3*a*). A plot of the reciprocals of the slopes of these titration curves (which in all cases are

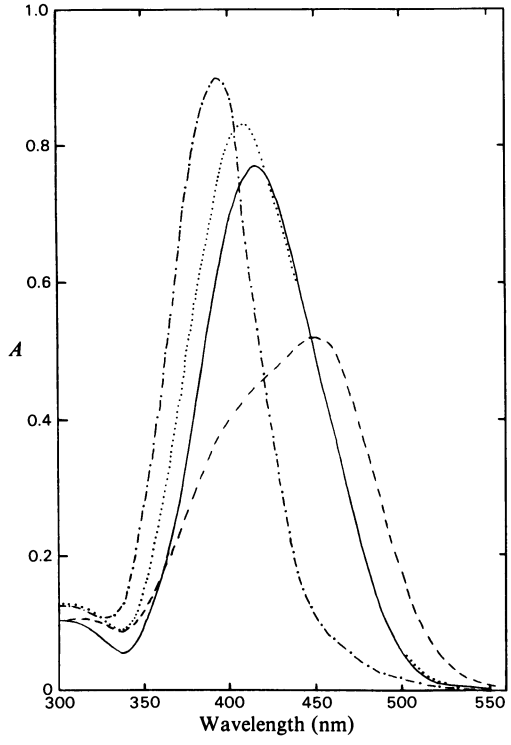


Fig. 2. *Spectra of* 4-*dimethylaminoazobenzene in* 1.9×10^{-3}M-*phosphatidylcholine* (——), *pH*7.4 *buffer* (– – – –), *methanol* (· · · ·) *and heptane* (– · – · –)

In each case the concentration of 4-dimethylaminoazobenzene was 9×10^{-6}M and measurements were made in 4cm path-length cuvettes. Quantitative binding measurements indicate that for the spectrum in phosphatidylcholine 97.5% of the total 4-dimethylaminoazobenzene was in the lipid, so the spectrum is essentially that of the bound chromophore.

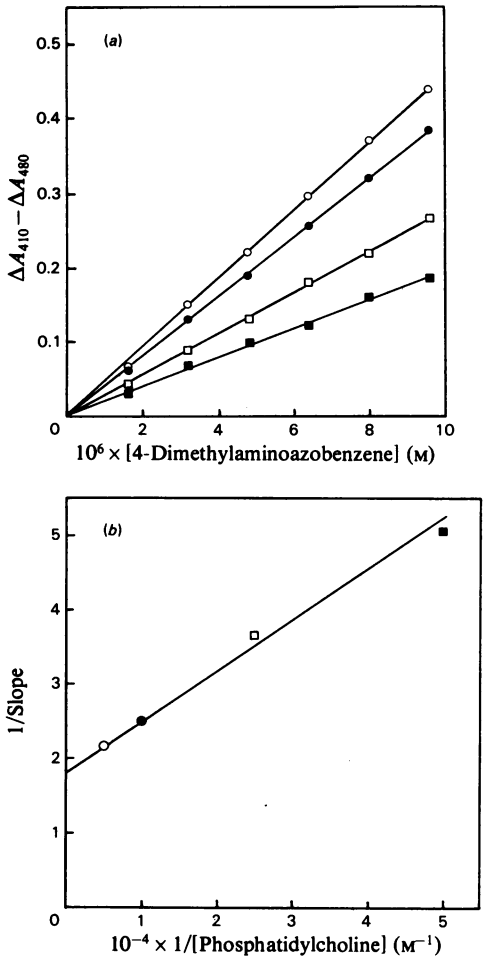


Fig. 3. *Difference spectrophotometric titrations of egg phosphatidylcholine with 4-dimethylaminoazobenzene* (*a*) Plots of $\Delta A_{410} - \Delta A_{480}$ against [4-dimethylaminoazobenzene] as a function of phosphatidylcholine concentration. Symbols: ○, 2×10^{-4}M-phosphatidylcholine; ●, 10^{-4}M-phosphatidylcholine; □, 4×10^{-5}M-phosphatidylcholine; ■, 2×10^{-5}M-phosphatidylcholine. The reciprocals of the slopes of these plots are plotted against the reciprocals of the phosphatidylcholine concentrations in (*b*). See the text for details.

linear with respect to concentration) against the reciprocals of the phosphatidylcholine concentrations (Fig. 3*b*) gives a limiting value of $(\Delta A_{410} - \Delta A_{480})$/[4-dimethylaminoazobenzene] as [phosphatidylcholine]$\rightarrow\infty$ of 5.46×10^4 litre·mol^{-1}, from which we obtain $\Delta\Delta\varepsilon = 1.37 \times 10^4$ litre·mol^{-1}·cm^{-1}. This quantity was used to calculate values of $\bar{\nu}$ (mol of 4-dimethylaminoazobenzene bound/mol of phospholipid) and c, the concentration of unbound 4-dimethylaminoazobenzene, from the equations:

$$\bar{\nu} = (\Delta A_{410} - \Delta A_{480})/(4\Delta\Delta\varepsilon[\text{phosphatidylcholine}]) \quad (1)$$

and

$$c = [\text{4-dimethylaminoazobenzene}]_{\text{total}} - (\Delta A_{410} - \Delta A_{480})/4\Delta\Delta\varepsilon \quad (2)$$

The values of $\bar{\nu}$ and c so obtained are presented as a double-logarithmic plot in Fig. 4, together with results from equilibrium-dialysis measurements. Within experimental error both sets of results give linear plots of log $\bar{\nu}$ against log c, with slopes essentially equal to unity. This means that in the concentration range studied the binding of 4-dimethylaminoazobenzene to egg phosphatidylcholine obeys a straightforward partition-type equilibrium, i.e. $\bar{\nu}/c$ is constant. The value of $\bar{\nu}/c$ at 25°C from the spectroscopic experiments was 2.65 ($\pm$0.08) $\times 10^4$ litre·mol^{-1}. A value of 1.7 ($\pm$0.5) $\times 10^4$ litre·mol^{-1} was obtained from equilibrium-dialysis experiments (Table 1). The results obtained by spectroscopy involved multilamellar liposomes only, whereas the equilibrium-dialysis results are from experiments on both multi-

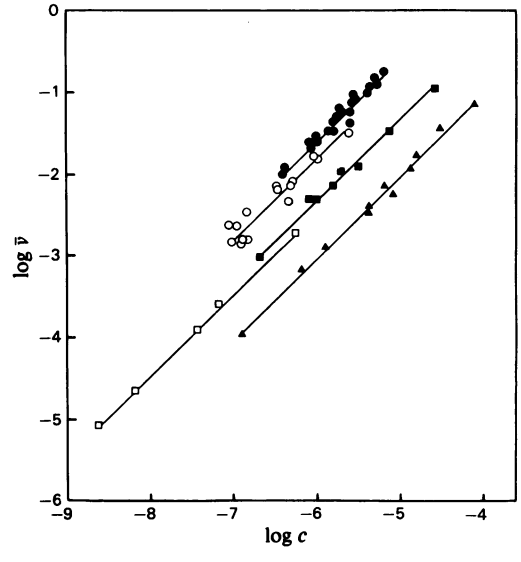


Fig. 4. *The binding of 2-acetylaminofluorene* (■), *4-dimethylaminoazobenzene* (○, ●), *oestrone* (□) *and testosterone* (▲) *by egg phosphatidylcholine at 25°C* Results are expressed in terms of $\bar{\nu}$ (mol of compound bound/mol of phosphatidylcholine) and c, the unbound concentration. For 4-dimethylaminoazobenzene the open circles refer to equilibrium-dialysis measurements and the filled circles to spectroscopic experiments (cf. Fig. 3). All plots are drawn with slopes of unity; the best slopes, from least-squares analysis, are shown in Table 2.

lamellar and unilamellar liposomes, the results from the two types of preparation being indistinguishable. The high degree of scatter in the equilibrium-dialysis results reflects the great tendency of 4-dimethylaminoazobenzene to adsorb to surfaces.

As at 25°C $\bar{\nu}/c$ is independent of concentration at 4°C, and has a slightly higher value, although the standard deviations of $\bar{\nu}/c$ at the two temperatures overlap (Table 1). The binding of 4-dimethylaminoazobenzene to dispersions of phosphatidylcholine/cholesterol (1:1, molar ratio) was not studied over a wide concentration range: instead binding was measured at a single low value of $\bar{\nu}$. In view of the results for phosphatidylcholine alone there seems no reason to expect $\bar{\nu}/c$ to vary with concentration. If $\bar{\nu}/c$ for the phosphatidylcholine/cholesterol mixture is expressed in terms of phospholipid only, then it is greater than the value for phosphatidylcholine alone. However, if it is expressed as mol bound/mol of bilayer component (i.e. phospholipid + cholesterol) then the value of $\bar{\nu}/c$ is nearly the same as for phosphatidylcholine alone (Table 1).

The constancy of $\bar{\nu}/c$ over a wide range of $\bar{\nu}$ (0.001–0.08) indicates that 4-dimethylaminoazobenzene in egg phosphatidylcholine behaves as an ideal 'solution' in that the binding of one 4-dimethylaminoazobenzene molecule does not affect the binding of the next.

The binding of 4-dimethylaminoazobenzene to protein A has not been measured, but is required for calculations of the subcellular distribution of 4-dimethylaminoazobenzene (see below). We therefore performed equilibrium-dialysis experiments with the 4-dimethylaminoazobenzene–protein A system and obtained a value for $\lim_{\bar{\nu}\to 0}(\bar{\nu}/c)$ of 1.2×10^5 litre·mol^{-1} at 4°C ($\bar{\nu}$ and c being defined as for the lipids). In common with the other compounds studied in the present work, 4-dimethylaminoazobenzene has low water solubility and we are obliged to express results in this form, rather than as more conventional association constants. Previous work has shown that both ligandin and protein A bind their hydrophobic ligands at a single site (Tipping *et al.*, 1976*a*,*b*,*c*, 1978; Ketterer *et al.*, 1976) and so limiting values of $\bar{\nu}/c$ can reasonably be taken as association constants (cf. Tipping *et al.*, 1976*a*).

2-Acetylaminofluorene

Fig. 4 shows the results of equilibrium-dialysis measurements of the binding of 2-acetylaminofluorene to egg phosphatidylcholine at 25°C. As with 4-dimethylaminoazobenzene straightforward partition-type binding is observed in the range of $\bar{\nu}$ 0.0009–0.11 with $\bar{\nu}/c = 5.0 \times 10^3$ litre·mol^{-1}. There is a somewhat greater temperature-dependence of 2-acetylaminofluorene binding than that of 4-dimethylaminoazobenzene, $\bar{\nu}/c$ decreasing by almost a half on an increase in temperature from 4°C to 25°C (Table 1). The $\bar{\nu}/c$ value for egg phosphatidylcholine/cholesterol is very close to that for egg phosphatidylcholine alone, when $\bar{\nu}$ is expressed in terms of phospholipid only (Table 1), but if it is expressed in terms of phospholipid + cholesterol the value is about a half that for phosphatidylcholine alone.

The u.v. spectrum of 2-acetylaminofluorene changes to a small extent on transfer from water to

Table 1. *The binding of 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone to dispersions of egg phosphatidylcholine and egg phosphatidylcholine + cholesterol*

The results are expressed as $\bar{\nu}/c$ where $\bar{\nu}$ = mol of ligand bound per mol of phospholipid, or per mol of phospholipid + cholesterol, and c is the unbound ligand concentration. The results for phosphatidylcholine at 4°C cover ranges of $\bar{\nu}$ and c similar to those at 25°C (Fig. 4). The values in italics are the slopes of the log $\bar{\nu}$ against log c plots, which are taken as unity to calculate $\bar{\nu}/c$ values. For phosphatidylcholine/cholesterol mixtures results refer to only one value of $\bar{\nu}$, which is indicated in each case. The uncertainties are ± 1 standard deviation.

	$10^{-3} \times \bar{\nu}/c$ (litre·mol^{-1})			
			Phosphatidylcholine + cholesterol (1:1)	
	Phosphatidylcholine at 4°C	Phosphatidylcholine at 25°C	$\bar{\nu}$ in terms of phospholipid only	$\bar{\nu}$ in terms of phospholipid + cholesterol
2-Acetylaminofluorene	9.1 (±0.1) *1.00*	5.0 (±0.7) *0.96*	5.4 (±0.4) [$\bar{\nu}$ = 0.00039]	2.7 (±0.2) [$\bar{\nu}$ = 0.002]
4-Dimethylaminoazobenzene	20.8 (±7.0) *0.94*	17.0 (±5.0) *0.95*	28.6 (±6.5) [$\bar{\nu}$ = 0.0021]	14.3 (±3.3) [$\bar{\nu}$ = 0.0011]
Oestrone	3.4 (±0.2) *0.97*	3.1 (±0.2) *0.96*	0.7 (±0.1) [$\bar{\nu}$ = 0.0030]	0.35 (±0.05) [$\bar{\nu}$ = 0.0015]
Testosterone	0.52 (±0.05) *1.01*	0.42 (±0.05) *1.00*	0.17 (±0.02) [$\bar{\nu}$ = 0.0011]	0.09 (±0.01) [$\bar{\nu}$ = 0.0006]

organic solvents, but the spectra in methanol, ethanol, dioxan and heptane are almost identical and consequently the spectrum of 2-acetylaminofluorene does not reflect the local environment as does the spectrum of 4-dimethylaminoazobenzene.

Equilibrium-dialysis measurements of the binding of 2-acetylaminofluorene by ligandin and protein A gave values for $\lim_{\bar{\nu}\to 0}(\bar{\nu}/c)$ of 1.0×10^5 and 7×10^4 litre·mol^{-1} respectively.

Oestrone and testosterone

These two compounds exhibit constant values of $\bar{\nu}/c$ over a large range of concentrations, as shown by the results of equilibrium-dialysis measurements (Fig. 4). Values of $\bar{\nu}/c$ of 3.1×10^3 litre·mol^{-1} for oestrone and 4.2×10^2 litre·mol^{-1} for testosterone were obtained at 25°C and at 4°C the values are marginally higher (Table 1). Since oestrone and testosterone have rather similar structures (cf. Fig. 1) the rather large difference in their $\bar{\nu}/c$ values shows that the binding of steroids to lipid bilayers cannot be thought of as a simple process of dissolution; orientation and specific head-group effects should also be taken into account. In contrast with the results for 2-acetylaminofluorene and 4-dimethylaminoazobenzene, the binding to lipid bilayers of both oestrone and testosterone is markedly diminished by the introduction of cholesterol. When $\bar{\nu}$ is expressed in terms of total bilayer component the $\bar{\nu}/c$ values for binding to phosphatidylcholine/cholesterol are 0.1–0.2 times those for binding to phosphatidylcholine alone. This suggests that the interactions of the two steroids with the bilayers differ qualitatively from those of 2-acetylaminofluorene and 4-dimethylaminoazobenzene. Munck (1957) has measured the interfacial activity of testosterone and some other steroids at a heptane/water interface and has shown that they concentrate at the interface with their hydrocarbon nuclei either flat against it (testosterone, progesterone and deoxycorticosterone) or edge on (cortisol), and with their hydrophilic groups in contact with water. As pointed out by Lucy (1968) a similar arrangement is possible in the lipid bilayers. On the other hand (+)-equilenin, a steroid with aromatic A and B rings, a 3-amino group and a 17-oxo group, is, according to the fluorescence polarization studies of Badley *et al.* (1973), oriented in phosphatidylcholine bilayers with its long axis essentially normal to the bilayer plane (although able to undergo molecular motion from 0–30° to the normal). Moreover cholesterol has no effect on the fluorescence properties of (+)-equilenin in the bilayers. It would clearly be interesting to carry out quantitative studies of the interactions of this steroid with phosphatidylcholine in the absence and presence of cholesterol. 2-Acetylaminofluorene and 4-dimethylaminoazobenzene each with one hydrophilic group (Fig. 1) would seem more likely to align themselves in the bilayers with their long axes parallel to the fatty acid hydrocarbon chains and to be able to undergo greater molecular motion than the steroids. In these terms the condensing effect of cholesterol on bilayers (see, e.g., Demel & de Kruyff, 1976) might be expected to result in the 'squeezing out' of oestrone and testosterone, but to have relatively little effect on the binding of 2-acetylaminofluorene and 4-dimethylaminoazobenzene.

Comparisons with previous work

There do not seem to have been any previous studies of the binding of 2-acetylaminofluorene or 4-dimethylaminoazobenzene to lipid bilayers or to membranes. On the other hand quantitative measurements of the binding of steroids to both artificial membranes (Snart & Wilson, 1967; Heap *et al.*, 1970) and microsomal fractions (Blyth *et al.*, 1971; Zakim & Vessey, 1977) have been made.

Our results for oestrone give higher values for binding to egg phosphatidylcholine than those for egg phosphatidylcholine/dicetyl phosphate (9:1, molar ratio) reported by Snart & Wilson (1967). The results given by these authors correspond to $\bar{\nu}/c = 6.4 \times 10^2$ litre·mol^{-1}, compared with our value of 3.1×10^3 litre·mol^{-1} (Table 1). Although Snart & Wilson (1967) found that cholesterol decreases the affinity of the bilayers for oestrone, the effect was small: for egg phosphatidylcholine/dicetyl phosphate/cholesterol (6:1:3, molar ratio), $\bar{\nu}/c = 5.2 \times 10^2$ litre·mol^{-1}. It does not seem likely that the presence of 10% dicetyl phosphate in the lipid dispersions could be responsible for the differences between our results and those of Snart & Wilson (1967), but there is no other obvious reason. We are in much better agreement with the results of Heap *et al.* (1970), who, like Snart & Wilson (1967) and ourselves, measured the binding of radioactively labelled steroids to egg phosphatidylcholine by equilibrium dialysis. At 37°C and pH 7.4 the results of Heap *et al.* (1970) for oestrone and testosterone correspond to $\bar{\nu}/c = 3.5 \times 10^3$ litre·mol^{-1} and $\bar{\nu}/c = 4.0 \times 10^2$ litre·mol^{-1} respectively, which are very close to our values (Table 1).

The binding of testosterone to rat liver microsomal membranes has been studied by Blyth *et al.* (1971), who found that the membranes contain many unsaturable 'loose' binding sites, which were ascribed to membrane lipid. The results of Blyth *et al.* (1971) give a value of 0.25 for the parameter (mol of testosterone bound/g of microsomal protein)/(unbound testosterone concentration). Taking the weight ratio of phospholipid/protein in rat liver microsomal fraction to be 1:3 and the molecular weight of a typical phospholipid to be 750 (DePierre & Dallner, 1975) this corresponds to a $\bar{\nu}/c$ value of 5.6×10^2 litre·mol^{-1}, which is very close to our result for egg phosphatidylcholine. (The molar ratio of cholesterol/

phospholipid in microsomal fraction is less than 0.1; DePierre & Dallner, 1975.)

Zakim & Vessey (1977) have studied the binding of oestrone to microsomal membranes and found a partition coefficient, in units of (mol of oestrone bound/mol of phospholipid)/(mol of oestrone unbound/mol of water), of 1.5×10^5. This value corresponds to a value of $\bar{\nu}/c$ of 2.7×10^3 litre·mol^{-1}, which is almost identical to our result with egg phosphatidylcholine (Table 1).

The subcellular distributions of 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone

A primary purpose of the present work was to provide data from which to estimate the distribution of ligands of the intracellular binding proteins ligandin and protein A between the aqueous and membrane parts of the cell. Our results enable us to calculate, for a given value of c, values of $\bar{\nu}$ for binding to phospholipid, and, by using data obtained previously (Tipping *et al.*, 1976*a*; Ketterer *et al.*, 1976), for binding to ligandin and protein A. With knowledge of the amounts of phospholipid and proteins in the cell, the distributions can be calculated. We are primarily interested in the liver of the rat, in which the cytosol concentrations of ligandin and protein A are both approx. 10^{-4}M (Litwack *et al.*, 1971; B. Ketterer, L. Christodoulides & E. Tipping, unpublished results). DePierre & Dallner (1975) have given a value of 30mg/g wet wt. for the phospholipid content of rat liver, which, taking the density of an average rat hepatocyte to be 1.067g/ml (Weibel *et al.*, 1969), and the molecular weight of an average phospholipid to be 750, corresponds to an overall cellular concentration of 0.0375M. We wish to know the distribution of our small molecule in the system cytosol + lipid bilayer. The volume of the lipid in 1 litre of liver cells is, taking the density of phospholipid bilayers to be 1.01g/ml (Huang, 1969), 32ml, and since the cytosol comprises 44% of the volume of a hepatocyte (Weibel *et al.*, 1969) the total volume of cytosol + phospholipid is 47.2% of the total cellular volume. In this volume the concentrations of ligandin and protein A are still approx. 10^{-4}M, whereas that of lipid bilayer is $0.0375\text{M} \times (100/47.2) = 0.079\text{M}$, which we round off to 0.08M for the sake of simplicity. At a given value of c the total concentration of small molecule in our system c_t is given by:

$$c_t = c\left\{1+\frac{10^{-4}K_L}{1+cK_L}+\frac{10^{-4}K_A}{1+cK_A}+0.08(\bar{\nu}/c)_c\right\} \quad (3)$$

where K_L and K_A are the association constants for ligandin and protein A respectively and, for binding to lipid, $(\bar{\nu}/c)_c$ is the value of $\bar{\nu}/c$ corresponding to the particular value of c. For the four compounds studied here $\bar{\nu}/c$ is independent of c (Fig. 4), but as shown in the following paper (Tipping *et al.*, 1979) this is not generally true. Since intracellular membranes have low cholesterol/phospholipid ratios (Rouser *et al.*, 1968) the appropriate values of $\bar{\nu}/c$ for calculating the subcellular distributions from eqn. (3) are those for pure phosphatidylcholine. The results of the calculations are shown in Table 2.

From Table 2 it is clear that intracellular membranes are important binders of the compounds studied. It is probable, however, that the percentages calculated to be in the membranes are overestimated because of the likelihood that not all the cellular phospholipid is able to bind small molecules in the same way as egg phosphatidylcholine bilayers. For example Stier & Sackmann (1973) have estimated that approx. 20% of the phospholipid in the endoplasmic reticulum is immobilized to some extent by cytochrome *P*-450 and such immobilization might

Table 2. *Calculated subcellular distributions of 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone*
The distributions are calculated by eqn. (3) for a dispersion consisting of ligandin (10^{-4}M), protein A (10^{-4}M) and phosphatidylcholine (0.08M). The free aqueous concentration is set equal to 0.1 μM in each case. Values of K_L and K_A are from Tipping *et al.* (1976*a*), Ketterer *et al.* (1976) and the present work. The concentrations, which are in μM, are calculated from binding data at 4°C. Values in parentheses are percentages of the total. See the text for details and comment.

	Concentration (μM)				
	Unbound aqueous	Bound to ligandin	Bound to protein A	Bound to phospholipid	Total
2-Acetylaminofluorene	0.1	0.99	0.70	72.8	74.6
	(0.1)	(1.3)	(0.9)	(97.6)	
4-Dimethylaminoazobenzene	0.1	0.79	1.19	166.4	168.5
	(0.1)	(0.5)	(0.7)	(98.8)	
Oestrone	0.1	0.89	2.34	27.2	30.5
	(0.3)	(2.9)	(7.7)	(89.1)	
Testosterone	0.1	1.69	0.99	4.2	7.2
	(1.4)	(27.2)	(13.7)	(57.7)	

modify the ability of the phospholipid to bind small molecules. Furthermore, in calculating the subcellular distribution of a small molecule with a view to considering its intracellular transport, the plasma membrane should be omitted. However even if we take a value of 0.04M for the 'effective' concentration of phospholipid (i.e. half the total concentration) the percentages of the compounds studied bound by the membrane lipid are still high: 95.3% for 2-acetylaminofluorene; 97.6% for 4-dimethylaminoazobenzene; 80.3% for oestrone; and 40.8% for testosterone.

From the results of the calculations shown in Table 2 it follows that lateral-diffusion fluxes in the membranes could be quite high, as long as the diffusion coefficients are not too low. Most current estimates of lateral-diffusion coefficients in membranes are between 10^{-8} and 10^{-7} cm^2/s (see e.g. Edidin, 1974; Vanderkooi & Callis, 1974). Although these values are 1–2 orders of magnitude lower than the diffusion coefficients of proteins with mol.wts. of 14000 (protein A) and 46000 (ligandin), and of unbound small molecules, the large concentration gradients in the membrane phase, relative to the free or protein-bound gradients in the aqueous phase, suggest that lateral diffusion in membranes of 2-acetylaminofluorene, 4-dimethylaminoazobenzene, oestrone and testosterone could be an important means of their intracellular transport.

We thank Dr. R. M. Hicks for carrying out examination of our liposomes by electron microscopy. B. K. is a Fellow of the Cancer Research Campaign, which we thank for a generous grant. We are grateful to the referee for drawing our attention to the paper of Heap *et al.* (1970).

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