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The influence of soluble binding proteins on lipophile transport and metabolism in hepatocytes

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A theory is presented that deals with the involvement of the intracellular binding proteins ligandin and aminoazodye-binding protein A (otherwise known as Z-protein or fatty-acid-binding protein) on the uptake and intracellular transport and metabolism of their ligands. Equations are derived that combine steady-state diffusional fluxes of small molecules that are (*a*) free in the aqueous phase of the cell, (*b*) bound to the two proteins and (*c*) partitioned into intracellular membranes, for model systems that resemble conditions in the rat hepatocyte. These equations are then combined with expressions for the enzyme-catalysed metabolic reactions undergone by these small molecules to assess the influence of diffusion rates on the overall metabolic rates. It is concluded that ligandin and protein A can enhance the rate of intracellular transport of their ligands by an order of magnitude or more and that this could make the hepatocyte several times more efficient in metabolizing these ligands. Various ways of testing this theory are discussed.

The cytosol of the rat hepatocyte has high concentrations of two proteins that share the ability to bind reversibly small molecules with hydrophobic moieties. These are ligandin (Litwack *et al.*, 1971) and aminoazodye-binding protein A (Ketterer *et al.*, 1967, 1976), which is probably identical with Z-protein (Levi *et al.*, 1969; Mishkin *et al.*, 1972) or fatty-acid-binding protein (Ockner *et al.*, 1972). Because of their rather non-specific binding capabilities and their abundance (each is present in the rat hepatocyte at a concentration of approx. 0.1 mM) it has often been suggested (see e.g. Levi *et al.*, 1969; Litwack *et al.*, 1971; Ketterer *et al.*, 1975*a*; Arias *et al.*, 1976) that the two proteins may play a role in cellular uptake and transport. This idea has been developed by Meuwissen *et al.* (1977), who pointed out that in the liver ligandin and protein A might enhance the rate of intracellular transport of bilirubin by facilitated diffusion in a manner analogous to the proposed mechanism for the transport of O_2 in muscle by myoglobin (Wyman, 1966; Wittenberg, 1966, 1970).

The basis of facilitated diffusion of this kind is that, given a constant concentration of the diffusing small molecule (diffusant) in the bloodstream and a constant concentration gradient within the cell, the flux due to the unbound diffusant is augmented by a second flux due to the protein-bound component. The latter diffuses more slowly than the former, because of the difference in size, and so for it to be a significant part of the total flux there must be significantly more bound than unbound diffusant. In the two extreme cases, this requirement could be met either by a relatively small amount of protein having a high affinity for the diffusant or by a high concentration of protein with a relatively low affinity. The second arrangement would be expected to be the more efficient, since a high affinity implies a slow rate of dissociation of the protein–diffusant complex, so that the rate of 'unloading' of the 'cargo', rather than its rate of movement, could be the limiting factor in the rate of the overall process. Ligandin and protein A in the rat hepatocyte, like myoglobin in muscle (cf. Wyman, 1966; Wittenberg, 1970) seem to meet these criteria of high concentration and moderate affinity.

In the present paper we attempt to extend the facilitated-diffusion concept as it applies to ligandin and protein A in liver cells. To do this we have considered not only the diffusion of protein-bound small molecules but also lateral diffusion in the phospholipid bilayers of intracellular membranes, into which ligands of the two proteins readily partition (Tipping *et al*, 1979*a*,*b*). Equations that describe steady-state diffusion in model arrange-

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ments of cytoplasm are first derived, based on those for gas transport by haemoproteins given by Collins (1961), Wang (1963) and Wyman (1966) and are used to get estimates of intracellular diffusion rates. The latter are then applied to models for diffusion-with-metabolism. Where possible we include critical discussions of the background data and of the assumptions involved. A preliminary account of the present work has appeared (Tipping & Ketterer, 1978).

Introductory information

The binding of small molecules to ligandin, protein A and phospholipid bilayers

A number of quantitative studies of the binding of small molecules to ligandin and protein A have been made (see e.g. Kamisaka *et al.*, 1975*a*,*b*; Ketley *et al.*, 1975; Ketterer *et al.*, 1975*a*, 1976; Tipping *et al.*, 1976*a*,*b*,*c*, 1978), from which it can be concluded that, in the majority of cases, association constants are in the range $0–10^7$ litre·mol^{-1}, although in unfractionated cytosol the values appear to be somewhat higher (Meuwissen *et al.*, 1975, 1977). The two proteins have similar binding specificities and each has a single primary binding site for compounds with a hydrophobic moiety. From presently available data there is little apparent temperature-dependence of binding. At equilibrium the fractional degrees of saturation of the proteins are given by the usual equation for a single binding site:

$$\bar{\nu} = \frac{\text{mol of ligand bound}}{\text{total mol of protein}} = \frac{Kc}{1+Kc} \qquad (1)$$

where K is the association (equilibrium) constant and c is the unbound aqueous concentration of ligand (see Table 1).

Most of the ligands of the two proteins have hydrophobic moieties and so they also bind to phospholipid bilayers. The membrane partition coefficient, χ, is defined by:

$$\chi = c_M/c \qquad (2)$$

where c_M is the concentration in the membrane phase. Values of χ are in the range $0–10^6$ and are independent of c under normal circumstances (Tipping *et al.*, 1979*a*,*b*).

Throughout this paper we shall use eqns. (1) and (2) to interrelate the concentrations of free, protein-bound and membrane-bound diffusant. Implicit in this procedure is the assumption that the rates of association and dissociation of the protein–diffusant and membrane–diffusant complexes are rapid compared with the diffusion processes in which we are interested (cf. Fig. 1). This assumption was also made by Collins (1961), Wang (1963) and Wyman (1966) in their treatments of haemoprotein-mediated transport of gases, and is forced on us here by the lack of kinetic data for the binding processes. Let us therefore consider how valid the assumption is.

Table 1. *Glossary of symbols*

A	(aminoazodye-binding) protein A, Z-protein, fatty acid binding protein
D	diffusion coefficient (cm^2·s^{-1}).
J	diffusive flux (mol·cm^{-2}·s^{-1}), i.e. mass flow per unit area.
K	protein association constant (litre·mol^{-1})
K_m	Michaelis constant (M); note that this is a dissociation constant.
L	ligandin
P	the 'combination' of protein A and ligandin, cf. eqn. (3).
R_E	a composite parameter for an enzyme, $R_E = V[\text{enzyme}]/K_m$ (s^{-1}).
V	enzyme-catalysed maximum velocity in mol·s^{-1}·(mol of enzyme)$^{-1}$.
c	unbound aqueous concentration of diffusant (M).
c_M	concentration of diffusant in membrane phase (M).
k_{on}, k_{off}	association and dissociation rate constants in litre·mol^{-1}·s^{-1} and s^{-1} respectively.
x	general distance in the direction of diffusion; g, h and q are specific values of x.
θ	aqueous fraction of cytoplasm; cf. eqn. (10).
$\bar{\nu}$	mol of ligand bound/mol of protein.
σ	cross-sectional area of model hepatocyte (see Fig. 4).
ψ	membrane permeability in cm·s^{-1}.
χ	membrane partition coefficient; cf. eqn. (2).
ϕ	the ratio g/h; cf. Fig. 2.

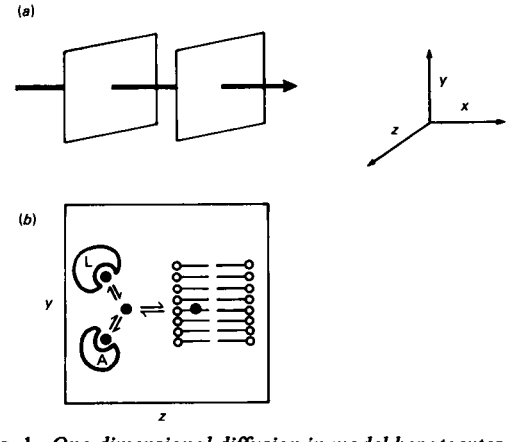

Fig. 1. *One-dimensional diffusion in model hepatocytes* It is assumed that at any given yz plane in (*a*), the four forms of the diffusant (unbound aqueous, bound to protein A, bound to ligandin and bound to lipid bilayer) are in equilibrium, but that there is a concentration gradient in the x-direction. The equilibria are represented in (*b*), in which the diffusant is denoted by ●.

If the rate of formation of the protein–ligand complex is diffusion-controlled (i.e. diffusion in the yz planes of Fig. 1), then the association rate constant k_{on} is approximately 10^9 litre $\cdot$ mol^{-1} $\cdot$ s^{-1} (Eigen & Hammes, 1963), so that for an association (equilibrium) constant K of 10^5 litre $\cdot$ mol^{-1} the value for k_{off} is 10^4 s^{-1}, and the time for 99% dissociation of the complex to occur is approx. 5×10^{-4} s. During this time a protein with diffusion coefficient $D_p = 10^{-6}$ cm^2 $\cdot$ s^{-1} (a typical value; see below) would diffuse in the x-dimension a root-mean-square distance of $\sqrt{2D_p t} \approx 3 \times 10^{-5}$ cm (see e.g. Setlow & Pollard, 1962), which is quite small compared with the radius of a hepatocyte (approx. 10^{-3} cm) and considerably smaller than the actual length of the tortuous intracellular diffusion path, which must be more like 10^{-2} cm. For association (equilibrium) constants of 10^6 and 10^7 litre $\cdot$ mol^{-1} then, for diffusion-controlled association and dissociation rates, distances for 99% dissociation are 10^{-4} and 3×10^{-4} cm respectively, which are still small relative to a diffusion path of 10^{-2} cm. When the rates are under diffusion control, therefore, the assumption of rapid binding and complex breakdown is justified, since for ligandin and protein A, $k_{off} \geqslant 10^2$ s^{-1}.

For k_{on} values corresponding to less than diffusion-controlled association rates, the distances for 99% dissociation become correspondingly greater; e.g. for $K = 10^7$ litre $\cdot$ mol^{-1} and $k_{on} = 10^6$ (3 orders of magnitude lower than the maximum rate) then $k_{off} = 10^{-1}$ s^{-1}, the distance for 99% dissociation is 10^{-2} cm and the assumption of rapid dissociation is untenable. A protein with these binding parameters would still facilitate diffusion, however, since some of its ligand would dissociate over a reasonable distance (e.g. a distance of 10^{-4} cm would allow 0.5% dissociation) and the low fractional dissociation would be offset by the high absolute amount of binding. It should be stressed that the steady-state facilitated-diffusion concept, relying as it does on the basic unbound flux being augmented by protein-bound and membrane fluxes, does not include the possibility of binding decreasing the total flux, since the unbound concentration gradient is the same regardless of protein and membranes.

To our knowledge rates of membrane-partitioning processes have not yet received the attention that has been given to protein binding, and for present purposes we assume they are sufficiently rapid for equilibrations to be considered instantaneous.

Diffusion coefficients

The ligands that bind with significant affinity to ligandin and protein A have molecular weights in the range of 200–1000 and so their diffusion coefficients are centred on a mean of approx. 4×10^{-6} cm^2 $\cdot$ s^{-1} (see e.g. Setlow & Pollard, 1962), which is the value we have adopted for all our calculations.

Ligandin (mol.wt. 46000) has a diffusion coefficient (D_L) of 7.5×10^{-7} cm^2 $\cdot$ s^{-1} in water at 20°C at infinite dilution (Ketterer *et al.*, 1975*b*), and under the same conditions protein A (mol.wt. 14000) would be expected to have a diffusion coefficient (D_A) of 1.1×10^{-6} cm^2 $\cdot$ s^{-1}, by comparison with other proteins of similar molecular weight (see e.g. Sober, 1968). At 37°C these values would be higher because of the lowered viscosity of water (Hardy & Cottington, 1949), but in the hepatocyte this increase is offset by the higher viscosity due to the high concentration of proteins (see e.g. Minton & Ross, 1978). We have therefore taken the results at infinite dilution at 20°C to characterize diffusion in the hepatocyte at 37°C.

Furthermore, as a convenient simplification we have chosen to treat the two proteins as a single entity in our derivations, a procedure that can be justified as follows. Their similar binding properties (see above) mean that we can, as a reasonable approximation, set their association constants for a particular ligand equal, denoting the generalized association constant by K_p. They also have similar cellular concentrations (both approx. 10^{-4} mol $\cdot$ litre^{-1}), which can be denoted by [P]/2, so that their sum is [P]. In the equations we derive in the present paper the association constants appear either alone, or as triple products with the concentrations and protein diffusion coefficients, in which case:

$$D_L[L]K_L + D_A[A]K_A = (D_L + D_A)\frac{[P]}{2}K_p = D_p[P]K_p \quad (3)$$

where $(D_L + D_A)/2 = D_p$

The lateral diffusion of small molecules in lipid bilayers and in intact biological membranes has been studied and/or reviewed by a number of authors (see e.g. Devaux & McConnell, 1972; Sackmann & Träuble, 1972*a*,*b*; Träuble & Sackmann; 1972; Cogan *et al.*, 1973; Galla & Sackmann, 1974; Edidin, 1974; Vanderkooi & Callis, 1974) and from the results presented it can be concluded that lateral-diffusion coefficients (D_M) are generally in the range 10^{-8}–10^{-7} cm^2 $\cdot$ s^{-1}, and as yet no obvious dependence of D_M on molecular weight or structure has emerged. For the calculations in the present paper we have used a value for D_M of 3×10^{-8} cm^2 $\cdot$ s^{-2}, the geometric mean of the range of values found experimentally.

The permeability of the sinusoidal plasma membrane

At the simplest level we can consider the crossing

of the sinusoidal plasma membrane to be the permeation of a lipid bilayer. Ruf *et al.* (1978) have estimated a permeability coefficient (ψ) of $0.15\,cm \cdot s^{-1}$ for the uncharged form of *o*-Methyl Red (4-dimethylaminoazobenzene-2'-carboxylic acid) for egg lecithin liposomes. At pH 7 the permeability is therefore (assuming the uncharged form to be the permeating species, and applying the pK value of 4.95) $1.3 \times 10^{-3}\,cm \cdot s^{-1}$. Hori *et al.* (1978), working with black lipid membranes composed of egg lecithin and cholesterol, found that, at pH 6.5, aniline (which is essentially neutral at this pH, having a pK of 4.6) has a permeability coefficient of $4.37 \times 10^{-4}\,cm \cdot s^{-1}$, and that salicylamide (2-amidophenol), which has a pK of 8.5 and is therefore also essentially uncharged at pH 6.5, has a permeability coefficient of $2.27 \times 10^{-4}\,cm \cdot s^{-1}$. These values are markedly lower than the value attained by Ruf *et al.* (1978) for neutral *o*-Methyl Red; on the other hand, Le Blanc's (1969) conductance studies of the anionic tetraphenylborate in egg, lecithin black lipid membranes yielded a diffusion coefficient across the bilayer of approx. $10^{-9}\,cm^2 \cdot s^{-1}$, which, taking a bilayer thickness of $5 \times 10^{-7}\,cm$, gives a permeability coefficient of approx. $2 \times 10^{-3}\,cm \cdot s^{-1}$, a value similar to that obtained by Ruf *et al.* (1978) for the anionic form of *o*-Methyl Red. Stein (1967) collected and analysed data for the permeabilities of membranes of algae and erythrocytes of uncharged compounds possessing hydrogen-bonding groups, and by extrapolating plots of $\log(\psi\sqrt{\text{mol.wt.}})$ against N (number of hydrogen-bonding groups in a given molecule) to $N = 0$ he estimated that the maximum value of $\psi\sqrt{\text{mol.wt.}}$ is of the order of 0.1, corresponding to a maximum value of ψ of about $0.01\,cm \cdot s^{-1}$ for neutral molecules with molar weights of several hundred. The range of permeability values estimated for small molecules typical of the ligands of ligandin and protein A thus covers about three orders of magnitude (10^{-4}–$10^{-1}\,cm \cdot s^{-1}$). The presence of microvilli in the sinusoidal plasma membrane means that this part of the plasma membrane contributes 72% of the total surface area (Blouin *et al.*, 1977), which must increase the permeabilities of the sinusoidal bilayers severalfold, but probably not by as much as an order of magnitude.

The above considerations apply to uptake by simple passive diffusion across lipid bilayers. However, a number of authors have suggested, on the basis of studies with isolated hepatocytes and plasma membrane fractions, that the uptake of small molecules might involve carriers in the sinusoidal membrane. Thus passive carrier-mediated uptake has been proposed for bilirubin, Indocyanine Green and bromosulphophthalein by Scharschmidt *et al.* (1975), for bile acids by Accatino & Simon (1976) and by Anwer *et al.* (1976), and for bromosulphophthalein by Schwenk *et al.* (1976). Energy-requiring carrier-mediated uptake has been claimed for taurocholate by Schwarz *et al.* (1975), for bromosulphophthalein by van Bezooijen *et al.* (1976) and for oestrone, oestradiol and testosterone by Rao *et al.* (1977). Accatino & Simon (1976) and Tiribelli *et al.* (1978) claim to have isolated the carrier proteins responsible for the uptakes of bile acids and bromosulphophthalein respectively. The systems employed by these workers are too complex for us to be able to extract values for membrane permeabilities simply from the uptake results as a whole, but we mention at this point that the presence of carriers in the sinusoidal membrane would by no means exclude, at least in the model systems we define and characterize in the present paper, a role for ligandin and protein A in the cellular uptake of their ligands. This matter is dealt with below in the Influence of the sinusoidal plasma membrane on uptake and metabolism section.

Enzyme activities

In our considerations of diffusion-with-metabolism (see below) we need values for the parameter R_E, defined by:

$$R_E = \frac{V[\text{enzyme}]}{K_m} \qquad (4)$$

where V is the maximum velocity in $mol \cdot litre^{-1} \cdot (mol\ of\ enzyme)^{-1}$, [enzyme] is the cellular concentration of enzyme and K_m is the free aqueous concentration of substrate corresponding to a velocity one-half that of V (i.e. the conventional Michaelis constant).

The calculation of R_E values is made difficult in cases where the enzymes involved are membrane-bound. For instance, the first step in the metabolic conversion of a lipophilic compound is often catalysed by the mixed-function oxidase system of the endoplasmic reticulum. Another type of reaction that involves ligands of ligandin and protein A is the hydrolysis of sulphate esters, e.g. steroid sulphates; this too is catalysed by membrane-bound enzymes. Literature values for the kinetic parameters of such enzymes are not always useful, because partitioning of the substrate between the aqueous and membrane phases is not taken into account in making the measurements. Thus the K_m component of R_E, as defined by eqn. (8), refers to the concentration of free substrate in the aqueous phase, but if assays are carried out with membrane-bound enzymes this concentration is not known, because the total substrate (which is normally equated with the free substrate in assays involving small amounts of enzymes) is distributed between the aqueous and membrane phases. This has been discussed more fully by Parry *et al.* (1976) and by Heirwegh *et al.* (1978).

One study in which partitioning was taken account of is that by Cumps *et al.* (1977). By measuring apparent K_m values over a range of values of enzyme concentrations and extrapolating to [enzyme] = 0, these authors found that the limiting value of K_m for the rat liver microsomal-fraction-catalysed hydroxylation of benzo[*a*]pyrene is 2.5×10^{-6} mol·litre^{-1}, and that the maximum velocity is 10^{-7} mol·s^{-1}·(g of microsomal protein)$^{-1}$. Since the velocity was measured by monitoring the formation of 3-hydroxybenzo[*a*]-pyrene, which constitutes only about 50% of the products, a better estimate of the overall maximum velocity is 2×10^{-7} mol·s^{-1}·(g of microsomal protein)$^{-1}$. Since there is approx. 0.045 g of microsomal protein per cm^3 of liver cells (DePierre & Dallner, 1975) the value of R_E we obtain from the data of Cumps *et al.* (1977) is approx. 4 s^{-1}.

Dolly *et al.* (1972) have given data for the oestrogen sulphatase of rat liver microsomal fraction ('microsomes'). For oestrone sulphate they find an apparent value for K_m of 2×10^{-5} mol·litre^{-1}, at a protein concentration of 0.011 g·cm^{-3}. The partition coefficient for oestrone sulphate in lecithin bilayers is approx. 10^3 (Tipping *et al.*, 1979*b*) and so by assuming that the weight ratio of phospholipid to protein in rat liver 'microsomes' is 1:2 (DePierre & Dallner, 1975) we obtain a 'true' value for K_m of approx. 3×10^{-6} mol·litre^{-1}. This, together with the maximum velocity of 8×10^{-8} mol·s^{-1}·(g of microsomal protein)$^{-1}$ gives a value for R_E of approx. 1 s^{-1}.

Models of the rat hepatocyte

The equations we derive apply to idealized versions of the rat hepatocyte based in quantitative terms on the properties of the real cell. We suppose diffusion to take place in the cytosol and in the lipid bilayers of the cellular membranes, and we need to know the relative volumes of these two phases. In the rat hepatocyte the membrane lipid is 90% phospholipid (Rouser *et al.*, 1968) and constitutes 3% of the total cell weight (DePierre & Dallner, 1976). The densities of the rat hepatocyte and of phospholipid bilayers are virtually the same, 1.067 g·cm^{-3} (Weibel *et al.*, 1969) and 1.01 g·cm^{-3} (Huang, 1969) respectively, and so the lipid constitutes 3% of the cell volume. We assume that all of the lipid bilayer is available for lateral diffusion. This is certainly an overestimate, first because the lipids of the plasma and nuclear membranes will not be involved in net transport. However, since these two membrane types constitute less than 10% of the total cellular membrane surface area (Blouin *et al.*, 1977), the overestimate is not serious. A second factor that should be considered is the permeability of the lipid bilayers. Generally it might be expected that charged diffusants would cross the bilayers less readily than would neutral diffusants, so that the former would diffuse to a greater extent in the cytosol-facing monolayers (although see above under 'The permeability of the sinusoidal plasma membrane').

The aqueous phase of the rat hepatocyte occupies 44% of the total cell volume (Weibel *et al.*, 1969) so the volume fraction of cytosol in the system (cytosol + intracellular lipid bilayer) is 44/(44 + 3) = 0.936 and the fraction of lipid is 3/(44 + 3) = 0.064.

Steady-state diffusion in an idealized arrangement of cytoplasm

In a previous publication (Tipping & Ketterer, 1978) we compared diffusion in three arrangements of the aqueous and membrane phases of the cytoplasm. Here we consider only the most realistic of these (previously called the mixed mode of diffusion), illustrated in Fig. 2, and derive more general diffusion equations than in our previous, preliminary analysis.

There are three types of diffusion for a small molecule in the model system of Fig. 2. Their steady state fluxes are given by Fick's First Law (Fick, 1855) as follows:

Unbound aqueous $J_U = -D_U(dc/dx)$ (5)

Protein-bound $J_P = -D_P[P]\,(d\bar{\nu}/dx)$ (6)

Membrane-bound $J_M = -D_M(dc_M/dx)$ (7)

For diffusion in the first section of the 'slab of cytoplasm' of Fig. 2, i.e. from $x = 0$ to $x = g$, the total flux is given by:

$$J_T' = J_U' + J_P' \tag{8}$$

Substituting J_U' and J_P' from eqns. (5) and (6) and, since we are assuming chemical equilibrium in the yz plane, for $\bar{\nu}$ from eqn. (1), followed by integration between $x = 0$ and $x = g$ gives:

$$J_T' = \frac{1}{g}\left\{D_U(c_0 - c_g) + D_P K_P[P]\left(\frac{c_0}{1 + K_P c_0} - \frac{c_g}{1 + K_P c_g}\right)\right\} \tag{9}$$

In the section of the slab from $x = g$ to $x = h$, the total flux is given by:

$$J_T'' = \theta(J_U'' + J_P'') + (1 - \theta)J_M'' \tag{10}$$

where θ is the aqueous fraction of the cross-sectional area of this part of the slab. [The value of θ depends on (*a*) the volume fractions of the aqueous and membrane phases and (*b*) the ratio $g/h(\phi)$. In order that the model arrangement of Fig. 2 represents the cell as a whole, the volume fractions must be 0.936 and 0.064 respectively (see the Background infor-

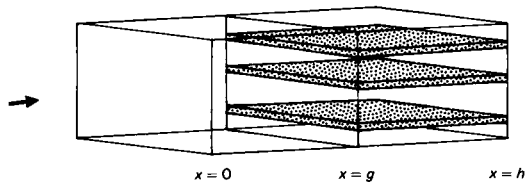

Fig. 2. *Model for intracellular diffusion*
Diffusion can take place either in the aqueous phase (open areas) or in the membrane phase (stippled areas) in the direction indicated by the arrow. The (fixed) concentration of unbound diffusant in the aqueous phase at the front face is c_0; that at the rear face is c_h.

mation section). It is readily shown that $\theta = (0.936-\phi)/(1-\phi)$.] Treating eqn. (10) in a similar way to eqn. (8) gives:

$$J_T'' = \frac{1}{(h-g)}\left[\theta\left\{D_U(c_g-c_h) + D_P K_P[P]\left(\frac{c_g}{1+K_P c_g} - \frac{c_h}{1+K_P c_h}\right)\right\} + (1-\theta)D_M \chi(c_g-c_h)\right] \quad (11)$$

In the steady state $J_T = J_T' = J_T''$, so that eqns. (9) and (11) can be combined. When this is done, a quadratic equation in the unknown intermediate concentration c_g is obtained of the form:

$$\alpha c_g^2 + \beta c_g + \gamma = 0 \quad (12)$$

where α, β and γ are combinations of the various constants involved (see the Appendix). Eqn. (12) can be solved for c_g, which can then be substituted back into either eqn. (9) or eqn. (11) to give J_T.

A convenient way to see how the various parameters influence transport is to define an effective diffusion coefficient, $D_{eff.}$:

$$D_{eff.} = \frac{J_T h}{c_0 - c_h} \quad (13)$$

$D_{eff.}$ is thus the value of the diffusion coefficient that would be obtained by a hypothetical experimenter who was able to measure the flux across the 'slab of cytoplasm' depicted in Fig. 2, when the concentrations of unbound aqueous diffusant at the two ends are c_0 and c_h. [It is not necessary to specify h to calculate $D_{eff.}$, since it cancels out when expressions for J_T are substituted into eqn. (13).] Fig. 3 shows some representative plots of $D_{eff.}$ against K_P for different values of χ and different concentrations.

The effects of the binding proteins at low diffusant concentrations are shown in Fig. 3(*a*). The interesting conclusion to be drawn from these plots is that there is very little dependence of $D_{eff.}$ on membrane

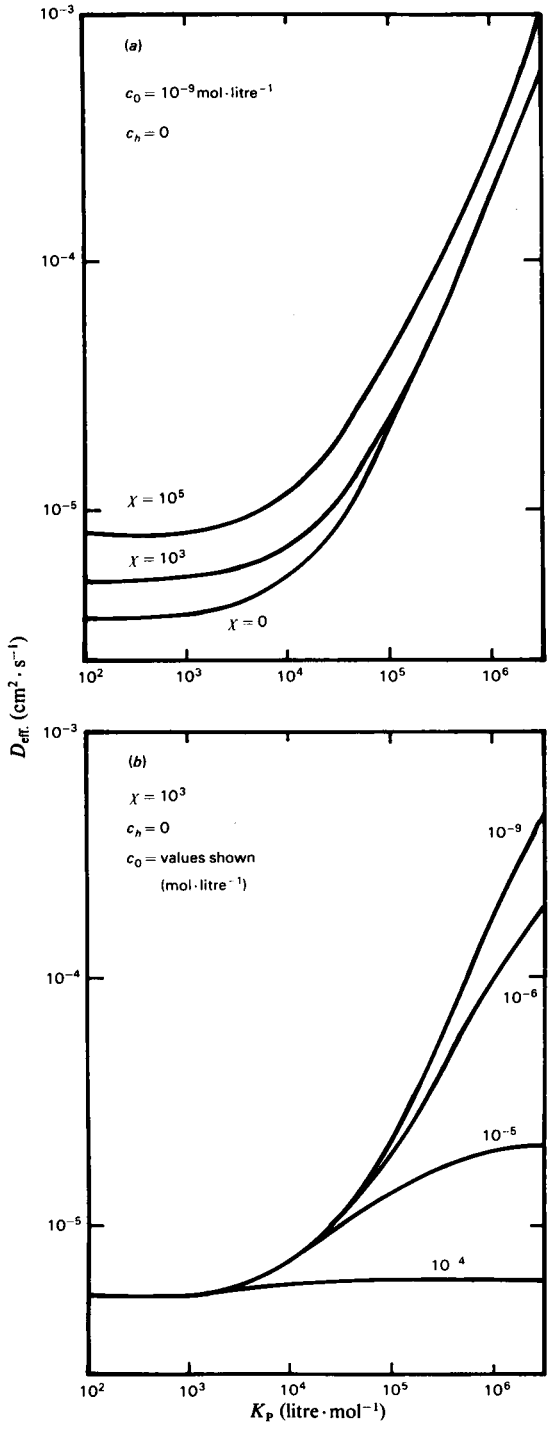

Fig. 3. *Dependence of the effective diffusion coefficient ($D_{eff.}$) on protein association constant (K_P), membrane partition coefficient (χ) and concentrations (c_0 and c_h) at the ends of the cellular model of Fig. 2*

partition coefficient χ, so that for a range of values of χ covering five orders of magnitude there is only a 2–3-fold variation in $D_{\text{eff.}}$ for a given value of K_P. For $K_P = 10^5$ litre · mol^{-1}, $D_{\text{eff.}}$ is approx. 5 times that for $K_P = 0$; for $K_P = 10^6$ litre · mol^{-1} the enhancement is about 40-fold. At high concentrations of diffusant the enhancements are less marked (Fig. 3*b*), as expected because of saturation effects, but the relative lack of dependence of $D_{\text{eff.}}$ on χ still holds. Variations in the parameter ϕ (the ratio g/h; cf. Fig. 2) have little effect on the plots in Fig. 3, nor does reversing the two sections (0–*g* and *g*–*h*) of the model system of Fig. 2.

From theoretical treatments of simpler model systems (Tipping & Ketterer, 1978) the lack of dependence of $D_{\text{eff.}}$ on χ can be explained as follows. At low values of χ the membrane diffusional flux is small since there is not much diffusant in the membrane. Therefore the total flux depends largely on the aqueous phase. At high values of χ the diffusional flux in the membranes is potentially very high, but cannot be attained because the membrane phase is discontinuous, so that the overall flux is dictated by diffusion in the intervening, continuous aqueous phase.

As might be expected from the general form of protein–ligand isotherms (e.g. eqn. 1) the proteins enhance transport less when the ligand concentrations are high enough to saturate them to a significant degree. It is therefore appropriate to consider what degrees of saturation of the proteins might be expected *in vivo*. Tipping *et al.* (1979*a*,*b*) have given equations for calculating subcellular distributions of various ligands for the two proteins. Such calculations show that since ligands with $K_P > 10^5$ litre · mol^{-1} (i.e. candidates for facilitated diffusion by the proteins) generally have partition coefficients $> 10^2$, then values of $\bar{\nu}$ as low as 0.01 correspond to high total cellular concentrations (1–100 μM) because of the large amount of membrane phase in the hepatocyte (see the Introductory information section). Therefore conditions of low saturation of ligandin and protein A are likely to be quite normal.

It is important to decide whether the model we have considered is sufficiently similar to the arrangements of membranes and cytosol found *in vivo* for our calculations to be of any use. In some electron micrographs of rat hepatocytes combinations of rough endoplasmic reticulum and cytosol resemble quite closely the arrangement shown in Fig. 2, but in general the arrangement is best regarded as a stretched-out (into one dimension) version of a tortuous diffusion path. For example it can be imagined that one route for a diffusant involves passage across some cytosol, followed by diffusion around a mitochondrion, some of which would occur in the lipid bilayer of its outer membrane; a conceptual stretching-out of the outer membrane would give a path similar to that in the arrangement in Fig. 2. In a similar manner, diffusion through regions of cytoplasm containing smooth endoplasmic reticulum can be 'one-dimensionalized'. In these terms our idealizations of cytoplasmic geometries are perhaps not too far-fetched.

Metabolism of the diffusant

In this section we consider the hepatic uptake and metabolism of a ligand of protein A and ligandin, using the results of the previous section to describe its diffusion. In the model system illustrated in Fig. 4 the diffusant has a constant free aqueous concentration in the plasma (c_{plasma}), and it is supposed that it cannot leave the cell except by undergoing metabolism. As a starting point we assume that the sinusoidal plasma membrane presents no barrier to uptake, i.e. its permeability is infinite. This means that in this case, but not in general, the free aqueous concentration immediately inside the cell, c_g, is equal to c_{plasma}.

The rate of change of the free aqueous concentration, c, at any point within the cell depends on the rate of diffusive transport and on the rate of metabolism. For Fickian diffusion and Michaelis–Menten kinetics (Michaelis & Menten, 1913) we have:

$$\frac{dc}{dt} = D_{\text{eff.}}(c)\frac{d^2c}{dx^2} - V\frac{[\text{enzyme}]c}{c + K_m} \qquad (14)$$

where V is the maximal enzyme-catalysed reaction velocity, K_m the unbound aqueous substrate concentration at half-maximal velocity and $D_{\text{eff.}}(c)$ indicates that the effective diffusion coefficient is a function of c, as shown above. In the steady state $(dc/dt) = 0$, and so:

$$D_{\text{eff.}}(c)\frac{d^2c}{dx^2} = \frac{V[\text{enzyme}]c}{c + K_m} \qquad (15)$$

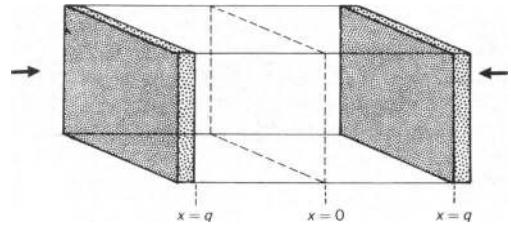

Fig. 4. *Model hepatocyte*
The stippled areas represent the sinusoidal plasma membrane and are exposed to plasma in which there is a constant free aqueous concentration of diffusant, c_{plasma}. Diffusion takes place in the direction indicated by the arrows.

As it stands eqn. (15) cannot be solved analytically, but it can be if we restrict ourselves to concentrations of diffusant sufficiently low that neither binding proteins nor enzymes are significantly saturated, a likely situation, as discussed above. Under these conditions $D_{\text{eff.}}$ is independent of c because terms like $K_P c_0$ in eqns. (9) and (11) become $\ll 1$, and concentration differences cancel out when eqns. (9), (11) and (13) are combined. For $c \ll K_m$ therefore:

$$\frac{d^2 c}{dx^2} = \frac{V[\text{enzyme}]c}{D_{\text{eff.}} K_m} = \frac{R_E}{D_{\text{eff.}}} c \qquad (16)$$

where R_E, a composite quantity describing the enzyme activity, is given by $V[\text{enzyme}]/K_m$.

Referring to Fig. 4 we see that the boundary conditions which the solution of eqn. (16) must fulfil are (i) $c = c_q$ at $x = q$, and (ii) $dc/dx = 0$ at $x = 0$. The required solution is:

$$c = c_q \cdot \frac{\cosh\sqrt{R_E x^2/D_{\text{eff.}}}}{\cosh\sqrt{R_E q^2/D_{\text{eff.}}}} \qquad (17)$$

From eqn. (17) we can calculate the unbound concentration distribution within the cell, i.e. the variation of c with x, given values of R, $D_{\text{eff.}}$, q and c_q. From the analysis of simple diffusion we have values of $D_{\text{eff.}}$ (which depend on the affinity of the diffusant for the binding proteins), and values of R_E have been discussed in the Introductory information section. At first sight we might assign to q the half-distance across a rat hepatocyte, i.e. about 10^{-3} cm, but this would require the contents of the cell to be arranged in neat stacks of membrane and cytosol so that transport would be linear from sinusoid to cell centre. The actual path is perhaps an order of magnitude greater; in our calculations we have used a value of 10^{-2} cm. Fig. 5 shows concentration–distance profiles calculated from eqn. (17).

In our model the rate of the enzyme-catalysed chemical reaction at any point in the cell is directly proportional to c, the unbound aqueous concentration, as long as we arrange that $c \ll K_m$. Thus the profiles of Fig. 5 show not only the dependence of concentration on distance but also the dependence of reaction rate on distance. The overall rates of metabolism under these conditions are therefore directly proportional to the areas under the curves of Fig. 5, and are obtained by integration of eqn. (17).

Rate of metabolism of whole cell (mol · s^{-1})

$$= 2\sigma\sqrt{R_E D_{\text{eff.}}}\left\{\frac{\sinh\sqrt{R_E q^2/D_{\text{eff.}}}}{\cosh\sqrt{R_E q^2/D_{\text{eff.}}}}\right\} c_q \qquad (18)$$

where σ is the uniform cross-sectional area of the cell. We wish to know how the rate of metabolism varies with R_E, $D_{\text{eff.}}$ and q. Table 2 shows the results

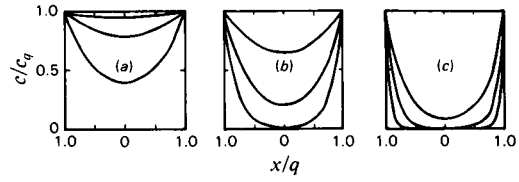

Fig. 5. *Concentration–distance profiles for the model hepatocyte of Fig. 4*

The curves are calculated from eqn. (17) for a value of q of 10^{-2}cm. The lowest curve in each case is for $D_{\text{eff.}} = 4 \times 10^{-6}cm^2 \cdot$s^{-1}, the middle curve for $D_{\text{eff.}} = 2 \times 10^{-5}cm^2 \cdot$s^{-1} and the highest curve for $D_{\text{eff.}} = 10^{-4}cm^2 \cdot$s^{-1}. The areas under the curves are proportional to the rates of metabolism of the cell as a whole. (*a*) $R_E = 0.1$ s^{-1}; (*b*) $R_E = 1$ s^{-1}; (*c*) $R_E = 10$ s^{-1}.

Table 2. *Calculated rates of metabolism in the model hepatocyte of Fig. 4*

According to eqn. (18) the rate of metabolism of the whole cell (in mol · s^{-1}) is directly proportional to σ, the area of the sinusoidal plasma membrane and to c_q, the concentration at $x = q$ (cf. Fig. 4). Since it is not necessary to specify either of these quantities to see the dependence of rate on $D_{\text{eff.}}$ (which is the object of the calculations), the values shown are the rates divided by $2\sigma c_q$ (cm · s^{-1}). They are thus values of:

$$\sqrt{R_E D_{\text{eff.}}} \cdot \frac{\sinh\sqrt{R_E q^2/D_{\text{eff.}}}}{\cosh\sqrt{R_E q^2/D_{\text{eff.}}}}$$

and have units of cm · s^{-1}; this parameter also occurs in eqn. (20), which relates ψ, the permeability of the sinusoidal plasma membrane, to intracellular diffusion and metabolism. The values corresponding to $D_{\text{eff.}} = \infty$ are the maximum rates. A value of 10^{-2}cm was used for q.

$D_{\text{eff.}}$ (cm$^2 \cdot$s^{-1})	Rate of metabolism/$2\sigma c_q$ (cm · s^{-1})		
R_E(s^{-1}) ...	0.1	1	10
4×10^{-6}	0.58×10^{-3}	0.20×10^{-2}	0.006
2×10^{-5}	0.86×10^{-3}	0.44×10^{-2}	0.014
10^{-4}	0.97×10^{-3}	0.76×10^{-2}	0.032
∞	1.00×10^{-3}	1.00×10^{-2}	0.100

of calculations based on eqn. (18), which provide this information. From Table 2 we see that for a given value of R_E, the rate of metabolism increases with $D_{\text{eff.}}$, although the increases are only significant for higher values of R_E. For $R_E = 1$ s^{-1} an increase in $D_{\text{eff.}}$ from 4×10^{-6}cm$^2 \cdot$s^{-1} to 10^{-4}cm$^2 \cdot$s^{-1} (the sort of enhancement that the proteins might bring about for a typical value of K_P of 10^6litre · mol^{-1}) causes an increase in the rate of metabolism of approx. 3.5-fold. For a more efficient enzyme with $R_E = 10$ s^{-1}, a greater-than-5-fold increase in rate is predicted.

Influence of the sinusoidal plasma membrane on uptake and metabolism

In the previous section we took no account of the sinusoidal plasma membrane, i.e. it was assumed that it presented no barrier to cellular uptake. If it does have a finite permeability for the diffusant, however, the membrane would be expected to influence the working of the cell. If the membrane has a permeability of ψ cm·s^{-1}, then the flux across it is given in terms of the concentration difference by:

$$J_{\text{membrane}} = \psi(c_{\text{plasma}} - c_q) \qquad (19)$$

(Note that J_{membrane} is the flux across the membrane and has nothing to do with J_M, the flux of lateral diffusion within the intracellular membranes.) Our intention here is to see what effect the finite permeability has on the rate of metabolism of the cell for a given value of c_{plasma}. In the steady state the rate of entry into the cell must equal the overall rate of metabolism. We may therefore combine eqns. (18) and (19) to obtain:

$$\frac{c_q}{c_{\text{plasma}}} = \frac{\psi}{\psi + \sqrt{R_E D_{\text{eff.}}}\,\dfrac{\sinh\sqrt{R_E q^2/D_{\text{eff.}}}}{\cosh\sqrt{R_E q^2/D_{\text{eff.}}}}}$$

$$= \frac{\psi}{\psi + \text{'metabolism parameter'}} \qquad (20)$$

so that for $\psi = \infty$ (i.e. an infinitely permeable membrane), $c_q = c_{\text{plasma}}$ and the plasma membrane has no influence, whereas for $\psi = 0$, $c_q = 0$ and the cell is completely sealed. Intermediate values of ψ give values of c_q between zero and c_{plasma}. Values for the 'metabolism parameter' of eqn. (20) are given in Table 2 and it can be seen that they cover the same range as the available values for lipid-bilayer permeabilities (10^{-4}–10^{-1} cm·s^{-1}) discussed in the Introductory information section.

Since the ranges of the 'metabolism parameter' and ψ are about the same, we are unable to decide, in general, whether it is the permeation of the sinusoidal plasma membrane or the subsequent intracellular diffusion and metabolism that limits the rate of uptake of a diffusant by the hepatocyte. Protein A and ligandin are expected to influence the working of the cell at relatively large ratios of ψ to the 'metabolism parameter' of eqn. (20), whereas at relatively low ratios the membrane permeability limits the cell's efficiency. It should be pointed out that the profiles of Fig. 5 are completely independent of the value of ψ, since it is the absolute value of c_q that is influenced by the membrane permeability not the ratio c/c_q. Thus, although the ratio of $c(x = 0)$ to c_{plasma} (cf. Fig. 5) is dependent on both ψ and the 'metabolism parameter', the ratio $c(x = 0)$ to c_q is dependent only on the 'metabolism parameter'.

Discussion

The general conclusion to be drawn from this work is that, given a number of assumptions, protein A and ligandin could make rat hepatocytes more efficient at taking up and metabolizing their ligands. The mechanism by which they do this consists of facilitated transport of the ligands within the cell, so that metabolizing enzymes far from the sinusoidal membrane receive more substrate than they would in the proteins' absence. Such a mode of action for the proteins was first put forward, in qualitative terms, by Gillette (1973).

Having set up a theory we are immediately concerned with how susceptible it is to tests. Since the ideas we have developed here depend to a large extent on the relative values of several different sets of data, the most direct way to attempt to refute the theory is by making accurate measurements of the most uncertain quantities. For example, if it could be shown that the actual rates of formation and dissociation of the protein–ligand complexes in question are substantially lower than the near diffusion-controlled rates that the theory requires, then the proteins could not facilitate the diffusion of their ligands. If the permeability of the sinusoidal plasma membrane to the diffusants in question could be shown to be invariably low, compared with the 'metabolism parameter' of eqn. (20), then the higher values of the effective diffusion coefficient that protein A and ligandin are predicted to bring about would make little or no difference to the overall rate of metabolism of the hepatocyte. If values of the enzyme parameter R_E turned out to be generally small (0.1 s^{-1} or less), i.e. if the metabolizing enzymes were poor catalysts or were present at low concentrations, then again high values of $D_{\text{eff.}}$ would be inconsequential, since reaction rate, rather than the rate of delivery of substrate, would be rate-limiting.

If, after suitable measurements, these questions of numerical values did not contradict the present theory, then the next step might be to attempt experiments on the cells themselves. One experiment might involve the passage of a constant concentration of diffusant (the various characteristic parameters of which were reliably known) through a liver, either intact or isolated, until a steady state was reached, followed by rapid fixing or freezing of the tissue and analysis of the concentration gradient of unmetabolized diffusant within the cells. Mutant animals lacking one or both of the binding proteins, but with other cellular contents as normal, would be ideal controls, but a more realistic approach might be to attempt to render the proteins ineffectual in

normal animals, perhaps by the use of a ligand highly specific to the proteins, but without effect on intracellular membranes or enzymes. Clearly these studies would be technically difficult and would require substantial developmental effort. A less ambitious method would involve the use of isolated hepatocytes, which would be relatively easy to remove from the diffusant-containing medium for the determination of the total cellular content of unmetabolized diffusant, but this would have the disadvantage that the polarity of the cells, as they exist in the intact liver, would be lost, so that uptake would not be confined to the sinusoidal plasma membrane.

While on the subject of experiments with intact tissues and cells it is as well to point out that the theory as it stands cannot be tested by single-dose elimination studies (i.e. by monitoring the decrease in plasma concentration of a pulse of diffusant with time), since they are by definition non-steady state. Although the steady state is theoretically easier to deal with at the molecular level than the disappearance of a single pulse, the reverse is true experimentally.

Until experiments with whole tissues or cells have been carried out, there is little point in trying to develop the present theory further, although in the light of the above, attempts to formulate a non-steady-state version might well be useful. When development of the steady-state theory is attempted, attention should be paid to dealing with diffusants that can leave the cell via the bile canaliculus without metabolism, or with only partial metabolism, and to describing the sequences of metabolic steps that are common in xenobiotic biotransformations.

The impetus for this work came from the discovery by Ketterer *et al.* (1967) that protein A and ligandin both interact covalently with metabolites of aminoazodye carcinogens *in vivo*, and so let us consider finally how the theory we have developed impinges on carcinogenesis.

The bulk of evidence at present available indicates that the induction of cancer by chemicals involves enzyme-catalysed transformations of the parent compound, which bring about the production of a highly reactive electrophile, the ultimate carcinogen, capable of attacking a range of nucleophiles. Reactions of the ultimate carcinogen with the nucleophilic centres in DNA, because of their potential for causing changes in the genome, are regarded by many workers as being of critical importance in the carcinogenic process, whereas those with small molecular nucleophiles such as glutathione are generally considered detoxifying (Miller, 1978; Orrenius & Jones, 1978). Ultimate-carcinogen molecules produced at the cell's periphery should have little chance of reaction with nuclear DNA because before reaching it they should undergo detoxification, whereas ultimate-carcinogen molecules originating in the vicinity of the nucleus are prime candidates for reaction with its contents. According to our theory protein A and ligandin enhance the rate of delivery of substrate to enzymes far from the plasma membrane, i.e. near the nucleus. Although this may improve the overall efficiency of metabolism of carcinogens, it also increases the vulnerability of the nucleus since the likelihood of the formation of ultimate carcinogen in its vicinity is increased.

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APPENDIX

Quadratic coefficients

The complete expressions for α, β and γ of eqn. (12) (see the main paper) are as follows:

$$\alpha = \frac{D_U K_P}{\phi} + \frac{D_U K_P}{1-\phi}$$

$$\beta = \frac{1}{\phi}\left[D_U(1-K_P c_0) + D_P[P]K_P\left(1-\frac{K_P c_0}{1+K_P c_0}\right)\right] + \frac{1}{1-\phi}\left[\theta\left\{D_U(1-K_P c_h) + D_P[P]K_P\frac{(1-K_P c_h)}{1+K_P c_h}\right\}\right.$$
$$\left. + (1-\theta)D_M\chi(1-K_P c_h)\right]$$

$$\gamma = -\left[\frac{c_0}{\phi}\left\{D_U + \frac{D_P[P]K_P}{1+K_P c_0}\right\} + \frac{c_h}{1-\phi}\left\{\theta\left(D_U + \frac{D_P[P]K_P}{1+K_P c_h}\right) + (1-\theta)D_M\right\}\right]$$