Albumin Helps Mediate Removal of Taurocholate by Rat Liver

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ABSTRACT Perfused rat liver removes 97% of the taurocholate from the afferent circulation when the perfusate albumin concentration is 0.5 g/dl. Increasing the albumin concentration 10-fold reduces the concentration of free taurocholate by a factor of five but produces only a 50% reduction in the apparent uptake coefficient. A similar discrepancy is evident from a model-independent analysis of the extraction fractions. From these observations we argue that uptake is not driven solely, or even predominantly, by the plasma concentration of free taurocholate but also depends on interaction between albumin and the cell surface. Nonequilibrium binding, saturation kinetics, and an inhomogeneous population of liver cells are considered as alternative explanations and excluded. The possibility that albumin exerts its effect by enhancing the diffusion of taurocholate across an unstirred layer in the Disse space appears improbable but cannot be eliminated.

INTRODUCTION

The mammalian liver appears specialized for the removal of solutes bound to protein. The most obvious structural feature subserving this specialization is the porous endothelial lining of liver sinusoids, permitting rapid exchange of albumin between blood and the Disse space, thus ensuring intimate contact between bound solutes and the surface of liver cells. An endothelium permeable to protein can hardly explain the removal process itself, however, because albumin is not removed in the uptake process (1).

No definitive study of this phenomenon has appeared, but it has generally been assumed that the plasma concentration of free (unbound) solute determines the uptake rate. Despite the fact that many solutes such as bilirubin and propranolol are efficiently removed in the face of virtually complete protein binding, this idea has gained wide acceptance. For example, a substantial pharmacologic literature based on it has recently been summarized and defended by Shand et al. (2) and Wilkinson (3).

The studies to be reported here were designed to test this assumption by examining the effect of albumin on the removal of taurocholate.

METHODS

The livers of 22 female Sprague-Dawley rats (250–300 g) were perfused *in situ* with Krebs-Ringer-HCO_3 buffer containing either 5.0 g/dl or 0.5 g/dl bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). The recirculating perfusion system consists of a stirred reservoir equilibrated with 95% O_2, 5% CO_2 through a coil of silastic tubing (Dow Corning Corp., Midland, Mich.), short lengths of silastic tubing that conduct perfusate to the portal vein and back to the reservoir from the thoracic vena cava, and a roller pump that circulates perfusate through the liver at a fixed flow rate. Perfusate temperature, pH, and pressure were monitored continuously and the preparation discarded if variations occurred outside the physiologic range. Sodium taurocholate (Calbiochem, La Jolla, Calif.) was added to the reservoir as a continuous infusion throughout each experiment to maintain a steady-state concentration of ~18 μM and a constant reservoir volume of ~95 ml. The hematocrit varied from 1 to 2% in individual experiments reflecting the presence of each rat's own erythrocytes washed from the liver during the initial stages of the perfusion. These erythrocytes were sufficient to ensure oxygenation of the liver but insufficient to cause appreciable hemolysis or to bind detectable amounts of taurocholate.

In 12 experiments we recorded the disappearance of radioactivity from the reservoir after an impulse injection of [^{14}C]-taurocholate (New England Nuclear, Boston, Mass.). An uptake coefficient was determined from each disappearance curve by using a compartmental model we have discussed elsewhere in detail (4, 5). Briefly, the model provides refined estimates of three first order transfer coefficients that relate the two flux rates at the sinusoidal face of liver cells (uptake and efflux) and the unidirectional flux at the canalicular face (excretion) to the solute concentration in the Disse space and the solute content of the cell interior. The model is fully distributed in the sense that its governing differential equations include both time and location as independent variables. In particular, the model takes explicit account of recirculatory mixing in the reservoir and of the intrahepatic concentration

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profiles that develop as solute removal proceeds. Estimates of the transfer coefficients obtained by this method are virtually independent of arbitrary assumptions about the distribution of flow to individual sinusoids and unaffected by convolutions arising in the tubing and the nonexchanging vasculature. The disappearance curves were fitted by a derivative-free, least squares, minimization algorithm (6) that matches the data to the model through numerical inversion of its Laplace transform (7). Goodness-of-fit is reported as the coefficient of determination for 14 data points obtained between 20 s after the impulse injection of [^{14}C]taurocholate and the time required for radioactivity in the reservoir to reach 5% of its initial value. The steady-state extraction fraction, E, was obtained from the area under the disappearance curve and the perfusate flow and is thus independent of model assumptions (4). Changes in perfusate albumin concentration were measured by the method of Bradford (8).

In 10 additional rats the distribution of transit times in extracellular fluid and the volume of this compartment were determined by recording the hepatic venous outflow curves (1) and the steady-state liver content of ^{125}I human serum albumin (Abbott Laboratories, North Chicago, Ill.). The perfusion system in these experiments was identical to that used in the uptake studies except that recirculation was interrupted temporarily to record the appearance of ^{125}I in hepatic venous effluent, and the impulse injection of the tracer was given into the portal vein cannula instead of the perfusate reservoir.

The purity of taurocholate, verified by thin-layer chromatography of both the labeled and unlabeled forms, was better than 98%. Concentrations of taurocholate were measured by the steroid dehydrogenase method (Nyegaard and Co., Oslo, Norway). The radioactivity of ^{14}C and ^{125}I was measured by liquid scintillation counting. Equilibrium dialysis of the perfusate was carried out at 4°C in bags of regenerated cellulose (Spectapor 4, Spectrum Medical Industries, Los Angeles, Calif.). Dialysis bags containing perfusate with either 0.5 or 5.0 g/dl albumin and various concentrations of taurocholate were gassed with 95% O_2 5% CO_2 and placed in sealed test tubes containing an identical solution free of protein. The equilibrium distribution of [^{14}C]taurocholate in this system was measured after 18 h of continuous agitation in a motor driven oscillator. Equilibrium was verified by the absence of a change in distribution between 15 and 18 h.

The governing equations for the distributed compartmental model, the calculations that underlie the interpretation of the binding data, and the mathematical basis for model-independent estimates of the uptake coefficients are summarized in an appendix.

RESULTS

As judged by perfusate flow, portal vein pressure, bile production, liver weight:body weight ratio, and the volume of distribution of labeled protein, livers perfused with 5.0 g/dl albumin were indistinguishable from those perfused with 0.5 g/dl albumin. The distribution of albumin transit times was also independent of the albumin concentration. Numerical data summarizing these results appear in Table I. As a further check on the functional integrity and similarity of the two groups, coded sections of each liver were examined by light microscopy. All the livers appeared normal, and no distinguishing histologic features could be identified. As summarized in Table I, changes in perfusate albumin concentration were trivial.

Scatchard plots of the binding data were linear up to a taurocholate concentration of 100 μM. Mean values (±SD) for the equilibrium constant, ϕ, the number of sites, n, and the correlation coefficient, r, determined from three separate binding studies were ϕ = 0.283 nM±33 μM, n = 2.9±0.1, and r = 0.999±0.001. A typical Scatchard plot appears in Fig. 1.

Given n and ϕ, the free fraction of taurocholate, ψ, is readily determined from the equilibrium relation. This calculation yielded values of ψ that were virtually independent of the taurocholate concentration over the range from 0.1 to 20 μM. The actual values of the free fraction over this range were from 0.114 to 0.115 when the albumin concentration was 5.0 g/dl, and from 0.563 to 0.573 when the albumin concentration was 0.5 g/dl. Thus a 10-fold increment in albumin concentration was associated with an 80% reduction in the free fraction of taurocholate, but in each case this fraction was constant over the entire range of bile salt concentrations that

TABLE I
Liver Perfusion Data

Perfusate albumin*		Perfusate flow	Portal vein pressure	Bile flow	Liver wt / Body wt	Extra-cellular volume‡	$\bar{t}_{ALB}$‡
g/dl	*% change per 15 min*	*ml/min per g*	*cm H_2O*	*μl/min per g*		*ml/g*	*s*
0.5	1.1±3.4 (5)	4.81±0.62 (6)	13±2 (11)	1.30±0.29 (11)	0.027±0.003 (11)	0.142±0.034 (5)	1.78±0.35 (5)
5.0	0.4±6.5 (5)	4.55±0.60 (6)	13±3 (11)	1.28±0.23 (11)	0.028±0.003 (11)	0.146±0.039 (5)	1.95±0.46 (5)

Mean±SD for (n) rats.
* Bovine serum albumin.
‡ Determined with ^{125}I human serum albumin. $\bar{t}_{ALB}$, mean albumin transit time corrected for cannula dead space.

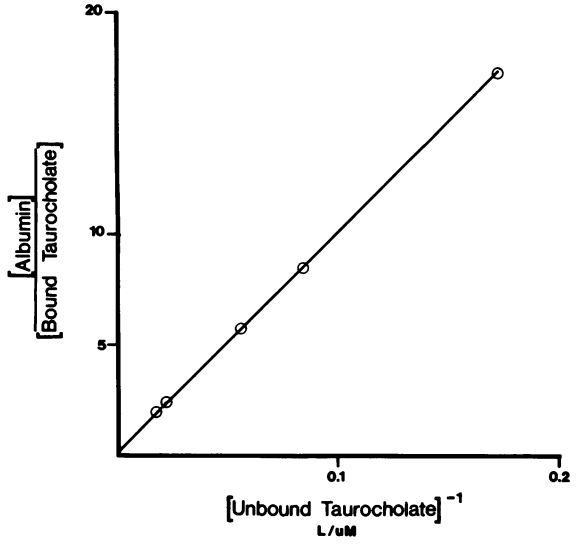

FIGURE 1 A representative Scatchard plot of equilibrium binding between albumin and taurocholate. Linearity of the plot is consistent with independent binding sites whose number (n = 2.9) is the reciprocal of the ordinate intercept. The equilibrium constant determined as n times the slope is 0.281 mM.

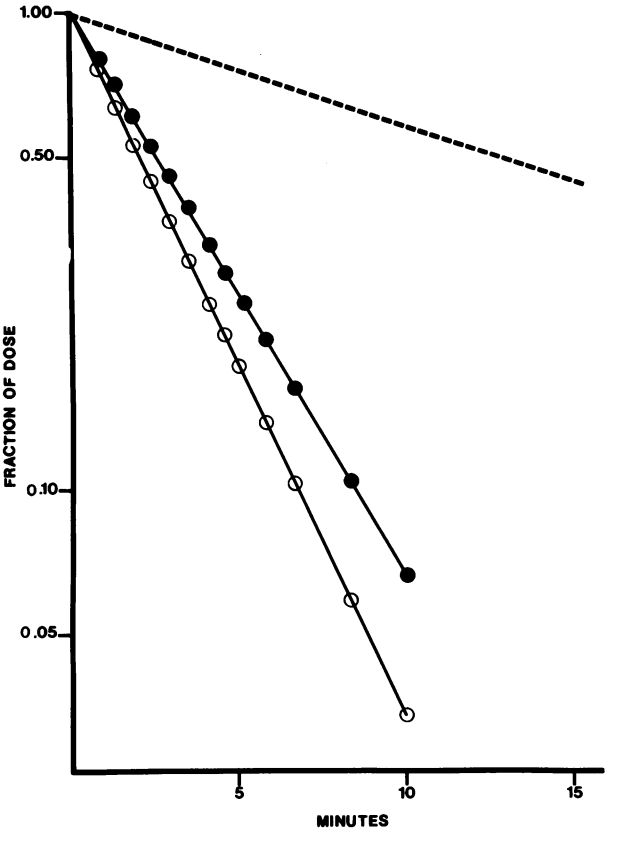

FIGURE 2 Representative taurocholate disappearance curves obtained when perfusate albumin concentration was 0.5 g/dl (○) and 5.0 g/dl (●). The broken line represents the predicted result for an albumin concentration of 5.0 g/dl based on the assumption that uptake is determined by the perfusate concentration of free bile salt.

could have occurred within the sinusoids or the Disse space. This is an important consideration, because our argument depends on knowing the binding ratio at every point within the liver lobule.

Two important results were apparent from simple inspection of the disappearance curves (Fig. 2). In every case the curves were log-linear suggesting that the removal process was not associated with measurable efflux of taurocholate from liver cells. This was confirmed by the fitted values of the efflux rate constants that averaged 0.00001 ± 0.00003 s^{-1}. Second, despite an 80% reduction in free taurocholate concentration, livers perfused with 5.0 g/dl albumin removed taurocholate only slightly less rapidly than did those perfused with 0.5 g/dl albumin.

The uptake constant, K, obtained from each curve is the uptake flux rate per unit of total taurocholate concentration and is therefore a falsely low estimate of the transfer coefficient that would apply to the uptake of free material. Accordingly, the conventional assumption that uptake is determined solely by the concentration of free taurocholate leads to a corrected uptake constant, K_c, defined by the relation, $K_c = K\psi$. Values of K_c together with the other kinetic data appear in Table II from which it is clear that K_c depends on the albumin concentration. The conventional assumption upon which K_c is calculated, i.e., that uptake is wholly determined by the plasma concentration of free taurocholate, therefore leads to a contradiction. Specifically, the higher value of K_c is associated with the higher albumin concentration, but the extraction fractions, and thus the actual removal rates, display the opposite relation. To appreciate the implication of this discrepancy, notice that increasing the concentration of albumin from 0.5 to 5.0 g/dl reduces the uptake rate, as expected, but that this effect is much less than that predicted from the concomitant change in taurocholate binding. This result is presented graphically in Fig. 2 by comparing representative disappearance curves obtained at each albumin concentration with the one predicted by a fivefold change in the free fraction.

DISCUSSION

The estimates of K_c are based on a model which assumes, in addition to the stipulation noted above, that all liver cells operate with the same transfer coefficient and that the binding reaction is everywhere at equilibrium.

To deal with the last assumption first, we require assurance that the binding reaction is rapid with respect to both the mean sinusoidal transit time and

TABLE II
Taurocholate Kinetic Data

Perfusate albumin	ψ	K	K_c	E	r^2
g/dl		*ml/min per g*	*ml/min per g*		
0.5	0.57	22.0±7.4 (6)	38.6±13.0 (6)	0.97±0.02 (6)	0.999±0.001 (6)
5.0	0.11	11.1±1.8 (6)	100.9±16.4 (6)	0.86±0.03 (6)	0.999±0.001 (6)
		$P < 0.01$	$P < 0.01$	$P < 0.01$	

Mean±SD for (n) rats. ψ, free fraction. K, uptake transfer coefficient. K_c, K corrected for binding. E, steady-state extraction fraction. r^2, coefficient of determination for disappearance curves.

the time characteristic of the membrane transport step. The sinusoidal transit time can be calculated from the albumin transit time, the flow, F, and the volume of the Disse space, ω, as $\bar{t}_{alb} - \omega/F$. The time characteristic for uptake is derived from the transfer coefficient as ω/K. On the assumption that ω is 7% of liver weight (9), we calculate a mean sinusoidal transit time of 950 ms and uptake times of from 190 to 380 ms depending on the albumin concentration. To estimate the speed of the binding reaction in relation to these values, we note that the association constant for a diverse group of similar binding equilibria is diffusion limited and $\sim 10^9$ $(M/s)^{-1}$ (10–12). This value and the equilibrium constant determined by dialysis were used to calculate the time required for the binding reaction to reach 99.5% of its equilibrium state. Because this calculation yields a value of only 5 μs, the assumption that binding is effectively at equilibrium everywhere along the sinusoids seems assured.

The assumption that the transfer coefficient for hepatic uptake is the same for all liver cells, while impossible to verify, can be shown to be unnecessary. For simplicity consider a single sinusoid in which u is the concentration of total solute, and x is the cumulative sinusoidal volume extending from zero at the portal inlet to v at the central vein. The equation governing the sinusoidal concentration profile under these circumstances is

$$F \frac{du}{dx} = - \frac{\psi K_c u}{v}. \quad (1)$$

If K_c is a constant, independent of u and x, Eq. 1 has a simple solution that may be expressed in terms of the extraction fraction, E, as

$$E = 1 - \exp - (\psi K_c/F). \quad (2)$$

The distributed model, though substantially more complicated than this, uses the assumptions embodied in Eq. 2 to estimate K_c from observations of E, ψ, and F. The sinusoidal concentration of taurocholate in these experiments was at most 18 μM, which is only 2–3% of the concentration required for half-saturation of hepatic uptake by rat liver (1, 13). We are confident, therefore, that K_c is independent of u. However, there are no experimental data to justify the assumption that K_c is independent of x. To avoid this uncertainty we abandon the constraint that K_c be constant, and instead allow K_c to be some unspecified function of x, say $K_c = g(x)$. The solution to Eq. 1 is then

$$\frac{F}{\psi} \ln (1 - E) = - \frac{1}{v} \int_0^v g(x)dx. \quad (3)$$

The right side of this relation is by definition the mean value of g(x) (i.e., the mean value of K_c), and its value can be computed from the independently measured values of F, ψ, and E. Since the integral in Eq. 3 is independent of the albumin concentration, an assumption that the concentration of free taurocholate is the sole determinant of uptake predicts that $F \ln (1 - E)/\psi$ will be constant. In the appendix this line of reasoning is developed for an array of sinusoids with a Gaussian distribution of flow rates consistent with the shape of the albumin outflow curves. Mean values for K_c computed by this model-independent approach were 33.1 ±5.8 and 86.4±19.8 for albumin concentrations of 0.5 and 5.0 g/dl, respectively. These values are similar to those in Table II derived from fitting the disappearance curve with K as a constant parameter.

In view of these considerations we suggest that the experimental data represent the net effect of two opposing phenomena. Since protein is not itself removed in the uptake process (Table I), only a fraction, ψ, of the total taurocholate in the sinusoids is available for transport. This retarding effect of protein binding on taurocholate removal is largely compensated for, however, by a second phenomenon in which the association

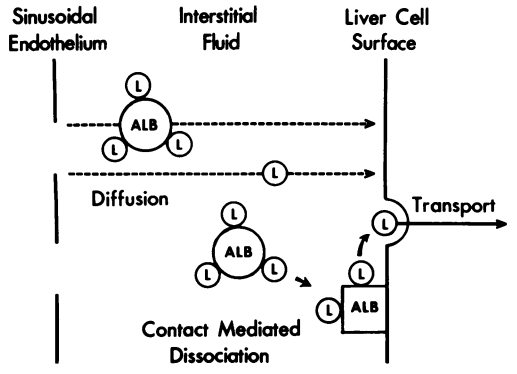

FIGURE 3 (Top) The net diffusional flux of taurocholate across an unstirred layer of interstatial fluid could be accelerated by protein binding if the diffusion coefficient of monomers (L) were less than three times the diffusion coefficient of albumin (ALB) with its three binding sites occupied. (Bottom) Contact of an albumin-ligand complex with the cell surface could produce a conformational change in the protein favoring local dissociation of the complex and subsequent presentation of free ligand to the transport site.

with protein evidently enhances the presentation of taurocholate to its carrier on the cell surface.

Two kinds of mechanisms, not necessarily mutually exclusive, might account for this effect. One possibility is that protein binding accelerates the diffusion of taurocholate across the unstirred fluid layer in the Disse space. The second is that dissociation of the taurocholate-albumin complex is enhanced by contact with the cell surface in such a way as to provide for a local concentration of free bile salt substantially higher than that predicted by the equilibrium distribution in free solution. These speculations are illustrated schematically in Fig. 3.

To evaluate the possibility that albumin exerts its effect by helping to overcome the diffusional resistance of unstirred fluid in the Disse space, we require an estimate of the diffusion time relative to the time characteristic of the membrane transport step. The reason for this is that changes in diffusion time can materially alter the overall uptake rate only if diffusion is rate limiting. Goresky (9) has estimated the time for albumin to reach diffusion equilibrium in the Disse space at 60 ms. If this estimate is correct, diffusion is substantially faster than the membrane transport step (190–380 ms) and is therefore unlikely to explain the present results. Because the diffusion coefficient for albumin in extracellular matrix is unknown, however, and because the geometric factors in Goresky's calculation are greatly simplified, a diffusional mechanism cannot be completely excluded. In any event, a rate limiting diffusion barrier in the Disse space would seem to be a maladaptive evolutionary development in view of the survival value of efficient hepatic extraction.

For these reasons a local phenomenon at the cell surface appeals to us as the more plausible explanation of how albumin binding promotes a relative enhancement of bile salt uptake. For example, contact between albumin and the cell membrane might induce a transient conformational change in the protein that could not only reduce its binding affinity for taurocholate but also present the free product of this reaction at a favorable location for transport into the cell. The specificity of such surface phenomena for albumin and the extent to which they might serve to promote the uptake of other protein-bound solutes are important questions amenable to future investigation. Meanwhile, it is clear that the binding of taurocholate to albumin has an importantly different effect on its hepatic removal than has been recognized heretofore.

APPENDIX

Distributed model equations. The model consists of a parallel array of identical sinusoids in series with a reservoir, P. Perfusate recirculates through this system via tubing and nonexchanging hepatic vasculature. Delay and dispersion in these nonexchanging conduits are modeled as a mixing compartment, C, in series with a simple delay, τ_d.

Let $u_i(x,t)$ be the solute concentration in the i^{th} sinusoid and the adjacent Disse space, $z_i(x,t)$ be the solute concentration in the corresponding cellular compartment, and F be the total perfusate flow. Let k_1, k_2, and k_3 be first order rate constants associated with uptake, efflux, and excretion, respectively. The compartmental volumes are designated as V_p, V_c, and V_u, for compartments P, C, and the total exchanging vasculature, respectively. The ratio of the intracellular volume to V_u is denoted by θ and the ratio of the interstitial volume (Disse space) to V_u by γ.

Consider m sinusoids grouped in j classes according to j values of f_i, the perfusate flow per sinusoid. The flow distribution scheme assigns a fraction, α_i, of the m sinusoids to each flow class. The governing differential equations are then

$$(1 + \gamma)\frac{\partial u_i}{\partial t} + \frac{\partial u_i}{\partial x} f_i + \theta \frac{\partial z_i}{\partial t} + \theta k_3 z_i = 0, \quad (4)$$

$$\theta \frac{\partial z_i}{\partial t} + \theta(k_2 + k_3)z_i - \gamma k_1 u_i = 0, \quad (5)$$

$$\frac{dP}{dt} V_p = FC(t - \tau_d) - FP, \quad (6)$$

$$\frac{dC}{dt} V_c = \sum_{i=1}^{j} (\alpha_i m f_i) u_i(V_u/m, t) - FC, \quad (7)$$

where x is the cumulative volume of each sinusoid.

There are 2j + 2 differential equations in all since Eq. 4 and 5 must be solved for each class of sinusoids. The solution to Eq. 4 is constructed by Laplace transformation using the following auxilliary conditions.

$$u_i(0, t) = P(t)$$

$$u_i(x, 0) = z_i(x, 0) = C(0) = 0$$

$$P(0) = 1.$$

The transformed solution for the concentration in the reservoir, P, is given by

$$\bar{P}(s) = \frac{\tau_p(1 + Y_c s)}{(1 + \tau_c s)(1 + \tau_p s) - \sum_{1}^{j} (\alpha_i m f_i / F) \exp - [\tau_d s + H_i(s)]}. \quad (8)$$

Here τ_p and τ_c are, respectively, V_p/F and V_c/F while $H_i(s)$ is defined as

$$H_i(s) = \left[V_u(1 + \gamma)s + V_u\gamma k_1 \frac{s + k_3}{s + k_2 + k_3} \right] \Big/ mf_i.$$

The parameters $V_u\gamma k_1(=K)$, k_2, and k_3 are determined by fitting the inverse of Eq. 8 to the disappearance curve recorded in compartment P. The fitting procedure uses numerical transform inversion since the inverse of $\bar{P}(s)$ does not exist in closed form. The flow distribution parameters (f_i, α_i) were assigned by assuming a Gaussian distribution of perfusate flows. We have shown previously (4) that when $\tau_p \gg \tau_d + \tau_c$ (a stipulation that is met in the present experiments) estimates of the transfer coefficients are insensitive to convolutions arising in the tubing and the nonexchanging vasculature. The assignment of τ_c and τ_d while arbitrary is thus of little numerical importance to the answer.

Binding equations. The equilibrium relation from which the Scatchard plot is constructed is

$$\frac{P_t}{L_p} = \frac{\phi}{nL} + \frac{1}{n}, \tag{9}$$

where P_t is the concentration of total protein, L_p and L are the concentrations of bound and free ligand, respectively, ϕ is the equilibrium constant, and n is the number of independent binding sites. If L_t is total ligand concentration and nP is the concentration of unbound sites, additional conservation relations are

$$nP_t = nP + L_p \tag{10}$$

$$L_t = L + L_p. \tag{11}$$

Solving Eq. 9–11 for the free fraction, $\psi(=L/L_t)$, yields

$$\psi = [L_t - P_t - \phi n + [[L_t - P_t - \phi n]^2 + 4\phi n]^{1/2}]/2.$$

The time-course of the binding reaction is governed by the nonlinear differential equation

$$\frac{dL}{dt} = \phi_1 L_p - \phi_2 n P L \tag{12}$$

in which ϕ_1 and ϕ_2 are, respectively, the "off" and "on" reaction rate constants.

The equilibrium dialysis data provide only the equilibrium constant, $\phi(=\phi_1/\phi_2)$, so that the solution to Eq. 12 requires an independent estimate of either ϕ_1 or ϕ_2. The references cited in the text suggest that ϕ_2 is diffusion limited and on the order of 10^8–10^{10} $M^{-1}s^{-1}$. Since ϕ is ~0.1 mM the appropriate range for ϕ_1 is 10^4–10^6 s^{-1}. These estimates were used in Eq. 10–12 to determine the time for L to reach 99.5% of its equilibrium value. The maximum equilibration time for the indicated ranges of ϕ_1 and ϕ_2 was 5 μs.

Model-independent equations for K_c. Eq. 3 in the body of the paper may be written for the ith sinusoid as

$$1 - E_i = \exp - \left[(\psi/f_i v) \int_0^v g(x)\, dx \right]. \tag{13}$$

The extraction fraction for the sinusoidal array as a whole is defined as

$$E = 1 - \frac{\sum_{i=1}^{j} \alpha_i m f_i u_i (V_u/m)}{Fu(0)} \tag{14}$$

where u(0) is the concentration at x = 0 common to all the sinusoids. Moreover by definition

$$1 - E_i = u_i(V_u/m)/u(0). \tag{15}$$

The transcendental system, Eq. 13–15, was solved by numerical iteration to find the integral in Eq. 13, which is by definition the average value of K_c. The values of K_c obtained in this way were similar to those obtained directly from Eq. 3.

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REFERENCES

1. Reichen, J., and G. Paumgartner. 1976. Uptake of bile acids by perfused rat liver. *Am. J. Physiol.* **231:** 734–742.
2. Shand, D. G., R. H. Gotham, and G. R. Wilkinson. 1976. Perfusion-limited effects of plasma drug binding on hepatic drug extraction. *Life Sci.* **19,** 125–130.
3. Wilkinson, G. R., and D. G. Shand. 1975. A physiological approach to hepatic drug clearance. *Clin. Pharmacol. Ther.* **18:** 377–390.
4. Forker, E. L., and B. Luxon. 1978. Hepatic transport kinetics and plasma disappearance curves: distributed modeling versus conventional approach. *Am. J. Physiol.* **235:** E648–660.
5. Luxon, B., and E. L. Forker. 1979. How to measure unidirectional solute flux rates in perfused rat liver by compartmental analysis. *Gastroenterology.* **76:** 1306.
6. Powell, M. J. D. 1965. A method for minimizing a sum of squares of non-linear functions without calculating derivatives. *Computer Journal.* **7:** 303–307.
7. Gaver, D. P. 1966. Observing stochastic processes and approximate transform inversion. *Oper. Res.* **14:** 444–459.
8. Bradford, M. D. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
9. Goresky, C. A. 1963. A linear method for determining liver sinusoidal and extravascular volumes. *Am. J. Physiol.* **204:** 626–640.
10. Jardetzky, O. 1966. The study of drug binding sites by nuclear magnetic relaxation. *In* Proceedings of the Third International Pharmacological Meeting. M. Rocha, E. Silva, editors. Pergamon Press, Inc., Elmsford, N. Y. **7:** 189–191.
11. Eigen, M. 1964. Zur Frage der Wechselwirkungen zwischen proteinen and substratmolekeln. *Colloquim Der Gesell Schaft Fuer Physiologesche Chemie.* **15:** 344–346.
12. Eigen, M., and G. G. Hammes. 1963. Elementary steps in enzyme reactions. *In* Advances in Enzymology. F. F. Nord, editor. John Wiley & Sons, Inc., New York. **25:** 1–38.
13. Reichen, J., and G. Paumgartner. 1975. Kinetics of taurocholate uptake by the perfused rat liver. *Gastroenterology.* **68:** 132–136.