British Journal of Pharmacology (1997) 120, 1215–1220 © 1997 Stockton Press All rights reserved 0007–1188/97 $12.00

Effect of binding protein surface charge on palmitate uptake by hepatocyte suspensions

[1]F.J. Burczynski, G.-Q. Wang & M. Hnatowich

Division of Pharmaceutical Sciences, Faculty of Pharmacy and Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

1 Studies were directed at determining whether hepatocytes, isolated from female Sprague-Dawley rats, facilitate the uptake of protein-bound long-chain fatty acids. We postulated one form of facilitated uptake may occur through an ionic interaction between the protein-ligand complex and the cell surface. These interactions are expected to supply additional ligand to the cell for uptake.

2 The clearance rate of [^{3}H]-palmitate in the presence of α_1-acid-glycoprotein (pI = 2.7), albumin (pI = 4.9) and lysozyme (pI = 11.0) was investigated. Palmitate uptake was determined in the presence of protein concentrations that resulted in similar unbound ligand fractions (= 0.03). The experimental clearance rates were compared to the theoretical predictions based upon the diffusion-reaction model.

3 By use of our experimentally determined equilibrium binding and dissociation rate constants for the various protein-palmitate complexes, the diffusion-reaction model predicted clearance rates were 4.9 μl s^{-1}/10^6 cells, 4.8 μl s^{-1}/10^6 cells and 5.5 μl s^{-1}/10^6 cells for α_1-acid-glycoprotein, albumin and lysozyme, respectively; whereas the measured hepatocyte palmitate clearance rates were 1.2 ± 0.1 μl s^{-1}/10^6 cells, 2.3 ± 0.3 μl s^{-1}/10^6 cells and 7.1 ± 0.7 μl s^{-1}/10^6, respectively.

4 Hepatocyte palmitate clearance was significantly faster ($P < 0.01$) in the presence of lysozyme than albumin which was significantly faster than α_1-acid-glycoprotein ($P < 0.01$). The marked difference in clearance rates could not be explained by considering differences in solution viscosity.

5 Our results are consistent with the notion that ionic interactions between protein-ligand complexes and the cell surface facilitate the ligand uptake by decreasing the diffusional distance of the unbound ligand and/or by facilitating the protein-ligand dissociation rate.

Keywords: Surface charge; pI; albumin; lysozyme; orosmucoid; α_1-acid-glycoprotein; palmitate; fatty acids; hepatic; uptake

Introduction

The role of binding proteins in the mechanism(s) of cellular uptake of protein-bound ligands is a contentious issue. Circumstantial evidence favours the view that hepatocytes (Burczynski *et al*., 1989; Pond *et al*., 1992; Burczynski & Cai, 1994) but not myocytes (Burczynski *et al*., 1995) facilitate the uptake of protein-bound ligands (see Burczynski & Luxon, 1995 for a review). Facilitated uptake in this context could occur by any of several mechanisms. One theory maintains that some type of surface-induced conformational change in the protein-ligand complex is responsible (Horie *et al*., 1988; Reed & Burrington, 1989). Such interactions are thought to increase the protein-ligand dissociation rate, thus, supplying more unbound ligand to the cell for uptake. Another plausible mechanism is the notion that protein-bound ligands are directly unloaded at the plasma membrane, perhaps due to site specific collisional exchanges. Direct transfer has been shown to occur in the transfer of metabolites via enzyme-enzyme complex formation (Srivastava & Bernhard, 1986). Alternatively, facilitated uptake may be due to ionic interactions between surface charged groups on the hepatocyte plasma membrane and those of the protein-ligand complex. This interaction is expected to result in a decrease in the diffusional distance that the unbound ligand must traverse in order to reach the cell surface.

Most studies aimed at elucidating facilitated uptake have focused on albumin as the binding protein. Proteins such as β-lactoglobulin (Nunes *et al*., 1988; Burczynski *et al*., 1990) also have been used but a common feature is that the isoelectric point (pI) of the protein of interest is often similar to that of albumin. Studies investigating the uptake of organic anions in the presence of binding proteins having pI values appreciably different from albumin have not been published.

Given the number of publications describing ionic interactions between cellular substrates and cell membranes, protein surface charge (pI) may be an important factor to consider when studying uptake processes. For example, cell membrane surface charge is known to affect the activity of membrane-bound enzymes, protein-membrane adsorption properties, transmembrane flux and capillary permeability properties of proteins (Norde & Lyklema, 1978; Park & Maack, 1984; Michel *et al*., 1985; Wojtczak *et al*., 1988; Smith & Borchardt, 1989; Ghitescu *et al*., 1992; Nishida *et al*., 1992; Wadhwani *et al*., 1992). Similarly, the surface charge of proteins affects their membrane adsorptive properties (Norde & Lyklema, 1978; Nishida *et al*., 1992). Thus, it is possible that ionic interactions between protein-bound ligands and cellular membranes provide additional unbound ligand to the cell surface for uptake.

If the uptake rate of ligands in the presence of their binding proteins is observed to be dependent upon the pI, this would provide compelling evidence that facilitated uptake is occurring. Thus, we examined the effect of protein surface charge on the uptake of a long-chain fatty acid (i.e. palmitic acid) by hepatocytes isolated from female rats. The hepatocyte ligand clearance rate in the presence of different binding proteins was compared with the predicted clearance rate based on the diffusion-reaction model of Bass & Pond (1988).

Methods

Purification of palmitate

Palmitate was purified by a modification of the ethanol extraction procedure of Borgstrom (1952) as previously described (Burczynski *et al*., 1993). Briefly, radiolabelled impurities were removed by twice extracting aqueous palmitate with

[1]Author for correspondence at: Faculty of Pharmacy, Room 410 Pharmacy Building, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

heptane. Following acidification of the palmitate solution with 5 M HCl, the solution was extracted once with heptane, the organic phase containing the palmitate harvested, evaporated, redissolved in ethanol and stored at −20°C.

Determination of unbound palmitate fraction and the protein-palmitate equilibrium binding constant

The purified palmitate (~1 nM) was added to solutions of phosphate buffered saline (PBS; mM: KCl 2.68, KH_2PO_4 1.65, NaCl 136.9, Na_2HPO_4 8.92, pH 7.4) containing $\alpha_1$acid glycoprotein (20 μM), albumin (5 μM) or lysozyme (50 μM). The unbound palmitate concentration was measured by equilibrium dialysis with thin polyethylene membranes following 48 h equilibrium according to Moran *et al*. (1987). The equilibrium association constant (K_a) for the different protein-palmitate complexes was calculated from equation 1

$$K_a = \frac{1-\alpha}{\alpha C_a} \quad (1)$$

where α is the unbound palmitate fraction and C_a is the binding protein concentration.

Determination of protein-palmitate dissociation rate

The dissociation rate constant for the various protein-palmitate complexes was determined by the albumin-agarose method previously described by Weisiger & Ma (1987). This method involved determining the transfer rate between protein-bound palmitate and albumin-agarose acceptor beads at 37°C. At $t=0$ s, α_1-acid glycoprotein-palmitate, albumin-palmitate or lysozyme-palmitate was added to the albumin-agarose suspension. At specified time intervals, the resulting mixture was sampled and vacuum filtered with GF/C glass fibre filters and the filtrate collected. Aliquots of filtrate and incubation mixture were sampled to determine radioactive content. The ratio of filtrate radioactivity to that of total radioactivity was plotted and data analysed according to the general exponential equation:

$$y = Ae^{(-k_{off}t)} \quad (2)$$

where k_{off} represents the protein-palmitate dissociation rate constant. Control experiments conducted with ^{125}I radio-labelled proteins produced no measurable protein binding to the albumin-agarose acceptor beads, in line with previous findings (Weisiger & Ma, 1987).

Preparation of hepatocyte suspensions

This study was approved by the University of Manitoba Animal Care Committee. Hepatocyte suspensions were prepared from female (250–300 g) Sprague-Dawley rats (University of Manitoba breeding stock) by the collagenase perfusion technique described previously (Burczynski & Cai 1994). Rats were housed in a temperature-light-controlled room (22°C; lights set on a 12 h on and 12 h off cycle starting at 06 h 00 min). Rats were allowed Agway Prolab Animal Diet (Agway County Foods, Inc. Syracuse NY) and water *ad libitum*. Cells were stored at room temperature and used within 2 h of isolation. Immediately before use, cells were equilibrated to 37°C. Cell viability, as assessed by trypan blue exclusion, was greater than 90% and not different before and after uptake experiments.

Uptake procedure

Hepatocytes were added to solutions of PBS (37°C) containing palmitate (10 nM) to which either 322 μM α_1-acid glycoprotein, 0.23 μM albumin or 2.1 mM lysozyme or the combinations 0.23 μM albumin plus 2.1 mM lysozyme was added. These protein concentrations resulted in an unbound palmitate fraction of $\alpha=0.03$ (calculated from equation 1). Thus, all uptake experiments were performed with the same unbound palmitate fractions and at a hepatocyte density of $\sim 2.8\times10^5$ hepatocytes ml^{-1}. Cells were prevented from sedimenting by gently agitating the incubating solution. At 30 s intervals, 0.4 ml aliquots of the cell suspension were immediately vacuum filtered through GF/C (Whatman GF/C membranes, 1.2 μm pore size, Fisher Scientific) and washed with 7 ml of ice-cold PBS. Previous work has shown this to be effective in preventing the transmembrane flux of palmitate (Pond *et al*., 1992). In some cases, experiments were conducted with 0.23 μM albumin in the presence and absence of dextran to alter the viscosity of the incubation medium. Viscosity measurements were made by a Cannon-Fenslee Viscometer. All uptake experiments were performed in duplicate.

Predicted palmitate clearance rates

Predicted palmitate clearance rates were calculated by use of the diffusion-reaction model (Bass & Pond, 1988) and compared with the experimental observations. Details of this model have been discussed previously (Burczynski *et al*., 1989; Weisiger *et al*., 1989; Cai *et al*., 1992; Pond *et al*., 1992). The model has been shown to reflect palmitate and oleate flux rates into polyethylene sheeting and decane, respectively (Burczynski *et al*., 1989; Weisiger *et al*., 1989). However, in the presence of hepatocytes the model underestimated the experimentally determined clearance rates (Burczynski *et al*., 1989; Pond *et al*., 1992). In essence the model predicts total clearance rate as:

$$Cl_1 = \frac{4\pi\alpha N R^2 P_f}{1+\frac{P_f R}{D_1}\left[\frac{\frac{\delta}{\delta+R}+\frac{\hat{\alpha}\hat{D}\tanh\lambda\delta}{\lambda R\tanh\lambda\delta}}{1+\hat{\alpha}\hat{D}}\right]} \quad (3)$$

where $\hat{D}=D_b/D_1$, D_1 and D_b are the diffusion coefficients of free and bound ligand respectively, $\hat{\alpha}=(1-\alpha)/\alpha$, $\lambda=[k_{off}(1+\hat{\alpha}\hat{D})/D_b]^{-1/2}$, P_f is the effective membrane permeability to unbound ligand, R is the hepatocyte radius, δ is the dimension of the unstirred fluid layer and N is the number of hepatocytes (10^6 cells). if we make the assumption that $P_f\to\infty$ and $\delta\to\infty$ equation 3 is reduced to

$$Cl_t = \frac{4\pi\alpha N R^2 D_1[1+\hat{\alpha}\hat{D}]}{1+\frac{\hat{\alpha}\hat{D}}{1+\lambda R}} \quad (4)$$

Parameter estimates used for calculating the predicted total palmitate clearance rates included the diffusion coefficient (temperature corrected) for palmitate ($D_1=6.4\times10^{-6}$ cm^2 s^{-1}; Patil *et al*., 1973), α_1-acid glycoprotein ($D_b=8.1\times10^{-7}$ cm^2 s^{-1}; CRC, 1976), albumin ($D_b=9.4\times10^{-7}$ cm^2 s^{-1}; CRC, 1976), and lysozyme ($D_b=1.7\times10^{-6}$ cm^2 s^{-1}; Sophianopoulos *et al*., 1962), and R was taken as 0.0012 cm (Pond *et al*., 1992). Parameter estimates for the unbound palmitate fraction and the dissociation rate constants for the different protein-palmitate complexes were determined in the present study.

Materials

Reagents and their sources were as follows: lysozyme, α_1-acid glycoprotein, fatty acid-free albumin (Sigma A-3803), collagenase, Swim's S-77 and Hams F-12 culture media were purchased from Sigma Chemical Co. [^{3}H]-palmitate (palmitate; radiopurity > 98.5%, 40 Ci mmol^{-1}) was purchased from New England Nuclear. All other chemicals were analytical grade and purchased from either Fisher Scientific or Sigma Chemical Co.

Data analysis

Cell associated radioactivity was determined by scintillation counting and the total palmitate space was calculated as the ratio of recovered cell-associated radioactivity to the concen-

tration of radioactivity in the bathing medium. Total palmitate clearance rates were obtained from the slope of the plot of palmitate space versus uptake interval. The predicted total palmitate clearance rates calculated from the diffusion-reaction model were compared to the total palmitate hepatocyte clearance rates. The data are presented as mean ± s.e.mean and the *n* value refers to the number of animals used for each study. Data were analysed according to Student's *t* test.

Results

Determination of unbound palmitate fraction and K_a

The unbound palmitate fraction in the presence of 20 μM α_1-acid glycoprotein, 5 μM albumin, and 50 μM lysozyme was determined to be 0.33 ± 0.03 ($n=6$), 0.0014 ± 0.0001 ($n=6$), and 0.58 ± 0.12 ($n=6$), respectively, resulting in calculated K_a values (from equation 1) of: $(1.0 \pm 0.14) \times 10^5$ M^{-1}, $(1.4 \pm 0.1) \times 10^8$ M^{-1}, and $(1.5 \pm 0.5) \times 10^4$ M^{-1}, respectively.

Determination of k_{off}

Regression analysis revealed that the transfer (corresponding to k_{off}) of palmitate from α_1-acid glycoprotein, albumin and lysozyme to albumin-agarose was 0.081 ± 0.024 s^{-1} ($n=7$), 0.078 ± 0.012 s^{-1} ($n=7$), and 0.129 ± 0.022 s^{-1} ($n=10$) for α_1-acid glycoprotein, albumin, lysozyme, respectively. There was no statistical difference between k_{off} values for α_1-acid glycoprotein-palmitate and albumin-palmitate complexes. However, the lysozyme-palmitate dissociation rate was significantly greater than for the other proteins ($P<0.01$).

Palmitate clearance rate

Uptake of palmitate by hepatocytes isolated from female rats in the presence of the three binding proteins is shown in Figure 1 and the calculated hepatocyte palmitate clearance rates summarized in Table 1. The slopes of palmitate space versus time differed significantly ($P<0.01$) for the different proteins.

Predicted palmitate clearance rate

The diffusion-reaction model predicted palmitate clearance rates in the presence of α_1-acid glycoprotein, albumin and lysozyme were calculated to be 4.9, 4.8 and 5.5 μl s^{-1}/10^6 cells, respectively (Table 1). The predicted clearance rates with α_1-acid glycoprotein and albumin as the binding proteins were significantly greater than the experimentally determined clearance rates ($P<0.01$). However, the predicted clearance rate with lysozyme as the binding protein was not different from the experimental clearance rate ($P>0.05$).

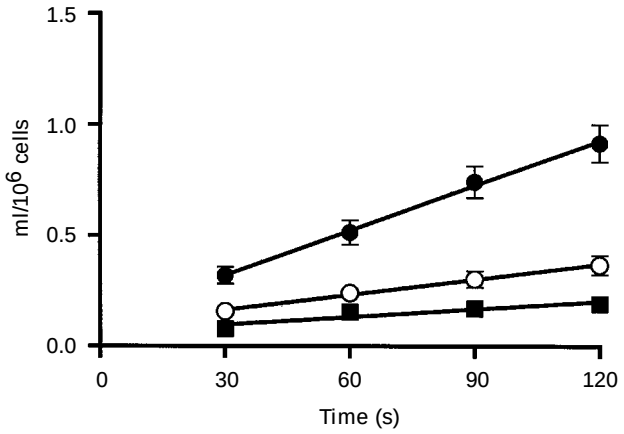

Figure 1 Plot of hepatocyte palmitate space: (■) 322 μM α_1-acid glycoprotein ($n=7$); (○) 0.23 μM albumin ($n=13$); (●) 2.1 mM lysozyme ($n=8$). The total palmitate clearance rate was calculated as the slope of each plot by use of linear regression analysis. Data are mean and vertical lines show s.e.mean.

Table 1 Hepatocyte palmitate clearance rate in the presence of α_1-acid glycoprotein, albumin, lysozyme

Protein[1]	*Clearance rate* (μl s^{-1}/10^6 cells) *Hepatocytes*	*Predicted*[2]
α_1-Acid glycoprotein	1.2 ± 0.1 ($n=7$)**	4.9
Albumin	2.3 ± 0.3 ($n=13$)**	4.8
Lysozyme	7.1 ± 0.7 ($n=8$)**	5.5

[1] In each case the free fraction (α) = 0.03. [2] Predicted clearance rate by use of the diffusion-reaction model (equation 4). **$P<0.01$ lysozyme versus albumin; albumin versus α_1-acid glycoprotein; lysozyme versus α_1-acid glycoprotein. Values represent mean ± s.e.mean.

If protein surface charge is an important consideration in the mechanism of palmitate uptake, then the clearance rate in the presence of a combination of 0.23 μM albumin plus 2.1 mM lysozyme ought to be higher than in the presence of 0.23 μM albumin alone, since the unbound palmitate fraction in the former case is expected to be lower than in the latter. Uptake experiments were conducted with the above protein combination and results were compared to those obtained with 0.23 μM albumin. Uptake of palmitate in the presence of the protein combination was significantly greater ($P<0.02$) than in the presence of 0.23 μM albumin alone (3.0 ± 0.4 ($n=6$) vs 2.2 ± 1.0 ($n=7$) μl s^{-1}/10^6 cells, respectively).

Effect of viscosity

The addition of dextran to 0.23 μM albumin (viscosity = 1.5 centistokes) did not affect the unbound palmitate fraction but significantly reduced the hepatocyte palmitate clearance rate (1.8 ± 0.5 ($n=6$) versus 2.2 ± 1.0 ($n=6$)) in the presence and absence of 0.5 mM dextran, respectively ($P<0.05$; paired *t* test). Higher concentrations of dextran (~1 mM; viscosity = 2.8 centistokes) could not be used due to aggregation of the hepatocytes (observed microscopically). Use of lower dextran concentrations (0.25 mM; viscosity = 1.0 centistokes) did not affect the palmitate clearance rate.

Discussion

Previous work done in an attempt to resolve the issue of whether cells facilitate the uptake of protein-bound ligands have focused on albumin as the binding protein. The decision as to whether facilitation occurs has traditionally relied upon a comparison of the ligand clearance rates obtained with hepatocyte suspensions or monolayers, with those predicted from various models (Sorrentino *et al*., 1989; Burczynski *et al*., 1989; Cai *et al*., 1992; Goresky *et al*., 1992; Pond *et al*., 1992; Burczynski & Cai, 1994), or with uptake media that are presumed to be lacking in facilitation mechanisms (e.g. polyethylene sheeting; Burczynski *et al*., 1989). In this study, we attempted to resolve this issue by examining the palmitate clearance rates in the presence of binding proteins that differed markedly in surface charge, i.e. α_1-acid glycoprotein (pI = 2.7; CRC, 1976), albumin (pI = 4.9; CRC, 1976) and lysozyme (pI = 11.0; Merck Index, 1983).

For proper inferences to be made regarding facilitated uptake, it was necessary to perform uptake experiments with protein concentrations that resulted in similar unbound ligand fractions, ensuring that the driving force for uptake was the same for all proteins studied. We calculated the unbound ligand fraction based on the K_a values obtained with low protein concentrations, an approach validated in previous studies (Sorrentino *et al*., 1989; Pedersen *et al*., 1990; Pond *et al*., 1992; Burczynski & Cai 1994). Accordingly, we showed that the

hepatocyte palmitate clearance rate in the presence of the basic protein lysozyme was 6.3 times greater than in the presence of the acidic protein α_1-acid glycoprotein and 3.2 times greater than albumin ($P<0.01$). Similarly, the palmitate clearance rate in the presence of albumin was 2.0 times greater than in the presence of α_1-acid glycoprotein ($P<0.01$). The relationship between palmitate uptake and pI suggested that an ionic interaction may exist between protein-ligand complexes and the hepatocyte surface. Although this might constitute an example of facilitated uptake, alternative explanations must be considered.

The concentrations of binding proteins in this study differed appreciably, consequently, so did their viscosity. Since viscosity may alter the diffusion coefficient for protein-bound and unbound ligands, it could affect the measured clearance rate. To determine the effect of diffusion on the uptake process we studied the clearance rate by use of the diffusion-reaction model (Bass & Pond, 1988). This model is particularly well suited to our analysis because it accounts for the possibility of rate-limiting codiffusion of protein-bound and unbound ligands, and the associated perturbation of the binding equilibrium in the unstirred fluid layer.

The diffusion-reaction model predicts that the palmitate clearance rate in the presence of α_1-acid glycoprotein or albumin will be similar, and that only a modest rate increase should be observed when lysozyme is used as the binding protein. The increased clearance rate reflects the lower diffusion coefficient and the faster ligand dissociation rate for the lysozyme-palmitate complex. Although the diffusion-reaction model predictions exceeded the values we observed experimentally for α_1-acid glycoprotein (4.4 fold) and albumin (2.2 fold) in a statistically significant fashion, they were not different from the experimental results obtained with lysozyme. Thus, when all three proteins are considered, we find a relationship between surface charge and palmitate uptake that is not predicted by current models.

In applying equation 4 we make the assumption that $P_f \rightarrow \infty$, i.e. the rate-limiting step in the overall uptake of palmitate is conductance through the unstirred fluid layer and not membrane conductance. Previous work with hepatocyte monolayers has shown this to be a reasonable assumption (Burczynski *et al*., 1989). Modelling of palmitate uptake by hepatocyte suspensions also assumes that $P_f \rightarrow \infty$ (Pond *et al*., 1992; Burczynski & Cai, 1994), although this has not been experimentally determined. We attempted to validate this assumption by altering the viscosity of the uptake medium, thereby altering the diffusion coefficient for both protein and ligand. The diffusion-reaction model predicts that decreasing the diffusion coefficient will decrease the total ligand clearance rate if membrane permeability is not rate-limiting. However, if membrane permeability is the rate-limiting step in the overall uptake process than viscosity should have no influence on the rate of palmitate clearance.

The addition of 0.5 mM dextran to 0.23 μM albumin produced no detectable effect on the unbound fraction. However, it did decrease the total palmitate clearance rate by 19%. Trypan blue exclusion measurements, following incubation in the dextran solution for 2 min, indicated that hepatocyte viability was unchanged. Attempts to increase the viscosity further by raising the dextran concentration to 1 mM or by the addition of methycellulose were not successful due to significant hepatocyte aggregation. Conversely, decreasing the dextran concentration to 0.25 mM had no detectable affect on the hepatocyte palmitate uptake rate, although this concentration may have been too low to cause any significant effect on diffusion. Thus, we conclude that the overall uptake of palmitate by hepatocyte suspensions is rate limited by diffusion through the unstirred fluid layer, and that the assumption $P_f \rightarrow \infty$ in equation 4 is valid.

Since the diffusion coefficient is inversely related to viscosity, it is possible that the decreased palmitate clearance rate in the presence of α_1-acid glycoprotein (322 μM), as compared with albumin (0.23 μM), may be the result of the protein concentration affecting viscosity. The Stokes-Einstein approximation to the diffusion coefficient predicts:

$$D_W/D_S = \eta_S/\eta_W \quad (5)$$

where D and η denote the diffusion coefficient and intrinsic viscosity of the protein, respectively; and the subscripts w and s stand for water and protein solution, respectively. The viscosity of protein solutions may be predicted from

$$\eta_s/\eta_w = 1 + \eta_i C + K\eta_i^2 C + \ldots \quad (6)$$

where η_i is the intrinsic viscosity of α_1-acid glycoprotein, which equals 0.069 (CRC, 1976), K is the electrostatic and nonelectrostatic interaction for dissolved protein (which equals 1.9; Tanford & Buzzell, 1956) and C is the concentration of α_1-acid glycoprotein in g dl^{-1}. The higher concentration of α_1-acid glycoprotein was estimated to increase the extracellular fluid viscosity by 10%. Assuming that the increased viscosity produces a 10% reduction in the α_1-acid glycoprotein and palmitate diffusion rates, the predicted clearance rate was expected to decrease by 6%. This value was too small to account for the much larger decrease (51%) in palmitate clearance rate with albumin and α_1-acid glycoprotein. Changes in extracellular fluid viscosity also cannot account for the increased palmitate clearance rate obtained with lysozyme since the change is in the wrong direction. A change in extracellular fluid viscosity, therefore, does not invalidate our conclusion of a facilitated uptake process.

Our conclusion for facilitated uptake was further supported by the experiments in which palmitate clearance rate in the presence of 0.23 μM albumin was compared with that in the combination of 2.1 mM lysozyme plus 0.23 μM albumin. The unbound palmitate fraction is expected to be lower in the presence of the protein combination than with albumin alone. However, the palmitate clearance rate was statistically greater in the presence of lysozyme plus albumin. The additional palmitate presented to the cell surface for uptake may have come from the lysozyme-palmitate complexes.

A curious finding in this study was that the K_a values for the protein-palmitate complexes differed considerably, yet the dissociation rate constants were similar. It is thought that the association process (i.e., k_{on}) for the protein-ligand reaction is diffusion limited, thus, large changes in K_a are expected to result from large changes in k_{off}. However, if the rate-limiting step for protein-palmitate association is not ligand diffusion through the unstirred fluid layer surrounding the protein but, rather, palmitate diffusion through the protein matrix to the binding site as occurs with carbon monoxide binding to myoglobin and haemoglobin (Hasinoff, 1981; Berg, 1985), k_{on} may differ considerably. Thus, since the three proteins differ markedly in their composition and structure, it is possible that they also differ in their k_{on} values.

The diffusion-reaction model rests on the assumption that a facilitation mechanism is absent. While it is true that the model-predicted clearance rate exceeded that observed, the predicted clearance rate is expected to be independent of the protein surface charge. However, experimentally the palmitate clearance rate differed by $\sim$6 fold between α_1-acid glycoprotein-palmitate and the lysozyme-palmitate complexes, by $\sim$3 fold between albumin-palmitate and lysozyme-palmitate complexes, and by $\sim$2 fold between α_1-acid glycoprotein-palmitate and albumin-palmitate complexes. We interpret these results to mean that ionic interactions between protein-bound ligands and the hepatocyte surface facilitate uptake by providing more unbound ligand than would otherwise be available. At physiological pH the negatively charged groups on the membrane surface attract positively charged species from the surrounding fluid to maintain electroneutrality. Proteins carrying a net positive charge (e.g. lysozyme) may thus be attracted to the cell surface thereby decreasing the diffusional distance of the protein-ligand complex and, hence, contribute more ligand to the uptake process. Moreover, since acidic

microenvironments are known to be associated with decreased binding of anthracyclines and long-chain fatty acids to albumin, protein-ligand complexes that enter the hepatocyte microenvironment will have a decreased K_a resulting in more ligand available for uptake (Shiau *et al*., 1990; Rivory *et al*., 1992). Variations in pH are also known to modulate albumin conformation through Neutral-Base, Neutral-Fast transitions (Bos *et al*.,1989; Carter & Ho, 1994) by which ligands may be released from the protein and made available to the cell surface.

Many examples of protein-membrane interactions have been described in the literature (Norde & Lyklema, 1978; Nishida *et al*., 1992). Of particular interest are the studies by Galis *et al*. (1988) and Raicu *et al*. (1991) showing that albumin-fatty acid complexes are preferentially extracted by capillary endothelial cells compared with the delipidated form of albumin. Presumably the binding of fatty acids to albumin promotes ionic interaction(s) between the albumin-fatty acid complex and the cell surface. The effect of protein surface charge on the transcellular protein flux rate across endothelial cells (Smith & Borchardt, 1989), hepatocytes (Wall & Maack, 1985) and proximal tubular cells (Park & Maack, 1984) is well documented, as is its role in capillary permeability (Michel *et al*., 1985). Ghitescu *et al*. (1992) and Wadhwani *et al*. (1992) showed that protein surface charge is an important factor determining the membrane protein permeability properties. Charged groups at the membrane surface also have been shown to affect the activity of membrane-bound enzymes by altering the local substrate and/or product concentration (Wojtczak *et al*., 1988). Moreover, the nature of the charged groups (anionic and cationic), net charge, and charge density are important considerations in elucidating the uptake kinetics of, and cell membrane permeability properties to, macromolecules (Wall & Maack, 1985).

Numerous studies describing ionic interactions between proteins and membrane surfaces, together with the data in this study, lead us to conclude that such interactions facilitate the uptake of protein-bound ligands, either by decreasing the diffusional distance of protein-bound ligands and/or by facilitating the protein-ligand dissociation rate. In any event more unbound ligand is delivered to the cell surface for uptake than would otherwise be expected.

This work was supported by the Canadian Liver Foundation, Manitoba Health Research Council and Medical Research Council of Canada.

References

BASS, L. & POND, S.M. (1988). The puzzle of rates of cellular uptake of protein-bound ligands. In *Pharmacokinetics: Mathematical and Statistical Approaches to Metabolism and Distribution of Chemicals and Drugs*. ed. Pecile A. & Rescigno A., pp. 241–265, London: Plenum Press.

BERG, O.G. (1985). Orientation constraints in diffusion-limited macromolecular association. *Biophys. J.* **47,** 1–14.

BORGSTROM, B. (1952). Investigation on lipid separation methods. Separation of cholesterol esters, glycerides and free fatty acids. *Acta Physiol. Scand.*, **25,** 111–119.

BOS, O.J.M., LABRO, J.F.A, FISCHER, M.J.E., WILTING, J. & JANSSEN L.H.M. (1989). The molecular mechanism of the neutral-to-base transition of human serum albumin. *J. Biol. Chem.*, **264,** 953–959.

BURCZYNSKI, F.J. & CAI, Z.-S. (1994). Palmitate uptake by hepatocyte suspension: effect of albumin. *Am. J. Physiol.*, **268,** G371–G379.

BURCZYNSKI, F.J., CAI, Z.-S., MORAN, J.B. & FORKER, E.L. (1989). Palmitate uptake by cultured hepatocytes: albumin binding and stagnant layer phenomena. *Am. J. Physiol.*, **257,** G584–G593.

BURCZYNSKI, F.J. & LUXON, B.A. (1995). Is there facilitated uptake of fatty acids by the liver? Interpretation and analysis of experimental data. *Can. J. Physiol. Pharmacol.*, **73,** 409–420.

BURCZYNSKI F.J., MORAN, J.B., CAI, Z.-S. & FORKER, E.L. (1990). B-lactoglobulin enhances the uptake of free palmitate by hepatocyte monolayers: The relative importance of diffusion and facilitated dissociation. *Can. J. Physiol. Pharmacol.*, **68,** 201–206.

BURCZYNSKI, F.J., MORAN, J.B., CAI, Z.-S., GEISBUHLER, T.P. & ROVETTO, M.J. (1995). Palmitate uptake by cardiac myocytes and endothelial cells. *Am. J. Physiol.*, **268,** H1659–H1666.

BURCZYNSKI, F.J., POND, S.M., DAVIS, C.K. & WEISIGER, R.A. (1993). Validity of albumin-fatty acid binding constants determined by heptane: water partition. *Am. J. Physiol.*, **265,** G555–G563.

CAI, Z.-S., BURCZYNSKI, F.J., LUXON, B.A. & FORKER, E.L. (1992). On the design and interpretation of experiments to elucidate albumin-dependent hepatic uptake. *Am. J. Physiol., 262,* G1127–G1137.

CARTER, D.C. & HO, J.X. (1994). Structure of serum albumin. *Adv. Protein Chem.*, **45,** 153–203.

CRC Handbook of Biochemistry and Molecular Biology. Proteins, Volume II (1976). Florida: Boca Raton: CRC Press Inc.

GALIS, Z., GHITESCU, L. & SIMIONESCU, M. (1988). Fatty acids bindings to serum albumin increased its uptake and transcytosis by the lung capillary endothelium. *Eur. J. Cell Biol.*, **47,** 358–365.

GHITESCU, L., DESJARDINS, M. & BENDAYAN, M. (1992). Immunocytochemical study of glomerular permeability to anionic, neutral and cationic albumins. *Kid. Int.*, **42,** 25–32.

GORESKY, C.A., PANG, K.S., SCHWAB, A.J., BARKER, F., III., CHERRY, W.F. & BACH, G.G. (1992). Uptake of a protein-bound polar compound, acetaminophen sulfate, by perfused rat liver. *Hepatology*, **16,** 173–190.

HASINOFF, B.B. (1981). Flask photolysis of myoglobin and hemoglobin with carbon monoxide and oxygen at low temperatures. Evidence for a transient diffusion-controlled reaction in supercooled solvents. *J. Phys. Chem.*, **85,** 526–531.

HORIE, T., MIZUMA, T., KASAI, S. & AWAZU, S. (1988). Conformational change in plasma albumin due to interaction with isolated rat hepatocyte. *Am. J. Physiol.*, **254,** G465–G470.

MERCK INDEX. (1993). *The Merck Index*, Rahway, N.J.: Merck & Co., Inc.

MICHEL, C.C., PHILLIPS, M.E. & TURNER, M.R. (1985). The effects of native and modified bovine serum albumin on the permeability of frog mesenteric capillaries. *J. Physiol.*, **360,** 333–346.

MORAN, J.B., BURCZYNSKI, F.J., CHEEK. R.F., BOPP, T. & FORKER, E.L. (1987). Protein binding of palmitate measured by transmembrane diffusion through polyethylene. *Anal. Biochem.*, **167,** 394–399.

NISHIDA, M., OOKUBO, Z., HASHIMURA, Y., IKAWA, A., YOSHIMURA, Y., OOI, K., SUZUKI, T., TOMITA, Y. & KAWADA, J. (1992). Interaction of bovine serum albumin with the surface of a microcrystalline aluminium oxide hydroxide compound: A possible new type of phosphate adsorbent. *J. Pharm. Sci.*, **81,** 828–831.

NORDE, W. & LYKLEMA, J. (1978). The adsorption of human plasma albumin and bovine pancreas ribonuclease at negatively charged polystyrene surfaces. 1. Adsorption isotherms. Effect of charges, ionic strength, and temperature. *J. Colloid Interface Sci.*, **66,** 257–265.

NUNES, R., KIANG, C.-L., SORRENTINO, D. & BERK, P.D. (1988). Albumin-receptor uptake kinetics do not require an intact lobular architecture and are not specific for albumin. *J. Hepatology*, **7,** 293–304.

PARK, C.H. & MAACK, T. (1984). Albumin absorption and catabolism by isolated perfused proximal convoluted tubules of the rabbit. *J. Clin. Invest.*, **73,** 767–777.

PATIL, G.S., MATTHEWS, R.H. & CORNWELL, D.G. (1973). Kinetics of the processes of desorption from fatty acid monolayers. *J. Lipid Res.*, **14,** 26–31.

PEDERSEN, J.B., PEDERSEN, S.M. & LINDUP, W.E. (1990). Bovine albumin-HABA interaction: re-analysis of earlier observations indicates that ligand-induced dimerization and a competitive contaminant operate simultaneously. *Biochem. Pharmacol.*, **39,** 1138 – 1141.

POND, S.M., DAVIS, C.K., BOGOYEVITCH, M.A., GORDON, R.A., WEISIGER, R.A. & BASS, L. (1992). Uptake of palmitate by hepatocyte suspensions: Facilitation by albumin. *Am. J. Physiol.*, **262,** G883 – G894.

RAICU, M., ALEXANDRU, D., FIXMAN, A. & SIMIONESCU, N. (1991). Albumin binding sites are expressed on the abluminal plasma membrane of capillary endothelium. *J. Submicrosc. Cytol. Pathol.*, **23,** 1 – 8.

REED, R.G. & BURRINGTON, C.M. (1989). The albumin receptor effect may be due to a surface-induced conformational change in albumin. *J. Biol. Chem.*, **264,** 9867 – 9872.

RIVORY, L.P., POND, S.M. & WINZOR, D.J. (1992). The influence of pH on the interaction of lipophilic anthracyclines with bovine serum albumin. *Biochem. Pharmacol.*, **44,** 2347 – 2355.

SHIAU, Y.-F., KELEMEN, R.J. & REED, M.A. (1990). Acidic mucin layer facilitates micelle dissociation and fatty acid diffusion. *Am. J. Physiol.*, **259,** G671 – G675.

SMITH, K.R. & BORCHARDT, R.T. (1989). Permeability and mechanism of albumin, cationized albumin, and glycosylated albumin transcellular transport across monolayers of cultured bovine brain capillary endothelial cells. *Pharmaceut. Res.*, **6,** 466 – 473.

SOPHIANOPOULOS, A.J., RHODES, C.K., HOLCOMB, D.N. & VAN HOLDE, K.E. (1962). Physical studies of lysozyme. I. Characterization. *J. Biol. Chem.*, **237,** 1107 – 1112.

SORRENTINO, D., ROBINSON, R.B., KIANG, C.-L. & BERK, P.D. (1989). At physiologic albumin/oleate concentrations oleate uptake by hepatocytes, cardiac myocytes and adipocytes is a saturable function of the unbound oleate concentration. Uptake kinetics are consistent with the conventional theory. *J. Clin. Invest.*, **84,** 1325 – 1333.

SRIVASTAVA, D.K. & BERNHARD, S.A. (1986). Metabolic transfer via enzyme-enzyme complexes. *Science*, **234,** 1081 – 1086.

TANFORD, C. & BUZZELL, J.G. (1956). The viscosity of aqueous solutions of bovine serum albumin between pH 4.3 and 10.5. *J. Phys. Chem.*, **60,** 225 – 231.

WADHWANI, K.C., SHIMON-HOPHY, M. & RAPOPORT, S.I. (1992). Enhanced permeabilities of cationized-bovine serum albumin at the blood-nerve and blood-brain barriers in awake rats. *J. Neurosci. Res.*, **32,** 407 – 414.

WALL, D.A. & MAACK, T. (1985). Endocytic uptake, transport, and catabolism of proteins by epithelial cells. *Am. J. Physiol.*, **248,** C17 – C20.

WEISIGER, R.A. & MA, W. (1987). Uptake of oleate from albumin solutions by rat liver. Failure to detect catalysis of the dissociation of oleate from albumin by an albumin receptor. *J. Clin. Invest.*, **79,** 1070 – 1077.

WEISIGER, R.A., POND, S.M. & BASS, L. (1989). Albumin enhances unidirectional fluxes of fatty acid across a lipid-water interface: theory and experiments. *Am. J. Physiol.*, **257,** G904 – G916.

WOJTCZAK, L., ADAMS, V. & BRDICZKA, D. (1988). Effect of oleate on the apparent K_m of monoamine oxidase and the amount of membrane-bound hexokinase in isolated rat hepatocytes: Further evidence for the controlling role of the surface charge in hexokinase binding. *Mol. Cell. Biochem.*, **79,** 25 – 30.

(Received June 4, 1996
Revised October 3, 1996
Accepted December 13, 1996)