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Relationship between pinocytic rate and uptake of transferrin by suspended rat hepatocytes

John R. Rudolph and Erwin Regoeczi

Department of Pathology, Room 4N66, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada L8N 3Z5

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Summary. The aim of this study was to compare quantitatively the capacity to transcytose (i.e. to uptake and release) transferrin (Tf) with the pinocytic activity of suspended adult rat hepatocytes. An oligodisperse preparation of ^{131}I-polyvinylpyrrolidone (PVP; M_r 36000) was used to measure the inward and outward aspects of the pinocytic process in separate experiments. Cell association of rat ^{125}I-Tf was measured at Tf concentrations approaching physiological, where ^{59}Fe uptake obeyed first-order kinetics. Release studies with both PVP and Tf were carried out under conditions which minimized the probability of *de novo* endocytosis of a molecule already released. Sets of experimental points representing cell-associated radioactivities were converted into continuous algebraic functions by fitting with two-term (release studies) or three-term (uptake studies) exponential equations. Transport of PVP and Tf through the cells was computed from these equations by deconvolution. This analysis showed that, under the present experimental conditions, the fractional transcytosis rates of Tf and PVP by hepatocytes were in the ratio of 1:0.77. These values imply that, in the physiological range of Tf concentrations, about 75% of the Fe taken up by hepatocytes may be due to a pinocytic mechanism (fluid-phase or mixed). Inclusion of chloroquine (1 mM) in the suspending medium, both in uptake and release experiments, resulted in more PVP and Tf passing through the cells, while Fe uptake was reduced. It is suggested that the base probably exerted its enhancing effect on transcytosis by shunting the subcellular transport of PVP and Tf to the outward leg through a shorter circuit.

Key words: Chloroquine - Hepatic iron metabolism - Pinocytosis - Polyvinylpyrrolidone - Transferrin

Offprint requests to: E. Regoeczi

Abbreviations. BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MEM, minimal essential medium; PVP, polyvinylpyrrolidone; Tf, transferrin

Introduction

Transferrin (Tf), the principal iron-binding plasma protein of the body, plays a key role in supplying iron to all eukaryotic cells (for a review see Bomford and Munro 1985). In most tissues, and especially in the erythron, cellular iron uptake proceeds overwhelmingly through transferrin receptors (for a review see Ward 1987). However, as pointed out previously by several investigators (Sibille et al. 1982; Cole and Glass 1983; Thorstensen and Romslo 1984; Page et al. 1984; Trinder et al. 1986; Holmes and Morgan 1989), the situation with the liver is different. On the one hand, hepatocytes, like erythroid cells, are endowed with high-affinity Tf receptors, detectable both immunochemically (Rudolph et al. 1988) and by kinetic studies of Tf binding (Young and Aisen 1981). On the other hand, these cells continue to take up iron in increasing quantities well beyond the saturation point of their Tf-receptor complement. From the concentration of diferric Tf prevailing in normal rat plasma, Rudolph (1988) estimated that the rat liver operates in vivo under conditions of Tf-receptor supersaturation.

The behaviour described above is clearly incompatible with receptor-mediated endocytosis as the exclusive mechanism by which iron bound to Tf gains access to hepatocytes. Therefore, additional routes, such as adsorptive pinocytosis via low-affinity binding sites and fluid-phase pinocytosis, have been postulated (Baker et al. 1985). Also, the possibility of reductive iron release from Tf in the vicinity of the hepatocyte's plasmalemma has been considered in several recent publications (reviewed by Thorstensen and Romslo 1990). In a quantitative sense, the relative contributions of the additional uptake mechanisms nevertheless remain obscure. This holds true particularly for fluid-phase pinocytosis, for which estimates range from a mere 5% (Page et al. 1984) to near exclusiveness (Sibille et al. 1982).

Prompted by this discrepancy, we thought that a fresh look at the quantitative relationship between rates of Tf transcytosis and pinocytic activity of hepatocytes

was warranted. A comparison of both parameters has been undertaken before by Page et al. (1984) on cultured hepatocytes using appropriate markers. However, these authors did not take into account a prominent feature of the pinocytic process, namely, that cells rapidly regurgitate a large portion of internalized material (Besterman et al. 1981; Swanson et al. 1985; Scharschmidt et al. 1986).

Our aim in the present study was to obtain data which would make possible the computation of the transcytotic movements in suspended hepatocytes of homologous Tf and the macromolecular pinocytosis marker polyvinylpyrrolidone (PVP). To this end, the uptake and release of both molecules were measured in parallel experiments using portions of the same cell preparations. The effect on these processes of chloroquine, an agent that depletes cell-surface receptors (Dean et al. 1984; Mellman et al. 1986), was also examined. Alternatively, human Tf, a ligand possessing superior binding affinity for the rat Tf receptor (Rudolph et al. 1986), was used to suppress receptor-mediated endocytosis of rat Tf in some experiments. From these measurements, an integrated picture of Tf and PVP transport through hepatocytes was derived by deconvolution, a mathematical procedure widely used in chemical engineering.

Materials and methods

Materials. Collagenase (type IV) and Hepes were obtained from Sigma. Minimum essential medium (MEM), sialidase (ex. *Vibrio cholerae*) and fetuin were from Gibco. Radioactive tracers were from New England Nuclear (Boston) and gel-filtration media from Pharmacia (Uppsala, Sweden). Bovine serum albumin (BSA) was from Boehringer (Mannheim, FRG), Desferal (deferoxamine mesylate) from Ciba and chloroquine phosphate from Sterling Drug Ltd (Aurora, ON). All chemicals used were of analytical grade, where available. A special narrow polymer range batch of PVP, with a mass-average M_r of 36000, was a gift from Dr W. Scholtan (Bayer AG, Leverkusen, FRG).

Proteins. Human and rat Tf were prepared as described elsewhere (Rudolph et al. 1986). Prior to use, they were converted to the diferric form. Fetuin (100 mg in 2 ml 0.1 M sodium acetate pH 6.0 containing 1 mM $CaCl_2$ and 0.1% sodium azide) was desialylated by incubation in dialysis tubing with sialidase (500 units) at 37° C for 20–24 h. The outer compartment, containing 100 ml of the above solution, was changed 1 h and 2 h after beginning the incubation. Asialofetuin was separated from the enzyme by anion-exchange chromatography (Regoeczi et al. 1978).

Isolation of hepatocytes. Adult female rats of the Sprague-Dawley strain served as liver donors. Freshly isolated hepatocytes were obtained by perfusion with collagenase as before (Rudolph et al. 1988). Viability of the cells, assessed by the exclusion of trypan blue, was better than 90%. Contamination with nonparenchymal cells was less than 2%.

Uptake studies. Hepatocytes ($8–12 \times 10^6$/ml), suspended in the presence of 1% BSA in either MEM or Hank's balanced salt solution (HBSS) to a volume of 3.4–5 ml, were warmed in 20-ml glass scintillation vials at 37° C for 10 min before beginning an experiment or preincubating with various compounds. After adding the test proteins (^{125}I-Tf, ^{59}Fe-Tf, ^{125}I-asialofetuin) or ^{131}I-PVP, referred to as the zero time in Results, cells were maintained in suspension at 37° C by shaking at 100 strokes/min in a shaking water bath. Duplicate or triplicate samples (200 µl) were taken at intervals, layered on top of 200 µl dibutyl phthalate contained in a 500-µl tube, and centrifuged in an Eppendorf microcentrifuge for 10 s. The tips of the tubes, containing the cell pellets, were removed to measure cell-associated radioactivities. Portions (100 µl) of the medium that remained above the oil were tested for acid-soluble products of proteolysis after incubation with 100 µl 20% trichloroacetic acid for 18 h at 4° C.

Release studies. Cells were incubated with radioligands as described above. After 15 or 45 min, the ligand still present in the medium was removed as follows. Ice-cold MEM or HBSS, containing 1% BSA, was added to a final volume of 30 ml, followed by immediate centrifugation at 100 *g* for 1 min in the cold. The cells were then resuspended in 30 ml cold medium and the procedure was repeated twice. The cells were finally resuspended to their original volume and reincubated for up to 15 min at 37° C. Samples (200 µl) were taken during reincubation at intervals in duplicate or triplicate for the determination of released radioactivity by microcentrifugation as outlined under *Uptake studies*. In order to minimize *de novo* internalization of radiotracers that had already passed through the cells, suspensions were sedimented at 100 *g* for 10 s after each sampling, followed by resuspension in a volume of fresh, prewarmed medium so that the cell concentration remained approximately constant.

Other techniques. Protein iodinations were catalyzed by tetrachloroglycoluril according to specifications given elsewhere (Rudolph et al. 1986). PVP (5 mg) was iodinated by using a published technique (Regoeczi 1976), except that the unbound radioactivity was removed by dialysis against phosphate-buffered saline. Saturation of Tf with iron and tagging with ^{59}Fe were accomplished as before (Rudolph and Regoeczi 1988). Radioactivities were assayed in a Packard model 5986 multichannel analyser. The possibility of chloroquine binding to PVP was examined as follows. A column of Sephadex G-100 and a sample of PVP (5 mg) were equilibrated with phosphate buffer pH 7.4 containing 10 µg/ml of chloroquine, a concentration giving $A = 0.20$ at the absorption maximum, 341 nm. After loading the sample on the column, the absorbance of the effluent was monitored in a Beckman DU-40 spectrophotometer, the expectation being that any chloroquine adsorbed on PVP would give rise to a 'blip' in the baseline coinciding with the eluting polymer. No chloroquine binding by PVP was detected by this method.

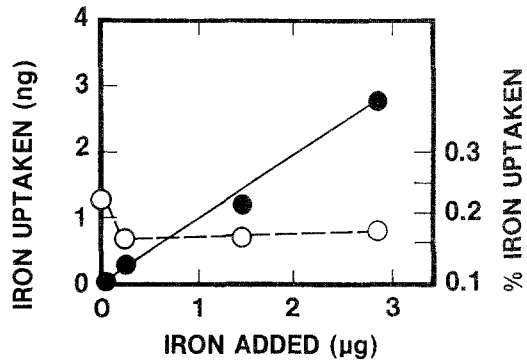

Fig. 1. Concentration dependence of iron uptake from 2Fe-Tf by suspended hepatocytes. Points are means of duplicate samples presented both as absolute values (●—●) and percentages (○- - -○) of the total Fe available. Hepatocytes (10^7/ml) were incubated for 45 min at 37° C with 2–1000 µg/ml of 2Fe-Tf in a total volume of 2 ml. Cell-associated ^{59}Fe was determined in 0.2-ml samples. Assuming 48000 Tf receptors on the surface of each hepatocyte (Rudolph et al. 1988), the Tf/receptor ratio was 30:1 at the lowest concentration tested, the corresponding value for the highest concentration being 15000:1

Analysis of uptake and release data. Each experiment described below (except the one in Fig. 1) was carried out on two or three preparations of hepatocytes with points measured in duplicate or triplicate. Preliminary processing of this raw data was undertaken with the IBM PC version of the statistical programme 'Minitab' (Ryan et al. 1985). Sets of mean values thus obtained were converted into continuous algebraic functions by exponential fitting. Based on the computer routine of Nelder and Mead (1965), the programme fitted a three-term exponential equation to the experimental values derived from uptake and a two-term one to those from release studies. Functions of this kind fitted to observed values need not have any interpretation in terms of a biological model. Fractions of PVP and Tf passing through the cells as a function of time were computed from these exponential equations by deconvolution (Shipley and Clarke 1972). In essence, the procedure calculates from the intracellular radioactivity levels, in conjunction with the release kinetics of the same substance as determined in a separate experiment, the total input into a system (the total number of tracer molecules taken up by cells) as if the system were closed, i.e. no release had taken place. The results are valid regardless of the nature of the underlying input and output processes at a molecular or cellular level.

Results

Preliminary experiments were conducted to decide if our hepatocyte preparations exhibited a relationship between iron uptake and Tf concentration similar to that reported by others (Sibille et al. 1982; Cole and Glass 1983). As seen in Fig. 1, the mass relationship was linear in such a way that the fractional uptake rate remained unchanged. The small deviation in fractional uptake rate observed at the smallest ligand/receptor ratio (30:1) is thought to have been due to preferential binding of Tf by high-affinity Tf receptor. These results agree with those of the authors cited above and show that acquisition of Fe by hepatocytes obeys first-order kinetics whenever the concentration of Tf in the medium exceeds a certain level.

Uptake and release of PVP by suspended hepatocytes

Due to its pronounced hydrogen-bonding capacity (De Duve et al. 1974), PVP may be expected to be present in the pericellular aqueous shell that accompanies cells during their passage through the hydrophobic dibutyl phthalate when separating endocytosed molecules from those in the medium by microcentrifugation. Therefore in order to distinguish between pinocytosed PVP and PVP held in the aqueous shell surrounding the plasmalemma, preliminary experiments were carried out with a combination of metabolic poisons which block pinocytosis (Ose et al. 1980) as well as receptor-mediated uptake of asialofetuin (Fig. 2). Under the conditions given in Fig. 2, 2.2% of PVP added to the medium centrifuged with the cells through the hydrophobic layer. This fraction was therefore considered as being trapped in the aqueous shell and all subsequent measurements were corrected correspondingly.

Final data on PVP uptake with or without 1 mM chloroquine in the medium are summarized in Fig. 3. Cell association of PVP was biphasic under both conditions with a rapid phase lasting for approximately 6–8 min, followed by a slower rise up to 60 min. Significantly more PVP became cell-associated in the presence of chloroquine than in its absence. Release of some 60–70% of cell-associated PVP took place rapidly irrespective of whether chloroquine was used (Fig. 4). However, the discharge then levelled off in control experiments, whereas it continued to a lower level with the drug present. The viability of chloroquine-treated cells was over 90% at the end of these experiments, as well as for those described in the section below.

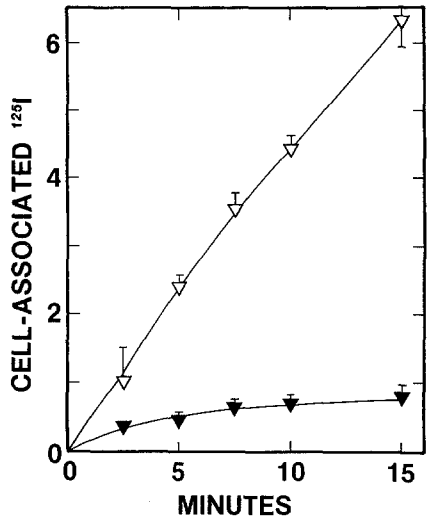

Fig. 2. Uptake of ^{125}I-asialofetuin (2 μg/ml) by suspended hepatocytes (10^7/ml) without (▽) or with (▼) 6 mM KF and 10 mM NaCN in the medium. Results are expressed as cell-associated ^{125}I (μg/10^8 cells). Points are means ±SE of four measurements

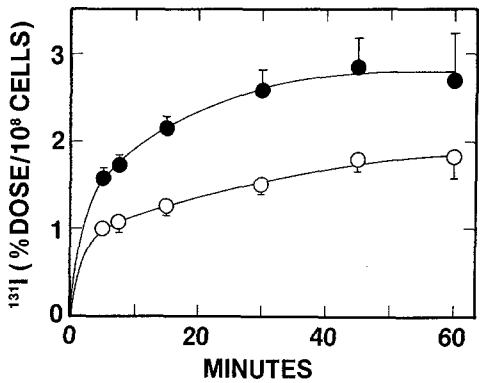

Fig. 3. Uptake of ^{131}I-PVP (200–250 μg/ml) by suspended hepatocytes (8–12 × 10^6/ml) in the absence (○) or presence (●) of 1 mM chloroquine. Incubation and subsequent separation of cell-bound and free ^{131}I were carried out as outlined in Materials and Methods. Points are means ±SE of measurements in triplicate from three experiments. Curves were fitted by computer using three exponential terms

Uptake and release of rat Tf by suspended hepatocytes

Experiments pertaining to uptake are summarized in Fig. 5. This study was conducted in the presence of a

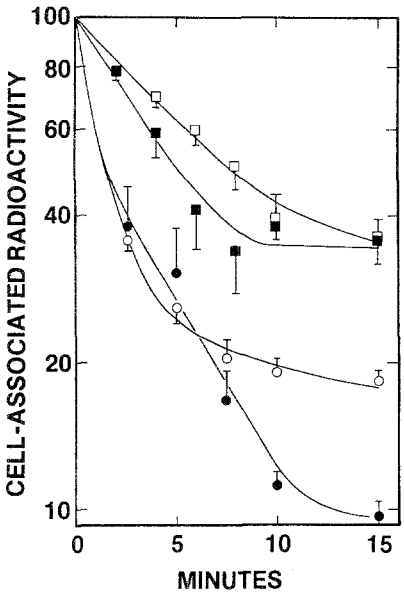

Fig. 4. Release of ^{131}I-PVP (○, ●) and ^{125}I-Tf (□, ■) from suspended hepatocytes in the absence (○, □) or presence (●, ■) of 1 mM chloroquine. Release studies were performed as outlined in Materials and Methods. Results are expressed as percentages of cell-associated ^{131}I and ^{125}I at the time commencing release. Preincubation with ^{131}I-PVP lasted 15 min and with ^{125}I-Tf 45 min. Ligand concentrations and numbers of experiments are given for ^{131}I-PVP in Fig. 3 and for ^{125}I-Tf in Fig. 5. Points are means ± SE. Curves were fitted by computer using two exponential terms

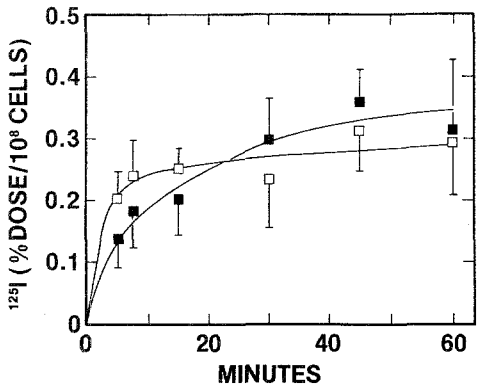

Fig. 5. Association of rat diferric ^{125}I-Tf (2 μg/ml) with suspended hepatocytes (8–12 × 10^6/ml) in the absence (□) and presence (■) of 1 mM chloroquine. Experiments were carried out as outlined in Materials and Methods, except that the medium also contained 1.55 mg/ml of unlabeled human 2Fe-Tf. Points are means ± SE of measurements in triplicate from two experiments. Curves were fitted by computer using three exponential terms

775-fold excess of human Tf in the medium which, as shown elsewhere (Rudolph et al. 1986), prevents binding of homologous Tf by rat Tf receptors nearly quantitatively. Yet the hepatocytes did acquire rat ^{125}I-Tf. When the medium contained chloroquine, accumulation of ^{125}I-Tf initially lagged behind, but it reached the level of control cells by 20 min and continued rising slowly over the control values thereafter. Of the ^{125}I activity prescent in the medium after 1 h of incubation, less than 0.2% was found to be soluble in trichloroacetic acid.

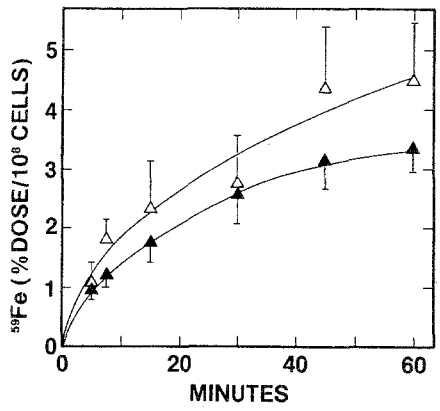

Fig. 6. Uptake of ^{59}Fe by suspended hepatocytes in the absence (△) and presence (▲) of 1 mM chloroquine. Cells (8–12 × 10^6/ml) were incubated with rat diferric ^{59}Fe-Tf (100 μg/ml) as outlined in Materials and Methods. Points are means ± SE of measurements in triplicate from two experiments. Curves were fitted by computer using three exponential terms

As is apparent from Fig. 4, close to 65% of the cell-associated Tf was released from hepatocytes over 15 min. Release occurred initially faster (half-life 5 min) when the medium contained 1 mM chloroquine than when it did not (half-life 8 min).

Curves representing iron uptake at a calculated ratio of Tf/exposed receptor of 1500:1 are presented in Fig. 6. It should be noted that hepatocytes accumulated, as expected, less ^{59}Fe from rat Tf when the medium contained 1 mM chloroquine. Measurement of iron release from hepatocytes was also attempted in complementary experiments. However, the data proved unreliable from a technical point of view because of the low levels of ^{59}Fe activity involved and therefore they were omitted.

Deconvolution analysis

Following conversion of the points obtained in uptake and release experiments into continuous algebraic functions, the resulting curves were deconvoluted using a standard mathematical procedure. As already explained under Materials and Methods, this treatment compensates uptake curves for losses due to concurrent partial release (or regurgitation) of endocytosed molecules, and thus provides quantitative information about the cumulative amounts of a probe that pass through the cells as a function of time. The graphical illustration of the outcome of this analysis is presented in Fig. 7. Apart from inflexions at the beginning, expressing initial conditions, the slope of such curves is expected to be linear whenever a system is in a steady state. Both the PVP and Tf curves have met this expectation in the studies not involving chloroquine. These curves show that hepatocytes transcytosed more Tf than PVP. The regression coefficients of the linear sections of these

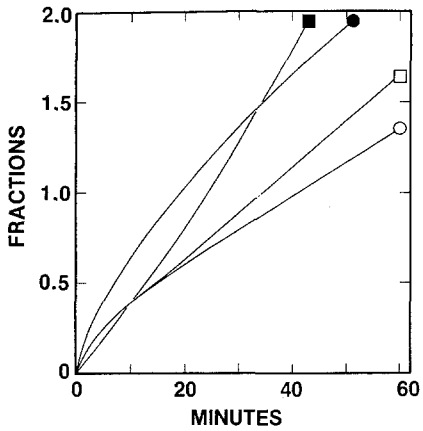

Fig. 7. Estimation by deconvolution of the cumulative amounts of ^{131}I-PVP (○, ●) and ^{125}I-Tf (□, ■) transcytosed by suspended hepatocytes as a function of incubation time. The principle of the method is outlined under Materials and Methods. Uptake data used for computation are the same as those in Figs. 3 (PVP) and 5 (Tf). Release values were derived from Fig. 4. Curves are normalized to 10^8 cells/ml. The ordinate is scaled in fractions of PVP or Tf in the medium, unity being the starting condition at zero time. (○, □) Studies without and (●, ■) studies with 1 mM chloroquine in the suspending buffer. For further explanations see the text

curves conformed to a ratio of Tf/PVP = 1:0.77. Assuming that endocytosed Tf surrenders both iron atoms during a single passage, newly 80% of the iron acquired by the hepatocyte from Tf could therefore be accounted for by a pinocytic process.

Deconvoluted curves of the experiments involving chloroquine were not entirely linear (Fig. 7). It is not clear whether this irregularity was due to a lack of steady state brought about by the compound or, perhaps, to our inability to secure better experimental values for subsequent computation. Be that as it may, the position of the curves leave no doubt that hepatocytes incubated with chloroquine transcytosed both PVP and Tf at elevated fractional rates. A rough estimate of the magnitudes involved here was obtained by fitting linear slopes to the curves and comparing their values with those from the control studies. According to this approximation, the transcytosis rates of Tf and PVP were 76% and 54% higher, respectively with chloroqine than without.

Discussion

For reasons explained in the Introduction, the prime aim of this study was to compare quantitatively the rate of pinocytosis and the transcytotic rate of Tf in suspended hepatocytes. The cells used acquired iron from Tf according to first-order kinetics (Fig. 1), thus attesting to their suitability for the purpose of the study. The computed rates of transcellular passage for Tf and PVP were in the ratio 1.0:0.77, the implication being that close to 80% of Tf entering the hepatocytes may do so by a pinocytic mechanism (see below). Our results therefore support the conclusion of Sibille et al. (1982) reached in studies on cultured hepatocytes.

A characteristic feature of fluid-phase pinocytosis is the kinetic compartmentation of the process, giving rise to rapidly and slowly recycling components (Besterman et al. 1981; Besterman and Low 1983). As seen in Fig. 4, the release of both Tf and PVP followed a kinetic pattern that was more complex than single-exponential. This is in keeping with the uptake mechanism suggested above. Albumin (Hoffenberg et al. 1970) and low-density lipoprotein in the receptor-independent pathway (Spady et al. 1985) are other examples of plasma protein uptake by the liver via pinocytosis.

Acidification of the endosomes (receptosomes) formed during receptor-mediated endocytosis is an essential prerequisite (Rao et al. 1983), even though not the sole one (Thorstensen and Romslo 1990), for the release of Fe from Tf. In order to be effective in protein-to-cell iron transfer, pinosomes, by inference, must possess a similar biochemical machinery. Indeed, as far as ATP-dependent acidification is concerned, both types of vesicles seem functionally equivalent (Mellman et al. 1986). Other putative components of the system (redox activity, low-molecular-mass iron binder, etc.) remain poorly understood in either pathway for the time being.

It is customary to distinguish several forms of pinocytosis, notably fluid-phase, adsorptive, and a combination of both (so-called 'mixed type') (Jacques 1973). In practice, identification of the pure forms is often difficult and, perhaps, arbitrary. For example, the endocytic index of PVP (reputedly a fluid-phase marker) varies for a given cell type with polymer size, and different cell types exhibit antagonistic preferences with respect to size-related uptake rates (Duncan et al. 1981). Thus pinocytosis of PVP, at least in some experimental arrangements, may well include an adsorptive component, however weak that might be. Similarly, pinocytosis of Tf by hepatocytes is likely to be of a mixed type. This preliminary conclusion bears out the observation that the hepatocyte plasmalemma contains several components with affinity for Tf, in addition to Tf receptor (Hu and Regoeczi, unpublished results).

Our original reason for using chloroquine was to reduce cell-surface receptors by segregating them in an internal pool (Tolleshaug and Berg 1979; Schwartz et al. 1984; Zijderhand-Bleekemolen et al. 1987). The marked promoting influence of this diamine on transcytosis of both PVP and Tf was unexpected. The phenomenon is probably best understood in conjunction with other known effects of the compound on the intracellular transport of macromolecules. Chloroquine inhibits the conversion of multivesicular bodies to secondary lysosomes (Hornick et al. 1984), thereby causing the regurgitation of intact epidermal growth factor (Wakshull et al. 1985). Although internalization of surface receptors is not inhibited *per se* (Knutson et al. 1985; Wakshull et al. 1985), endocytosis of ferritin is impeded because of an increase in endosomal volume density (Stenseth and Thyberg 1989). Molecules of endogenous origin are also channelled by the drug into

the secretory pathway. For example, fibroblasts secrete newly synthesized lysosomal enzymes, rather than transport them to lysosomes (Hasilik and Neufeld 1980), and pituitary cells discharge newly synthesized adrenocorticotropin, instead of storing it in secretory granules (Moore et al. 1983). Based on the above, chloroquine may be perceived as an agent which inhibits vesicular traffic directed toward the depth of the cell, on the one hand, and promotes exocytosis (discharge) on the other. By so doing, the compound essentially shunts vesicular traffic to a shorter circuit in the periphery of the cell.

Several other findings support the notion that shunting of pinosomes could give rise to an increased turnover of their contents. First, pinosomes can be resolved into an early and a later population (Casey et al. 1986). The early population is of low buoyance, is highly exocytic and is the precursor of the later, buoyant pinosomes. If chloroquine reduced, as postulated, the formation of later pinosomes, larger fractions of the endocytosed material would be available for accelerated discharge from early pinosomes. The release curves of Tf and PVP in Fig. 4 support this argument. On the other hand, the content of those later pinosomes which still form (albeit in lesser numbers) would be expected to sequester in the cell interior, as receptors do (see above). This is a likely explanation of the higher cell-associated radioactivity content of the treated cells seen in Fig. 3 for PVP and in Fig. 5 for Tf. Second, the importance of the cell periphery in understanding these events is underlined by electron microscopic studies (Townsend et al. 1984), showing that internalized ligands which are destined to be released intact are predominantly localized in vesicles in the peripheral cytoplasm of hepatocytes.

The concentration of chloroquine used in this study was some 100-fold higher than that considered effective (Dean et al. 1984). Yet the uptake of iron was only reduced, not suspended (Fig. 6). This suggests that factors other than a decrease in pH play a role in the release of iron from Tf. Furthermore, if pinosome shunting is a reality, this release, or at least a sizeable portion of it, must take place in the peripheral aspect of hepatocytes, perhaps in vesicles which never move far from the plasmalemma. Similar conclusions were reached in a recent review by Thorstensen and Romslo (1990).

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