Hyperosmolarity Inhibits Galactosyl Receptor-mediated but Not Fluid Phase Endocytosis in Isolated Rat Hepatocytes*

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We have investigated the effects of hyperosmolarity induced by sucrose on the fluid phase endocytosis of the fluorescent dye lucifer yellow CH (LY) and the endocytosis of 125I-asialo-orosomucoid (ASOR) by the galactosyl receptor system in isolated rat hepatocytes. Continuous uptake of LY by cells at 37 °C is biphasic, occurs for 3–4 h, and then plateaus. Permeabilized cells or crude membranes do not bind LY at 4 or 39 °C. Intact cells also do not accumulate LY at 4 °C. The rate and extent of LY accumulation are concentration- and energy-dependent, and internalized LY is released from permeabilized cells. Efflux of internalized LY from washed cells is also biphasic and occurs with half-times of approximately 38 and 82 min. LY is taken up into vesicles throughout the cytoplasm and the perinuclear region with a distribution pattern typical of the endocytic pathway. LY, therefore, behaves as a fluid phase marker in hepatocytes. LY has no effect on the uptake of 125I-ASOR at 37 °C. The rate of LY uptake by cells in suspension is not affected for at least 30 min by up to 0.2 M sucrose. The rate of endocytosis of 125I-ASOR, however, is progressively inhibited by increasing the osmolality of the medium with sucrose (>98% with 0.2 M sucrose; Oka and Weigel (1988) J. Cell. Biochem. 36, 169–183). Hyperosmolarity completely inhibits endocytosis of 125I-ASOR by the galactosyl receptor, whereas fluid phase endocytosis of LY is unaffected. Cultured hepatocytes contained about 100 coated pits/mm of apical membrane length as assayed by transmission electron microscopy. In the presence of 0.4 M sucrose, only 17 coated pits/mm of membrane were observed, an 83% decrease. Only a few percent of the total cellular fluid phase uptake in hepatocytes is due to the coated pit endocytic pathway. We conclude that the fluid phase and receptor-mediated endocytosis processes must operate via two separate pathways.

Many inhibitors have been used as perturbants to dissect the multistep pathways of receptor-mediated endocytosis and receptor recycling (Besterman and Low, 1983; Schwartz, 1984; Goldstein et al., 1985; Weigel, 1987). Agents such as monensin, lysosomotropic amines, cytoskeletal drugs, and metabolic energy poisons have allowed investigators to define and affect discrete processing steps in this pathway. We recently found that hyperosmolarity induced by increasing concentrations of sucrose, sodium chloride, or other osmolites blocks the dissociation of internalized receptor-ligand complexes and completely stops receptor recycling (Oka and Weigel, 1988). In the presence of sucrose, the internalization of surface-bound ligand was also incomplete. Not all the receptor-ligand complexes on the cell surface could be internalized by cells under hyperosmotic conditions. Heusser and Anderson (1987) also found that exposure of fibroblasts to high concentrations of sucrose effectively stopped the continuous internalization of low density lipoprotein. These investigators concluded that the coated pit pathway involving the recycling of clathrin and the formation of coated pits was inhibited under hyperosmolar conditions. In earlier studies, Daukas and Zigmond (1985) concluded that hyperosmolarity decreased the endocytosis of chemotactic peptide in polymorphonuclear leukocytes. Even though peptide uptake was decreased, however, the cells were still able to undergo fluid phase endocytosis as judged by their ability to continue to internalize [125I]sucrose in the presence of high concentrations of sodium chloride. This observation has not been followed up by other investigators.

The prevailing understanding in the literature is that fluid phase endocytosis is probably accounted for by the receptor-mediated clathrin-coated pit pathway (Marsh and Helenius, 1980; Steinman et al., 1983). That is, all the fluid uptake in a cell could be due to the coincident volume internalized via the coated pit pathway. According to this notion, cells essentially possess only one mechanism by which vesicles and fluid are internalized. This view of fluid phase endocytosis is based primarily on studies performed with fibroblasts. Recent results from a number of investigators examining different receptors including those for asialoglycoproteins (Oka and Weigel, 1983; Weigel et al., 1986), insulin (Smith et al., 1987; McClain and Olesiak, 1988), transferrin (Stein and Susman, 1986), chemotactic peptide (Zigmond and Tranquillo, 1986), and low density lipoprotein (Edge et al., 1986) indicate that receptor-bound ligands are endocytosed and processed by multiple cellular pathways not just a single pathway (Weigel, 1987). The question of whether these pathways all involve coated pits needs to be reexamined. For example, insulin uptake in hepatocytes has been shown to occur by two separate pathways, one of which involves a coated pit pathway and the other which does not involve coated pits (McClain and Olesiak, 1988; Smith et al., 1987). Similarly, we have documented over the last several years the existence of two separate ligand-processing and receptor-recycling pathways in the galactosyl receptor system in isolated rat hepatocytes (Weigel, 1987). In this case, it is not yet known whether these are both coated pit pathways. The major galactosyl receptor pathway, which has been studied by other investigators as well, is a coated pit pathway (Wall et al., 1986). The purpose of the present study was to determine whether the receptor-mediated coated pit pathway in hep-
tocytes solely accounts for the fluid phase uptake capacity of these cells or if there is more than one pathway by which fluid volume can be taken up. The results indicate that hepatocytes take up fluid in a fluid phase pathway and to a far greater rate than fluid is taken up by any coated pit pathway.

**Materials**—Human orosomucoid from Sigma was desialylated and iodinated as described before (Weigel and Oka, 1982). NaCl (10-20 mCi/μg of iodine) was from Amersham Corp. Digitonin from Sigma or Eastman Kodak was dissolved to 25% (w/v) in dimethyl sulfoxide or to 1.4% in 100% ethanol with warming. Lucifer yellow CH (lithium salt) from Sigma was dissolved in medium 1/BSA, usually at a stock concentration of 1 mg/ml, and filtered (0.2-μm pore size). BSA was from Armour (CRG-7) or Sigma (fraction V). Collagenase was from Sigma (type IA), Serva Fine Biochemicals Inc. (17449), or Boehringer Mannheim (type D). Medium 1/BSA contains a modified Eagle’s medium (GIBCO) supplemented with 2.4 g/liter Hepes, pH 7.4, 0.22 g/liter NaCl, and 0.1% BSA.

**Cell Preparation**—Hepatocytes were prepared from male Sprague-Dawley rats (~250 g) by a modification (Clarke et al., 1987) of the collagenase liver perfusion procedure of Seglen (1976). Final cell pellets were suspended in medium 1/BSA. The cells were routinely 85-90% single cells and viable as judged by trypan blue exclusion. Viability by trypsin blue was not compromised by 0.25 m sucrose for cells in suspension or 0.4 m for cells in culture. Experiments were performed in the absence of serum; 0.1% BSA was present for most experiments. Cells were cultured in William’s E medium as described previously (Oka and Weigel, 1987). For experiments with cells in suspension, the cells were first incubated at 37 °C for 60 min to increase and stabilize the number of surface receptors (Weigel and Oka, 1983). Cell suspensions in different experiments were incubated in 50-250 ml Erlenmeyer flasks at 2-3 °C accumulated virtually no LY, nor did cells that had first been permeabilized with digitonin and then incubated at either 4 or 37 °C. LY does not bind significantly to membranes either in live cells at 4 °C or in permeable cells at 4 or 37 °C.

**Osmolarity**—Sucrose was dissolved to a concentration of 1.6 M in double distilled water. Medium 1 was concentrated 2-fold in a Buchi water bath. Osmolarity could be changed without also altering the osmolality of the medium.

**EXPERIMENTAL PROCEDURES**

**Validation of LY as a Fluid Phase Marker in Hepatocytes**—Since LY has not been used previously with hepatocytes, it was necessary to demonstrate that this molecule behaves as a bona fide fluid phase molecule with these cells. The kinetics of uptake of LY at 37 °C by cells in suspension is biphasic (Fig. 1). This was more obvious at higher LY concentrations. There is an initial rapid rate of uptake for approximately 20 min and then a slower rate of uptake for 3-4 h until a steady-state intracellular concentration of LY is achieved. The rate of internalization increased linearly with the concentration of LY (r = 0.985), indicating that the uptake is not saturable at least up to a concentration of 1.09 mM LY (0.5 mg/ml). Likewise, the extent of LY uptake was linear with increasing concentration (r = 0.990). LY uptake is, therefore, concentration-independent and time-dependent. LY accumulation only occurred with intact cells at 37 °C (Fig. 2). Cells incubated at 4 °C accumulated virtually no LY, nor did cells that had first been permeabilized with digitonin and then incubated at either 4 or 37 °C. LY does not bind significantly to membranes either in live cells at 4 °C or in permeable cells at 4 or 37 °C.
Other investigators have also concluded that LY is impermeant to membranes and does not bind to cells or membranes (Bowman and Tedeschi, 1983; Steinberg et al., 1987; Mir et al., 1988). A live cell is required in order to obtain the time-dependent accumulation at 37 °C. If hepatocytes are incubated in the presence of LY at 37 °C, chilled to 4 °C, and then washed and resuspended with the nonionic detergent digitonin, the cells virtually immediately lose all of the accumulated LY into the medium (Fig. 3). This result indicates that the internalized LY is not bound to cellular components and is free to leave the cell after the vesicular organelles have been made permeable.

The initial rate of fluid uptake was calculated from data obtained from experiments such as in Fig. 1 to be $5.7 \pm 2.2$ (n = 8) pl/cell/h. The estimated intracellular volume for isolated rat hepatocytes in suspension (average diameter ~28 μm) is approximately $12 \times 10^{-12}$ liters/cell. This estimated rate of fluid volume uptake therefore corresponds to approximately 93% of the cell volume per h and is consistent with values obtained in other cell types. As indicated above (Figs. 2 and 3), the rate of internalization decreases with time. The final steady-state extent of internalized fluid volume corresponded to about $7 \times 10^{-12}$ liters/cell or about 60% of the estimated cellular volume.

Reversibility of LY Accumulation—As a further indication that the internalization of LY represents a fluid phase process and not a partition of LY into membrane or its binding to cellular components, the ability of the internalized LY to be released from live hepatocytes was examined. Cells were incubated with LY at 37 °C, washed, and resuspended at 37 °C in medium without the dye (Fig. 4A). The rate of release of LY from the cells was also biphasic and went to completion in approximately 4 h. The efflux kinetics, therefore, correspond closely to the influx kinetics. In a similar series of experiments, cells were allowed to internalize LY for various times at 37 °C, washed, and the rate of efflux at 37 °C was then determined (Fig. 4B). As expected, the biphasic character of the efflux curve was most pronounced when the LY influx time had been brief. At long influx times (e.g. 90 min, Fig. 4B), the efflux curves were less noticeably biphasic. The rapidly filling/emptying compartment has a relatively small volume compared with the more slowly exchanging compartment. The approximate $k_0$ values for release of the inter-

![Fig. 2. LY uptake by intact or permeable cells. Cells were treated with (■) or without (○) 0.055% digitonin and then incubated at 37 °C (■) or 4 °C (○) with 0.2 mg/ml LY. Cell samples were washed, and cell-associated LY was determined as described under “Experimental Procedures.”](image-url)

![Fig. 3. Digitonin releases internalized LY. Cells were allowed to endocytose 0.1 mg/ml (○, ■) or 0.4 mg/ml (□, □) LY at 37 °C. Samples were taken at the indicated times, chilled to 4 °C, washed, and treated with (■) or without (□) 0.055% digitonin for 30 min. Cell-associated LY was then determined after washing the cells.](image-url)

![Fig. 4. Efflux of internalized LY. A, cells were allowed to internalize 0.4 mg/ml LY at 37 °C for 2 h. The cells were then chilled, washed twice by centrifugation, resuspended in fresh medium 1/BSA without LY, and put back at 37 °C. Fluorescence in the medium (▲), cell-associated fluorescence (■), and their sum (○) were measured. At the indicated times. At $t = 0$, the total fluorescence measured was equivalent to 1150 ng of LY/10^6 cells. B, cells were first incubated with LY as in A for 15 (○), 30 (■), 60 (□), or 90 (▲) min and then washed and resuspended without LY at 37 °C. Cell samples were taken, washed, and cell-associated LY determined at the indicated times.](image-url)
ized LY were determined from semilog plots of the kinetic data (Table I). The average $t_{0.5}$ values for the fast and slow efflux components were, respectively, $37.9 \pm 2.9$ (n = 3) and 82.3 $\pm$ 6.6 (n = 4) min. As the time of internalization increased, the time required for efflux of a constant percentage (e.g. 50%) of the intracellular LY also increased. These characteristics are compatible with the conclusion that LY is taken up by hepatocytes by a fluid phase process.

**Intracellular Accumulation of LY**—Although hepatocytes have a high level of endogenous fluorescence, the LY uptake

<table>
<thead>
<tr>
<th>Duration of LY uptake</th>
<th>Fast component $t_{0.5}$</th>
<th>Slow component $t_{0.5}$</th>
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<tbody>
<tr>
<td>min</td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td>15</td>
<td>34.5</td>
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<td>39.3</td>
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<td>60</td>
<td>39.8</td>
<td>82.9</td>
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<tr>
<td>90</td>
<td></td>
<td>83.7</td>
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Mean (S.E.) $37.9 \pm 2.9$ $82.3 \pm 6.6$

**TABLE I**

**Effect of loading time on the efflux of LY from hepatocytes**

The data from the early and later portions of the efflux curves shown in Fig. 4B were analyzed by least squares linear regression to estimate the first order rate constants and half-times. Correlation coefficients were all $20.98$.

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**Fig. 6. Effect of sucrose on LY and $^{125}$I-ASOR endocytosis.**

Cells were allowed to endocytose continuously 2 $\mu$g/ml $^{125}$I-ASOR (A) and 0.4 mg/ml LY (B) simultaneously in the absence (C) or presence of 0.1 (○), 0.15 (△), or 0.2 (■) M sucrose in medium 1/BSA.

was clearly revealed to be intracellular using fluorescence microscopy. Intense punctate staining was observed in vesicles and possibly tubules throughout the cytoplasm after 30 min in the presence of LY (Fig. 5B). In the absence of LY, the level of fluorescence was much less (Fig. 5A). The distribution of labeled vesicles was very similar to what has been observed with other endocytic markers such as fluorescein-labeled dextran. Prominent staining was often seen close to the nucleus, presumably in the Golgi-lysosomal region. This result confirms the above biochemical studies.

**Effect of Hyperosmolarity on $^{125}$I-ASOR and LY Internalization**—In order to examine the possible differential effect of hyperosmolarity on the uptake of the receptor-mediated ligand versus the fluid phase ligand LY, hepatocytes in suspension were incubated simultaneously with $^{125}$I-ASOR and LY in the presence of increasing amounts of sucrose (Fig. 6). The continuous uptake of $^{125}$I-ASOR was unaffected by the simultaneous presence and uptake of LY (not shown). The continuous uptake of $^{125}$I-ASOR was progressively inhibited with increasing concentrations of sucrose (Fig. 6A) and 0.2 M sucrose gave virtually complete inhibition of endocytosis (99%). At the same time, the internalization of LY was unaffected for at least 20 min by all the concentrations of sucrose tested (Fig. 6B). Only after approximately 30 min was there any change in the rate of LY accumulation at the highest concentration of sucrose. The effect of increasing osmolarity on the inhibition of $^{125}$I-ASOR uptake was extremely rapid (Fig. 7A). Hepatocytes continuously internalizing asialoglycoprotein at 37 °C were essentially completely shut down within 1–2 min after the addition of sucrose to a final concentration of 0.2 M. The uptake of LY in these same cells was unperturbed (Fig. 7B).

**Effect of Metabolic Energy Poisons on LY Uptake**—Hyperosmolarity induced with sucrose at 37 °C did not significantly alter cellular ATP levels. For example, after 30 and 60 min in the presence of 0.2 M sucrose, the ATP content of cells was, respectively, 101 and 89% of the initial control value. We examined previously the sensitivity of $^{125}$I-ASOR uptake and receptor recycling to ATP depletion in isolated hepatocytes (Clarke and Weigel, 1985) and observed that a single round
of uptake of receptor-bound ligand was unaffected even when ATP pools were depleted greater than 98%. Recycling of galactosyl receptors, however, was inhibited if the cellular ATP levels were decreased below a threshold of about 60%. Recycling was completely inhibited if ATP levels fell to 40% or less of controls (Clarke and Weigel, 1985). LY uptake by hepatocytes was examined in the presence of a nitrogen atmosphere or different concentrations of sodium azide (Fig. 8). As anticipated for a cellular process that involves vesicular trafficking and also requires membrane recycling, LY accumulation which continued for over 1 h. In contrast, during the same period of time, there was virtually no uptake of 125I-ASOR. We conclude from this result that LY uptake is energy-dependent as expected and that LY and ASOR are taken up by pathways that have slightly different sensitivities to ATP depletion. Steinman et al. (1974) also observed the relative resistance of horseradish peroxidase uptake to ATP depletion in fibroblasts.
case, it could not be reliably quantitated for cells in suspension. In the presence of 0.2 M sucrose, the surface of these cells showed extensive microvilli and membrane convolutions (Fig. 10B) compared with the untreated cells (Fig. 10A). The pericellular region in the sucrose-treated cells also contained numerous vesicles and large vacuoles. Coated pits were evident on the control cells but were not apparent on the treated cells, which often contained broad diffusely coated regions. In fact, the cell surface morphology made quantitation essentially impossible.

The morphology of the cultured hepatocytes exposed to sucrose was very different. Receptor-mediated endocytosis in cultured cells was more resistant to increasing sucrose concentration, requiring about 0.4 M sucrose to achieve maximal inhibition. The cells attached to the substratum were, therefore, exposed to a 50% greater osmolarity compared with hepatocytes in suspension in the presence of 0.2 M sucrose (~850 versus ~560 mmol/kg). Despite this osmotic stress, the cell surface morphology of the cultured hepatocytes (Fig. 10D) was very similar to the untreated cells (Fig. 10C). The sucrose-treated cultured cells did not have extensive microvilli, surface membrane convolutions, or dramatically increased intracellular vesicles and vacuoles. With cells in suspension or in culture, an obvious difference was seen in the appearance and organization of mitochondria in the presence of sucrose.

It was possible to quantitate the frequency of normal coated pits in cultured hepatocytes even in the presence of sucrose (Table II). Control cells after culture overnight had 99.6 ± 18.8 coated pits/mm of apical membrane length. Coated pits were found over the whole apical cell surface (Fig. 11), often clustered near regions of cell-cell contact (Fig. 11a). Coated pits at the base of a microvillus were very common (Fig. 11, c and d). The basolateral surface was essentially devoid of coated pits. In the presence of 0.4 M sucrose, the number of coated pits was 16.9 ± 6.2, an 83% decrease (Fig. 11, e–i). Hyperosmolarity, therefore, interferes with the normal coated pit cycle in hepatocytes as it does in fibroblasts (Heuser and Anderson, 1987).

![Figure 9. Effect of nigericin and K+ depletion on the endocytosis of [125I]ASOR or LY.](image)

**Fig. 9. Effect of nigericin and K+ depletion on the endocytosis of [125I]ASOR or LY.** Cells were incubated at 37 °C with 1.5 μg/ml [125I]ASOR (A) and 0.2 mg/ml LY (B) in the absence (C) or presence of 0.5 (●), 1.0 (□), 2.0 (■), 4.0 (△), or 7.5 (▲) μM nigericin. Medium 1 for this experiment was formulated as in the GIBCO catalog but without K+.

![Figure 10. Effect of hyperosmolarity on hepatocytes in culture and in suspension.](image)

**Fig. 10. Effect of hyperosmolarity on hepatocytes in culture and in suspension.** Hepatocytes in suspension (2 × 106 cells/ml) or after overnight in culture (1 × 107/35-mm dish) were incubated at 37 °C for 25 min with medium 1/BSA alone or containing, respectively, 0.2 or 0.4 M sucrose. The cells were then fixed, washed, and processed for electron microscopy as described under "Experimental Procedures." A, suspension cells minus sucrose; B, suspension cells plus sucrose; C, cultured cells minus sucrose; D, cultured cells plus sucrose. The bar is 1 μm.

**Table II**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Length of apical surface analyzed</th>
<th>Coated pits</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1001</td>
<td>99.6 ± 18.8 (100%)</td>
</tr>
<tr>
<td>0.4 M Sucrose</td>
<td>809</td>
<td>16.9 ± 6.2 (17%)</td>
</tr>
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**DISCUSSION**

LY has been used as a small fluorescent molecule to demonstrate electrical coupling between cells (Stewart, 1978), to stain neurons (Zimmerman, 1986), and to label selectively certain cell types in developing embryos (Sarthy and Hilbush, 1983). More recently, LY has been used to measure fluid phase endocytosis in macrophages (Swanson et al., 1985, 1987), yeast (Riezman, 1985), proximal tubular cells (Goligorsky and Hruska, 1986), and CV-1 kidney cells (Doxsey et al., 1987). LY seems well suited for this use since it is highly fluorescent, is impermeable to membranes (Bowman and Te-
FIG. 11. Effect of hyperosmolarity on coated pits in cultured hepatocytes. Hepatocytes cultured overnight were incubated at 37 °C in medium 1/BSA in the absence (a-d) or presence (e-i) of 0.4 M sucrose for 25 min. The cells were fixed and processed for electron microscopy as described under "Experimental Procedures." The bar is 1 μm. Examples of coated pits are indicated by arrowheads.

deschi, 1983), and does not bind to membranes (Mir et al., 1988; Steinberg et al., 1987). Other fluid phase markers including sucrose, fluorescein-labeled dextran, horseradish peroxidase, and polyvinylpyrrolidone have been used in different cell systems. Different cell types may bind a possible fluid phase marker, which then precludes its use. For example, although horseradish peroxidase has been widely used and in many cases is a suitable fluid phase marker, Esfahani et al. (1986) showed that it behaved differently in its uptake characteristics compared with LY in human monocytes or the macrophage-like cell line U937. Presumably, problems arise if cells have a mannose or N-acetylglucosamine receptor that can interact with the horseradish peroxidase. Klein and Satre (1986) determined that amebae were able to accumulate fluorescein-labeled dextran to a concentration greater than that in the medium. Therefore, a given fluid phase probe must first be shown to be suitable in a given cell type.

In the studies presented here, the evidence that LY is taken up in a fluid phase manner by hepatocytes was the following. 1) Crude membranes or permeabilized cells did not bind LY at 4 or 37 °C. 2) Intact cells did not accumulate LY at 4 °C, only at 37 °C. 3) The rate and extent of LY accumulation were not saturable and were energy-dependent. 4) Internalized LY was accumulated in a typical endocytic pattern, particularly localized in the perinuclear region and could be rapidly released from permeable cells. Using LY, the calculated initial rate of fluid uptake by hepatocytes was 5.7 ± 2.2 pl/cell/h (n = 8). The reported values using different markers for fluid phase uptake in isolated rat hepatocytes range from 0.04 to 30.0 pl/cell/h (Munnikma et al., 1980; Ose et al., 1980; Gordon et al., 1987; Scharschmidt et al., 1986; Sasaki et al., 1987). The reason for this wide range in rate of fluid uptake is unclear, although it is known that the growth state of cells can dramatically influence their fluid phase uptake capability (Wiley and Cunningham, 1982). It has also been reported that fluid phase endocytosis capability changes in the rat with age (Horbach et al., 1986). It is possible that the wide variation from different laboratories also reflects the different methodologies used to isolate or culture hepatocytes.

The complex process of fluid phase endocytosis involving membrane vesicle formation, fusion, and recycling continued in cells under a severe osmotic stress. Despite a 100–200% increase in the osmolality of the medium, the cells remained viable for hours with normal ATP levels, and the rate of fluid phase uptake was virtually unchanged. This is the first report of a treatment that completely blocks a receptor-mediated pathway (>98%) without affecting (≤2% inhibition) a fluid phase pathway. Hepatocytes may be a unique cell type in this regard. Thirion and Wattiaux (1988) observed that 10 μM monensin had no effect on the fluid phase uptake of sucrose in rat hepatocytes but that sucrose-labeled asialofetuin uptake was inhibited about 70%.

The kinetics of coated pit recycling cessation in the presence of hyperosmolar sucrose was extremely rapid. ASOR uptake was stopped within 1–2 min after making the medium hyperosmolar. Under these same conditions, the uptake of LY was virtually unaffected, suggesting that fluid phase endocytosis occurs by a different pathway than the receptor-mediated uptake of the ligand.

Hyperosmolality inhibits receptor-mediated endocytosis of transferrin (Bowen and Morgan, 1988), asialoglycoprotein (Oka and Weigel, 1988), low density lipoprotein (Heuser and Anderson, 1987), and chemotactic peptide (Daukas and Zigmond, 1985). 0.2 M Sucrose added to complete medium increases the osmolality approximately 2-fold (from 264 to 555 mmol/kg) and completely inhibits continuous endocytosis of 125I-ASOR by hepatocytes in suspension (Oka and Weigel, 1988). All of the effects on cells in suspension could also be
observed with cells in culture. In both cases, the same qualitative results were obtained in terms of the sensitivity of ligand uptake and processing steps to increasing osmolarity. However, cells in suspension were more susceptible to increasing osmolarity than were cells in culture. For cells in suspension, the optimal sucrose concentration necessary to get a maximum inhibition was 0.2 M, whereas 0.4 M sucrose was required for cells in culture.

The same results were observed for the uptake of low density lipoprotein in fibroblasts by Heuser and Anderson (1987), who concluded that hyperosmolarity interfered with receptor-mediated endocytosis by disrupting the coated pit cycle and causing the generation of abnormal nonfunctional coated pits. Our finding that coated pit frequency decreased by >80% in cultured hepatocytes treated with sucrose agrees with this conclusion. Since hyperosmolarity decreased the number of coated pits on the cell surface, it is also likely that the coated pit cycle is constitutive. The relative resistance of cultured cells to the effect of hyperosmolarity may be due to their attachment to the substratum or the higher surface/volume ratio. The anchored cells tolerated the physical stress with less deformation of the cell surface. At the electron microscopy level, the plasma membrane of the suspension cells had numerous microvilli and pericellular vacuoles induced by the hyperosmolar sucrose. This was not the case with the cultured cells.

Depletion of intracellular K+ has also been used to arrest coated pit formation and to stop receptor-mediated endocytosis in fibroblasts (Larkin et al., 1983; 1985). In the latter studies, potassium-depleted fibroblasts or hepatocytes had 70% fewer coated pits than the control cells. Fibroblasts had 30 coated pits/mm of membrane, whereas hepatocytes in perfused livers contained ~150 coated pits/mm of sinusoidal membrane. We found ~100 coated pits/mm of apical membrane in cultured hepatocytes. The difference in coated pit frequency between hepatocytes and fibroblasts may be related to a greater amount of endo/exocytic activity in hepatocytes or to a different surface/volume ratio.

The mechanism by which hyperosmolarity induced by sucrose blocks the coated pit recycling pathway is not known. Hyperosmolarity decreases the transmembrane potential in hepatocytes (Howard and Wondergem, 1987). Hepatocyte volume was essentially constant at 37 °C but not at 4 °C with up to a 50% increase in osmolality. Henderson et al. (1986) linked a decreased membrane potential in hepatocytes with intracellular acidification secondarily caused by inhibition of a membrane Na+/H+ exchange activity. Sandvig et al. (1987) showed that cytoplasmic acidification induced by several techniques blocks receptor-mediated uptake via coated pits, although the number of coated pits was not reduced. Heuser et al. (1987) reported that nigericin-induced K+ depletion, hyperosmolarity, and ATP depletion all cause cytoplasmic acidification to pH 6.2-6.5. They proposed that the low pH induces aberrant clathrin assembly to give microcages on the plasma membrane and in the cytoplasm, thus stopping the coated pit cycle. The reason that the coated pit number decreases with hyperosmolarity but not necessarily with cytoplasmic acidification remains to be reconciled.

Both the internalization and the externalization, or efflux, of LY were biphasic, suggesting the involvement of more than one kinetic step in these processes. Multiple kinetic steps in fluid phase endocytosis have also been described in macrophages and fibroblasts (Besterman et al., 1981; Murphy, 1985; Swanson et al., 1985). Our results are consistent with the idea that the uptake kinetics represent a gradual sequential filling of intracellular compartments further into the cell with time.

The ability of the internalized LY to be released indicates that the transfer of material among these various compartments and their communication with the extracellular medium are reversible. The results indicate that a large compartment that requires a long time to fill may equilibrate slowly with a smaller compartment that fills more quickly. The larger compartment in turn takes a longer time to empty. Alternatively, the results are explained by two parallel compartments: a small one that fills rapidly and a large one that fills slowly.

Cells can have separate pathways for the uptake of fluid components and membrane-adsorbed components (Gonnella and Neutra, 1984; Gonatas et al., 1984). Brown et al. (1987) reported that endocytosis of horseradish peroxidase by kidney collecting duct intercalated cells occurred by a nonclathrin-coated vesicle pathway. The alternative pathway in this cell type also involves a cytoplasmic coat on the inner side of the plasma membrane, but the protein involved is not clathrin. Other studies suggest that the inhibition of the coated pit recycling pathway by depletion of intracellular K+ does not necessarily block the uptake of other molecules. For example, ricin but not diphtheria toxin was transported into HepG2 cells after hypotonic shock and K+ depletion (Moya et al., 1988). Under the same conditions, these cells were unable to internalize transferrin or low density lipoprotein. In this case, ricin is not a fluid phase marker but a membrane marker, presumably an adsorbed membrane component following bulk membrane recycling.

Many of the above studies suggest the operation of alternate pathways for both membrane-bound and fluid phase components other than a receptor-mediated coated pit pathway. The relative contribution of different pathways within a given cell type has been investigated in only a few cases. The coated pit pathway appears to account for approximately 50% of the fluid phase uptake capacity in CV-1 African green monkey kidney cells (Doxsey et al., 1987) and about 16% in polymorphonuclear leukocytes (Daukas and Zigmond, 1985). Intracellular K+ depletion markedly decreased the uptake of horseradish peroxidase in human fibroblasts (Larkin et al., 1983), indicating that virtually all of the fluid uptake may be accounted for by the coated pit pathway. Marsh and Helenius (1980) calculated that the fluid uptake into coated vesicles in baby hamster kidney-21 cells is similar to the fluid phase uptake rates in macrophages and fibroblasts. Although this conclusion was supported by indirect evidence, it may nonetheless apply to the situation in fibroblasts. The major finding in the present study was that conditions that almost completely inhibited galactosyl receptor-mediated endocytosis via the coated pit pathway did not decrease total fluid phase uptake in hepatocytes (<2%). This result indicates that the major route of fluid internalization in isolated hepatocytes is not the coated pit pathway but rather a separate independent pathway(s).

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Hyperosmolarity Inhibits Receptor-mediated Not Fluid Phase Endocytosis

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