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Sugar uptake by fluid-phase pinocytosis and diffusion in isolated rat hepatocytes

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Measurements of sugar pinocytosis (fluid-phase endocytosis of radiolabelled sucrose, lactose and raffinose) in freshly isolated rat hepatocytes are disturbed by sugar diffusing into the cells through plasma-membrane blebs. Non-pinocytic entry may be even more pronounced at 0 °C, and is a major contributor to 'background' radioactivity. By electrodisruption of the plasma membrane, a distinction can be made between pinocytotically sequestered sugar and free sugar that has entered the cytosol by diffusion. Pinocytosis proceeds at a rate of 2% /h (relative to the intracellular fluid volume), whereas the rate of sucrose entry by diffusion is more than twice as high. Three pinocytotic compartments are distinguishable in isolated hepatocytes: (1) a rapidly recycling compartment, which is completely destroyed by electrodisruption, and which may represent pinocytic channels continuous with the plasma membrane; (2) a non-recycling (or very slowly recycling) electrodisruption-resistant compartment, which allows accumulation of the lysosomally hydrolysable sugar lactose, and which therefore must represent non-lysosomal vacuoles (endosomes?); (3) a lysosomal compartment (non-recycling, electrodisruption-resistant), which accumulates raffinose and sucrose, but which hydrolyses lactose. The last two compartments can be partially resolved in metrizamide/sucrose density gradients by the use of different sugar probes.

INTRODUCTION

Pinocytosis is frequently applied as a collective term to the uptake of soluble (non-particulate) extracellular components by means of plasma-membrane invagination. In the present context we will use the word in a more restricted sense (and more in accordance with its etymology), as a term for fluid-phase endocytosis only (i.e. excluding receptor-mediated endocytosis).

Pinocytosis is usually studied as the uptake of an inert non-adsorbing marker molecule, such as radiolabelled sucrose or polyvinylpyrrolidone (for review, see Pratten *et al.*, 1980; Besterman & Low, 1983). Both compounds have been used to assess pinocytosis in the rat liver, both *in vivo* (Forker, 1970; Munniksma *et al.*, 1980) and in isolated hepatocytes (Ose *et al.*, 1980). The pinocytic rates measured were remarkably similar with either compound *in vivo*, or with polyvinylpyrrolidone in isolated cells, i.e. about 10 μl/h per g wet wt. (0.08 μl/h per 10^6 hepatocytes). However, in isolated hepatocytes the sucrose uptake was found to be 3 times higher than the uptake of polyvinylpyrrolidone, even after removal of radiochemical contaminants (Ose *et al.*, 1980). Since sucrose is so widely used in pinocytosis studies with isolated cells (Besterman *et al.*, 1981; Dean, 1984; Gibbs & Lienhard, 1984; Pauw *et al.*, 1986), it is obviously important to find out the reason for this discrepancy.

During our own work with isolated rat hepatocytes, using electroinjected [^{14}C]sucrose as a probe of intracellular autophagy (Gordon & Seglen, 1982), we found that sucrose can also enter the cells by diffusion through the surface blebs forming immediately after cell isolation (Gordon *et al.*, 1985). The limiting membrane of such blebs is apparently hyperpermeable compared with the normal plasma membrane, allowing transmembrane diffusion of small molecules such as sucrose to take place.

Since bleb-like lesions are readily seen (in the microscope) to be continuously generated and repaired during incubation of hepatocytes as shaking suspensions, the possibility would have to be considered that extracellular sucrose might be capable of entering the cells through a direct diffusional route as well as by pinocytosis. In the present paper this possibility is examined and substantiated, and it is shown that pinocytotic and diffusional uptake can be distinguished by means of electrodisruption (Gordon & Seglen, 1982).

EXPERIMENTAL

Animals and cells

Isolated hepatocytes were prepared from 18 h-starved male Wistar rats (250–300 g) by two-stage collagenase perfusion (Seglen, 1976). The cells were incubated as shaking suspensions in 15 ml centrifuge tubes (0.4 ml in each tube) at 37 °C. The incubation medium was suspension buffer (Seglen, 1976) supplemented with Mg^{2+} (to 2 mM) and 20 mM-pyruvate (as an energy substrate). After incubation, the cells were washed (unless otherwise stated) in 3 × 4 ml of ice-cold wash buffer (Seglen, 1976) to remove extracellular radioactivity.

Electrodisruption and radioactivity measurements

Before electrodisruption, the cells were washed and resuspended in a non-ionic medium (10% sucrose). The cells were then disrupted by a single high-voltage pulse (2 kV/cm) as previously described (Gordon & Seglen, 1982; Seglen & Gordon, 1984). The resulting cell corpses, containing intact cell organelles (including endosomes), were separated from the cytosol (and hence from [^{14}C]sucrose that had entered by diffusion) by centrifugation through metrizamide/sucrose cushions. Their contents of sequestered radioactivity were then measured by liquid-scintillation counting. All measure-

ments were done in triplicate, i.e. from three parallel cell samples.

Density-gradient analysis

Metrizamide/sucrose gradients (1.03–1.21 g/ml) were prepared and centrifuged as described previously (Seglen & Solheim, 1985). Cell corpses were homogenized with a hand-operated Dounce glass homogenizer and layered on top of the gradients. Acid phosphatase and cytochrome oxidase were used as biochemical markers for lysosomes and mitochondria respectively (Tolleshaug & Seglen, 1985).

Chemicals

[^{14}C]Sucrose (554 Ci/mol; 1 Ci/l) and [^{14}C]lactose (59 Ci/mol; 0.2 Ci/l) were purchased from Amersham International, Amersham, Bucks., U.K., and [^{3}H]raffinose (7800 Ci/mol; 1 Ci/l) from New England Nuclear Co., Dreieich, Germany. Metrizamide was from Nyegaard, Oslo, Norway, and other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

RESULTS AND DISCUSSION

Raffinose uptake by hepatocytes at 37 °C

The trisaccharide [^{3}H]raffinose entered hepatocytes at 37 °C with markedly biphasic kinetics (Fig. 1). An initial rapid uptake phase, lasting for only a couple of minutes, was followed by a slow continuous uptake lasting for the duration of the incubation. In addition, a relatively high background of cell-associated radioactivity was evident already at zero time.

Since we routinely maintain hepatocyte suspensions on ice while preparing for experiments, the cells had already been exposed to radiolabelled raffinose for some time at 0 °C before the start of the actual experimental incubation at 37 °C. It therefore seems reasonable to ascribe the background radioactivity to influx through lesions generated during the cell purification procedure (repeated filtrations and centrifugations, performed at 0 °C), and remaining unrepaired at low temperature. At least some lesions, such as cell surface blebs (Gordon *et al.*, 1985), are known to be permeable to raffinose and other sugars (Seglen *et al.*, 1986), and are not repaired at 0 °C (Gordon & Seglen, 1982). Raffinose may therefore be able to penetrate into the cells to some extent for as long as they are maintained on ice.

The rapid initial sugar uptake seen on warming to 37 °C (Fig. 1) indicates that extensive membrane perturbations occur when the temperature is raised. This phase is very transient, suggesting rapid repair at 37 °C of the lesions that cause sugar influx at 0 °C. It is known that surface blebs are rapidly repaired at 37 °C (Gordon *et al.*, 1985), and this may well be true of other lesions as well.

During subsequent incubation at 37 °C, dynamic equilibrium (steady state) between bleb formation and rapid resealing will presumably be established, accounting in part for the slow phase of sugar uptake from 5 min onwards. The rate of uptake is lower than in cells maintained at 0 °C (cf. Fig. 2), indicating that other types of lesion (e.g. preparation-inflicted membrane punctures) could contribute under the latter conditions. In addition, the slow uptake at 37 °C will include true pinocytosis.

Our presumptions concerning the nature of the three uptake phases are indicated in Fig. 1.

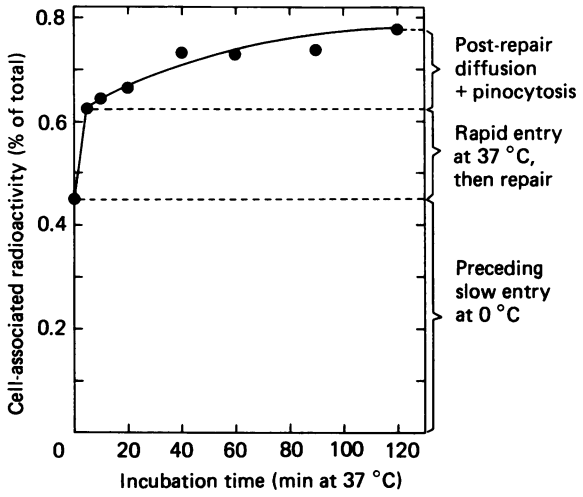

Fig. 1. Uptake of raffinose by hepatocytes at 37 °C

Hepatocytes were maintained at 0 °C for approx. 1 h in the presence of [^{3}H]raffinose (0.4 μCi/ml) before the start of incubation at 37 °C. At different subsequent time points, cells were rapidly washed three times at 0 °C and the cell-associated radioactivity was measured and expressed as a percentage of the total radioactivity in the system (cells + medium). The various modes of sugar entry assumed to contribute to this radioactivity are indicated.

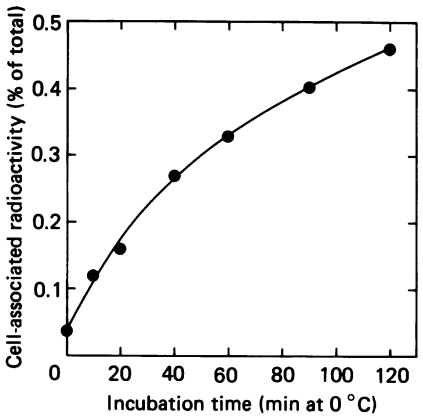

Fig. 2. Raffinose entry into hepatocytes at 0 °C

Hepatocytes were incubated with [^{3}H]raffinose at 0 °C for the length of time indicated, and the amount of cell-associated radioactivity (as percentage of total) was measured.

Raffinose uptake at 0 °C

In order to test the above presumptions, raffinose uptake at 0 °C was investigated. As shown in Fig. 2, the uptake was appreciable, amounting to 0.5% of the total (extracellular) radioactivity within 2 h. About 85% of this radioactivity was non-sedimentable after electrodisruption (results not shown), indicating that it was not sequestered in cellular vesicles. Cellular organelles sediment with the cell corpses after electrodisruption, as shown by the structural appearance of the corpses and, e.g., by the complete sedimentability of the lysosomal marker enzyme acid phosphatase (Seglen & Gordon, 1984).

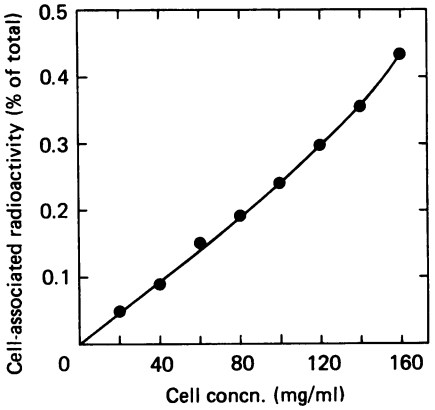

Fig. 3. Raffinose uptake at 0 °C as a function of cell concentration

Hepatocytes at the concentration indicated (mg wet wt./ml) were incubated with [^{3}H]raffinose for 1 h at 0 °C.

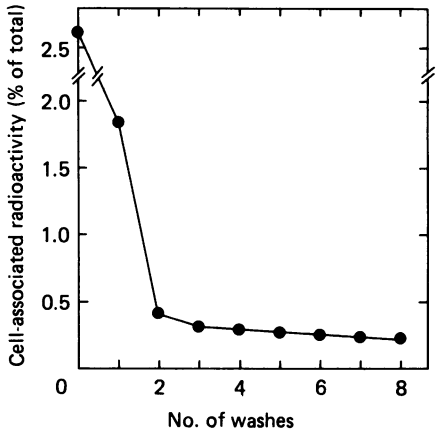

Fig. 4. Removal of extracellular radioactivity by washing

Hepatocytes were incubated with [^{3}H]raffinose for 1 h at 0 °C, sedimented by centrifugation, and washed with ice-cold buffer (4 ml) for the number of times indicated.

The amount of cell-associated radioactivity was proportional to the cell concentration (i.e. the radioactivity per cell was constant) within the concentration range usually employed (100 mg of cells/ml or less) (Fig. 3). The slightly upward curvature at higher cell concentrations could reflect increasing cell damage as a mechanical result of cell crowding (collision frequency?).

To ensure that the cell-associated radioactivity did not represent carry-over of extracellular medium during centrifugation, repeated washes with a large volume of non-radioactive medium were performed. The radioactivity in the sedimented cell pellet declined precipitously during the first three washes (standard procedure), and then reached a relatively stable value (Fig. 4). The slight decline observed at each subsequent wash probably reflected cell loss, plus some additional mechanical damage.

The cell-associated radioactivity which cannot be washed away is unlikely to represent extracellular contamination. Strong adsorption to the cell surface might be considered, but this has been ruled out in the

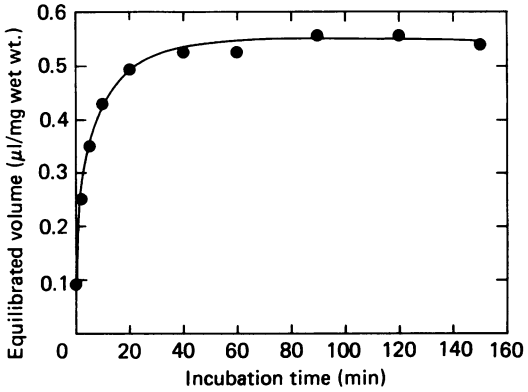

Fig. 5. Estimation of intracellular fluid space by [^{14}C]valine equilibration

Hepatocytes were incubated with 20 mM-[^{14}C]valine (0.4 μCi/ml) at 37 °C for the length of time indicated. The cell-associated radioactivity was measured and expressed as fluid equivalents (μl/mg cellular wet wt.) on the basis of total radioactivity and fluid volume (cells + medium).

case of sucrose, which behaves like raffinose in this context. Radioactive sucrose associating with hepatocytes at 0 °C could not be competed out by non-radioactive sucrose even at very high concentrations (50 mM); sucrose metabolites (glucose, fructose) were likewise without effect. We therefore have to conclude that radiolabelled di- or tri-saccharides associating with hepatocytes at 0 °C are located intracellularly, having entered by influx through hyperpermeable cell-surface lesions.

Determination of intracellular fluid volume

In order to calculate intracellular sugar concentrations, knowledge of the intracellular fluid volume is necessary. The parameter of interest is the functional volume, accessible to an inert solute (such as a sucrose or raffinose) entering the cell.

We routinely use the wet weight of a packed hepatocyte pellet as a reference base in biochemical studies of these cells; this parameter has been found to be almost exactly equivalent to liver tissue wet weight (Seglen, 1976). To determine the intracellular fluid volume as a fraction of this wet weight, we have used [^{14}C]valine as a fluid marker. This amino acid is not significantly metabolized by hepatocytes, it equilibrates across the plasma membrane at 37 °C by means of a non-concentrative transport system (the L system), and it remains inside the cells during washing at 0 °C (Seglen & Solheim, 1978).

At a high concentration of valine (20 mM), its consumption by protein synthesis is negligible, and at 37 °C it eventually reached a constant intracellular concentration (Fig. 5). The final equilibration volume corresponded to about 0.5 μl/mg wet wt., which is the same value as previously found by using a non-metabolizable glucose analogue as a probe (Kletzien *et al.*, 1975). We have used this value in the calculation of uptake rates, expressing sugar uptake as fluid equivalents, with the intracellular fluid volume as a reference base (i.e. as % of the latter). Although this is appropriate for pinocytosis, it is not strictly correct for diffusion, which does not involve a bulk phase uptake of fluid. However,

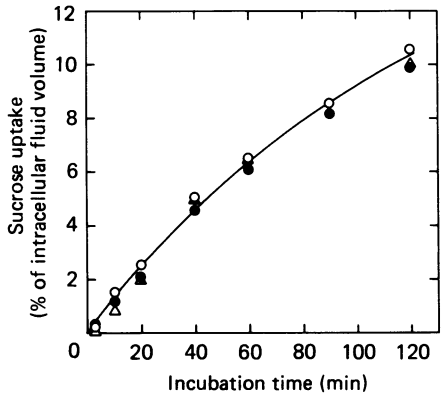

Fig. 6. Concentration-independence of sucrose uptake at 37 °C

Hepatocytes were incubated at 37 °C with [^{14}C]sucrose (0.4 μCi/ml) alone (○; tracer dose), or with an additional 1 mM (●) or 10 mM (△) of unlabelled sucrose. The net uptake of radioactivity (2 min value subtracted) was measured and expressed (in %) as fluid equivalents relative to intracellular fluid volume.

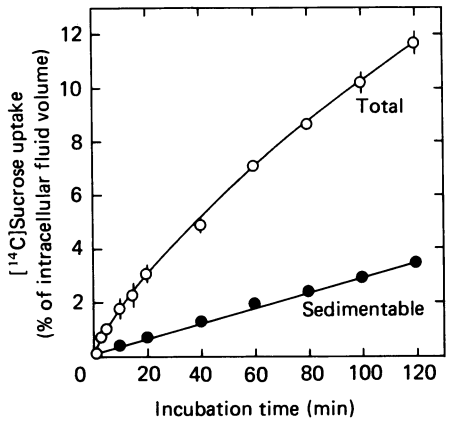

Fig. 7. Uptake and sequestration of sucrose at 37 °C

Hepatocytes were incubated with [^{14}C]sucrose at 37 °C for the length of time indicated. The cells were then washed and electrodisrupted, and radioactivity was measured in the unfractionated system ('total'; ○) as well as in the sedimented cell corpses ('sedimentable'; ●). Net uptake (2 min value subtracted) is expressed (in %) as fluid equivalents relative to intracellular fluid volume. Each value is the mean ± S.E.M. for three separate experiments. Most of the S.E.M. values are so small as to be concealed by the symbols.

if all the values given are expressed as fluid equivalents, they may facilitate a direct quantitative comparison between true pinocytosis and other modes of sugar entry.

Sucrose uptake: use of electrodisruption to distinguish between sedimentable and non-sedimentable radioactivity

Since sucrose is more frequently used as a pinocytosis probe than is raffinose, we conducted some studies on the uptake of [^{14}C]sucrose. The background radioactivity as well as the uptake during the first 2 min at 37 °C were subtracted, in order to exclude as much of the diffusional entry as possible.

Sucrose entered hepatocytes at a rate equivalent to 6% of the intracellular volume/h (Fig. 6). Addition of unlabelled sucrose to the medium (at 1 or 10 mM) had no effect on uptake, indicating that carrier-mediated transport mechanisms were not involved.

When hepatocytes were electrodisrupted at various time points to separate cytosolic from sequestered sucrose (the latter presumably residing in endocytic vesicles and lysosomes), it was found that only one-third of the radioactivity sedimented with the cell corpses (Fig. 7). The sedimentable (sequestered) sucrose accumulated at a nearly constant (very slowly declining) rate, with no evidence of biphasic kinetics. It is likely that the accumulation of sedimentable radioactivity reflects true pinocytosis, and the remainder represents diffusion. The initial pinocytotic rate was equivalent to 2% of the intracellular volume/h, or 10 μl/h per g wet wt. (0.08 μl/h per 10^6 cells). This is the same rate as previously observed *in vivo* (Munniksma *et al.*, 1980) and in isolated hepatocytes pinocytosing polyvinylpyrrolidone (Ose *et al.*, 1980).

The rate of sucrose uptake by diffusion was 2–3 times higher than the pinocytotic uptake (Fig. 7). This diffusional uptake may fully account for the difference between sucrose and polyvinylpyrrolidone pinocytosis previously observed (Ose *et al.*, 1980). Diffusion through blebs is limited to molecules of M_r below 1000 (Gordon & Seglen, 1982); hence polyvinylpyrrolidone would enter by pinocytosis only. Polyvinylpyrrolidone is therefore more suitable than sucrose as a pinocytosis probe, unless diffusion is excluded from the measurement, e.g. by electrodisruption of the cells.

Pinocytosed sucrose: stable or recycling?

Upon re-incubation of the cells in non-radioactive medium, sucrose was lost at an initially very high rate (40%/h during the first 20 min). The rate of loss then slowed down to about 10%/h (Fig. 8). The fraction of sedimentable (sequestered) sucrose was, however, remarkably stable, exhibiting no detectable loss.

A rapid initial loss of pinocytosed markers has previously been observed both in hepatocytes (Ose *et al.*, 1980) and in other cell types (Roberts *et al.*, 1977; Besterman *et al.*, 1981; Daukas *et al.*, 1983; van Deurs *et al.*, 1984; Gibbs & Lienhard, 1984; Swanson *et al.*, 1985), and has been ascribed to recycling from a peripheral pinocytotic compartment (Besterman *et al.*, 1981). This would probably apply to the present case as well, since the initial rate of sucrose loss is too high to be accounted for by diffusion via blebs. On the other hand, a diffusion rate equivalent to 4–5% of the intracellular fluid volume/h (Fig. 7) would contribute heavily to the second, slow, phase of sucrose loss (at 10%/h), assuming diffusion to proceed at the same rate in both directions across the surface bleb membrane.

Previous experiments with [^{14}C]sucrose electroinjected into the cytosol of hepatocytes demonstrated a long-term radioactivity loss of about 6%/h (Seglen & Gordon, 1984). The loss was attributed to cell death, but, in the light of the present results, diffusion may have been a major contributor. The initial loss in those experiments was 17%/h during the first 20 min after resealing, i.e. less than half the rate of loss of pinocytosed sucrose during an equivalent period. This further supports the view that the extensive initial loss of sucrose in the present experiments must represent recycling from a peripheral pinocytotic compartment rather than transmembrane

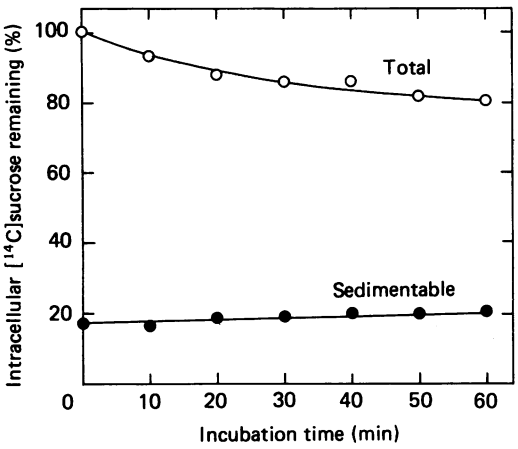

Fig. 8. Temporal stability of sucrose internalized by hepatocytes

Hepatocytes were allowed to internalize [^{14}C]sucrose for 1 h at 37 °C. The cells were then washed and reincubated at 37 °C, and at various subsequent time points they were removed, washed and electrodisrupted. Radioactivity in whole cells (unfractionated system; ○) and sedimented cell corpses (●) was measured and expressed as a percentage of the total radioactivity at zero time.

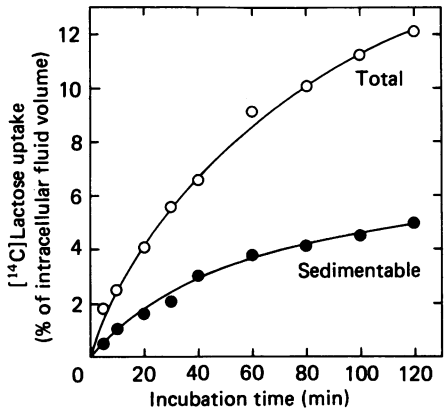

Fig. 9. Uptake and sequestration of lactose at 37 °C

Hepatocytes were incubated with [^{14}C]lactose at 37 °C for the length of time indicated. The cells were then washed and electrodisrupted, and radioactivity was measured in whole cells (unfractionated system; ○) as well as in the sedimented cell corpses (●). Net uptake (2 min value subtracted) is expressed (in %) as fluid equivalents relative to intracellular fluid volume.

diffusion of cytosolic sucrose. On the other hand, the initial loss of electroinjected sucrose previously observed (Seglen & Gordon, 1984) was also more rapid than expected from diffusion rates alone, perhaps indicating a contribution from recycling of sucrose, pinocytosed during the 30 min resealing period.

The fact that no loss occurred from the sedimentable sucrose fraction indicates that electrodisruption specifically preserves a stable, non-recycling, pinocytotic compartment, while destroying the peripheral, recycling, compartment, as previously suggested (Seglen & Solheim, 1985). Electrodisruption is assumed to break up the plasma membrane selectively, while leaving intracellular organelles unafflicted (Gordon & Seglen, 1982; Seglen & Gordon, 1984). The disappearance of the recycling compartment after electrodisruption thus suggests that this compartment is intimately associated with the plasma membrane, perhaps in the form of pinocytic channels continuous with the cell surface. Recycling may simply represent a 'regurgitation' from such channels, i.e. an equilibration between two basically extracellular compartments with very restricted intercommunication (which stop communicating altogether, e.g., at 4 °C; cf. Ose *et al.*, 1980; Besterman *et al.*, 1981). The quasi-intracellular nature of these putative pinocytic channels may bear some analogy to the upper alimentary tract, from which the contents can readily be discharged by dribbling or vomiting. It has been shown by electron microscopy that endocytic channels can be both long and tortuous (Goldenthal *et al.*, 1984; van Deurs *et al.*, 1984), making them plausible candidates for a quasi-intracellular compartment.

Lactose uptake

It has commonly been assumed that the rapidly recycling pinocytotic compartment represents endosomes and the non-recycling (or slowly recycling) compartment lysosomes (van Deurs *et al.*, 1984; Swanson *et al.*, 1985), although a three-compartment model has also been

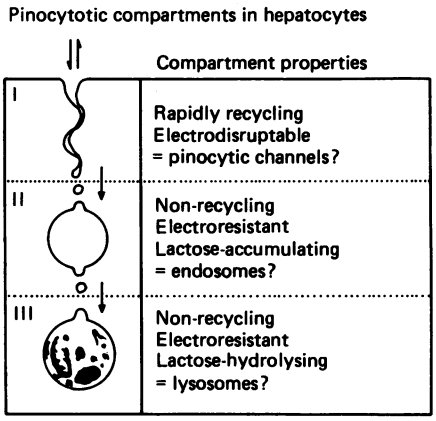

Fig. 10. Putative pinocytotic compartments in hepatocytes

For further explanation and discussion, see the text.

considered (Besterman *et al.*, 1981). If the two-compartment model were applicable to hepatocytes, we would have to equate the electrodisruption-sensitive compartment with endosomes and the electrodisruption-resistant compartment with lysosomes.

To investigate the nature of the resistant (sedimentable) compartment, we introduced [^{14}C]lactose as a pinocytosis probe. This sugar is rapidly hydrolysed inside lysosomes (Høyvik *et al.*, 1986), and may therefore serve as a marker of non-lysosomal compartments.

Lactose was taken up by hepatocytes at a somewhat higher rate than sucrose, suggesting that adsorptive endocytosis might contribute to a certain extent. The higher rate of uptake was particularly evident in the sedimentable (electrodisruption-resistant) fraction (Fig. 9).

The fact that lactose accumulated in the sedimentable fraction at all shows that a major part of the non-recycling compartment must be non-lysosomal. However, the rate of accumulation declined more

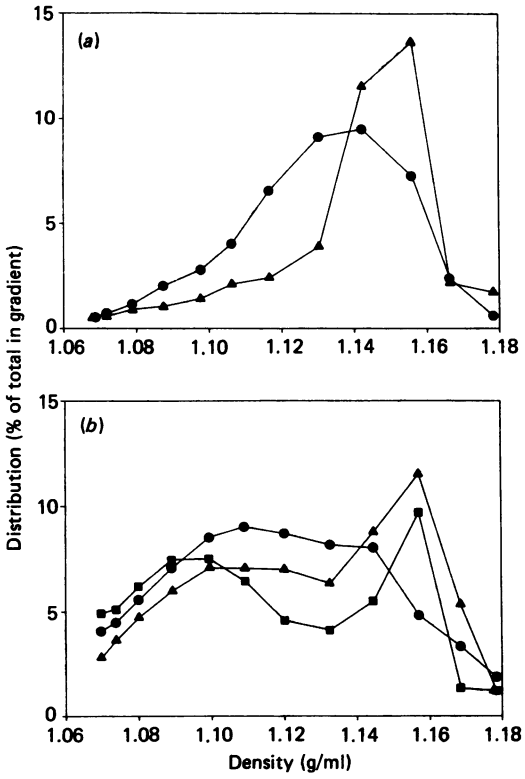

Fig. 11. Subcellular distribution of pinocytosed sugars

Hepatocytes were incubated with a radiolabelled sugar for 1 h at 37 °C. The cells were then electrodisrupted, and the sedimented cell corpses homogenized and centrifuged on metrizamide/sucrose density gradients. (*a*) Subcellular marker enzymes: ●, acid phosphatase (lysosomal marker); ▲, cytochrome oxidase (mitochondrial marker). (*b*) Density distribution of (●) [^{3}H]raffinose, (▲) [^{14}C]sucrose and (■) [^{14}C]lactose.

rapidly than with sucrose, as would be expected if lactose were gradually transferred from a non-lysosomal compartment (endosomes?) to the lysosomes (Fig. 9). Our results therefore suggest that the non-recycling compartment comprises both endosomes and lysosomes, and that a three-compartment model is necessary to describe adequately pinocytosis in hepatocytes (Fig. 10).

Subcellular localization of sedimentable radioactivity

The subcellular localization of the sedimentable electrodisruption-resistant fraction of pinocytosed sugars was investigated by density-gradient analysis. When homogenized cell corpses were centrifuged to equilibrium on metrizamide/sucrose gradients, raffinose, sucrose and lactose (pinocytosed for 1 h) were all found to distribute heterogeneously (Fig. 11). Raffinose displayed two broad and overlapping peaks, one in the region of the lysosomes (1.14 g/ml) and one in a lighter region (1.10 g/ml). Sucrose and lactose both displayed the light peak, but the heavy region of these gradients was obscured by mitochondrial radioactivity, probably taken up from the cytosolic (diffusion-generated) sugar pool (mitochondria can take up both sucrose and lactose, but not raffinose; cf. Seglen *et al.*, 1985, 1986). It was nevertheless obvious that there was less radioactivity in the lysosomal region with lactose than with sucrose, consistent with the ability of lysosomes to hydrolyse lactose.

The gradient distributions thus support the notion that sugar sequestered into the non-recycling pinocytotic compartment may reside both in lysosomes and in non-lysosomal (probably endosomal) vacuoles. With time, an increasingly greater fraction will be found in the lysosomes, which represent the end-station of pinocytosis (Besterman *et al.*, 1981). It is therefore not surprising that the distribution profile of pinocytosed polyvinylpyrrolidone at 3 h was markedly more lysosomal than the 1 h distribution profile of raffinose reported here (Ose *et al.*, 1980).

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