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Use of digitonin extraction to distinguish between autophagic–lysosomal sequestration and mitochondrial uptake of [^{14}C]sucrose in hepatocytes

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In isolated rat hepatocytes, electroinjected [^{14}C]sucrose is sequestered both by mitochondria and by autophagosomes/lysosomes. Radioactivity can be selectively extracted from the latter organelles by low concentrations of digitonin, thereby providing a specific bioassay for autophagic sequestration. By including a digitonin extraction step in the assay procedure, autophagic [^{14}C]sucrose sequestration could be shown to be virtually completely (> 90%) suppressed by the autophagy inhibitor 3-methyladenine (10 mM), whereas mitochondrial sugar uptake was unaffected. An amino acid mixture likewise suppressed autophagic sequestration very strongly, while having no detectable effect on the mitochondria.

INTRODUCTION

The autophagic–lysosomal pathway is a major route of endogenous macromolecule degradation in living cells. Material to be degraded along this pathway appears to be sequestered by a specialized membraneous organelle called a phagophore (Seglen, 1985), the resulting vesicle (autophagosome) subsequently entering lysosomes by a 'fusion' process of unknown nature (Holtzman, 1976; Kovács *et al.*, 1982). These early steps have previously only been studied by means of morphological (electron microscopic) methods, but in recent years several investigators have begun to apply isotope microinjection or other biochemical techniques to the study of autophagic sequestration (Stacey & Allfrey, 1977; Hendil, 1981; Gordon & Seglen, 1982; Kominami *et al.*, 1983).

In our own laboratory we have developed methods for electropermeabilization and electrodisruption of isolated rat hepatocytes, making it possible to measure intracellular sequestration of the membrane-impermeant, inert probe [^{14}C]sucrose in large numbers of small cell samples (Gordon & Seglen, 1982; Seglen & Gordon, 1984). Somewhat surprisingly, [^{14}C]sucrose turned out to accumulate in mitochondria as well as in autophagosomes and lysosomes (Tolleshaug *et al.*, 1984; Seglen & Solheim, 1985). While the mitochondrial sugar uptake appears to be a hitherto unknown biological process, interesting in its own right, it is a disturbance factor from the point of view of obtaining a specific assay for autophagic sequestration. We have therefore sought a means of separating mitochondrial radioactivity from the radioactivity sequestered in autophagosomes/lysosomes. As will be shown in the present communication, such separation can be obtained by specific solubilization of the latter organelles with a detergent (digitonin).

EXPERIMENTAL

Chemicals

[^{14}C]Sucrose (554 Ci/mol; 1 Ci/l) was obtained from Amersham International; metrizamide was from Nyegaard, Oslo, Norway; 3-methyladenine (6-amino-3-methylpurine) was from Fluka AG, Buchs, Switzerland; digitonin, amino acids and other biochemicals were from Sigma.

Hepatocytes

Hepatocytes were isolated from 18-h starved, adult male Wistar rats (250–300 g) by means of collagenase perfusion (Seglen, 1976), and incubated in pyruvate/Mg^{2+}-fortified suspension buffer (Seglen & Gordon, 1984). For sequestration measurements, the cells were incubated as small-volume (0.4 ml) aliquots in rapidly shaking centrifuge tubes (Seglen, 1976); cells to be homogenized for gradient analysis were incubated as large-volume (4–8 ml) aliquots in slowly shaking Petri dishes (Tolleshaug *et al.*, 1984).

[^{14}C]Sucrose loading

Hepatocytes were electropermeabilized with five high-voltage pulses and diffusion-loaded with [^{14}C]sucrose (5–10 μCi/ml) for 1 h at 0 °C, followed by 30 min resealing at 37 °C and washing at 0 °C (Gordon & Seglen, 1982; Seglen & Gordon, 1984).

Cell disruption and homogenization

After incubation, the cells were suspended in a non-ionic medium (10% sucrose) and electrodisrupted (as 0.5 or 1 ml aliquots) with a single high-voltage pulse (Gordon & Seglen, 1982). The resulting (irreversibly permeabilized) cell corpses were separated from cytosol

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(and non-sequestered [^{14}C]sucrose) by centrifugation through metrizamide/sucrose cushions. The cell corpse pellets have been shown to contain all the cell's lysosomes in an intact state (Seglen & Gordon, 1984).

For sequestration measurements, the radioactive cell corpse pellet was dissolved (in 0.5 ml of 0.1 M-NaOH/0.4% sodium deoxycholate) and counted either directly, or after digitonin extraction and recentrifugation (see below). For the preparation of homogenates (approx. 10%, wet wt./volume), several cell corpse pellets were pooled and homogenized to completion (checked in the light microscope) in a Dounce hand-operated homogenizer.

Digitonin extraction

A stock solution of digitonin (15 mg/ml in water/dimethyl sulphoxide, 1:1 v/v) was diluted with buffered 10% sucrose (2 mM-Hepes, 1 mM-EDTA, pH 7.4) to the desired final digitonin concentration (useful range for selective extraction, 0.2–0.5 mg/ml). The cell corpse pellet was suspended in 1 ml of digitonin/sucrose solution, and gently agitated for 5–10 min at 0 °C. The cell corpses were resedimented (10 min at 5000 rev./min in the HG-4L rotor of the Sorvall RC-3 centrifuge, i.e. approx. 90000 $g \cdot$ min), and the supernatant was completely aspirated for separate measurements of radioactivity in the total supernatant and the total pellet, respectively. The pellet was well-packed and very small compared to the supernatant volume, making corrections for reciprocal volume cross-contamination unnecessary.

Total cellular radioactivity, when given, represents the sum of the radioactivities measured in the pellet and in the supernatant.

Metrizamide gradients

Cell corpses were homogenized (in a tight-fitting Dounce homogenizer) either directly, or after digitonin treatment and re-sedimentation. In the latter case, the supernatant was first aspirated, i.e. the digitonin-extracted material was not included in the gradient. Any nonsedimentable gradient material has been generated secondarily by the homogenization procedure (organelle destruction).

The iso-osmotic metrizamide (0–40%)/sucrose (0.25–0 M) density gradients were generated and centrifuged as described previously (Seglen & Solheim, 1985). Gradient recoveries were close to 100% both for radioactivity (100.0 ± 0.9%; mean ± S.E.M. of 32 gradients), acid phosphatase (97.2 ± 1.0%) and protein (98.8 ± 0.9%).

Analytical procedures

Radioactivity was measured by liquid-scintillation counting, and net sequestration of [^{14}C]sucrose calculated as previously described (Seglen & Gordon, 1984). Acid phosphatase was measured, in the presence or absence of 0.5% Triton X-100, according to Barrett (1972), and total protein by the Bio-Rad protein assay (Technical Bulletin 1051 E, April 1977; Bio-Rad Laboratories, Richmond, CA, U.S.A.).

RESULTS

Concentration-dependent selectivity of digitonin extraction

Hepatocytes have been found to transfer cytosolic [^{14}C]sucrose to two different sedimentable structures

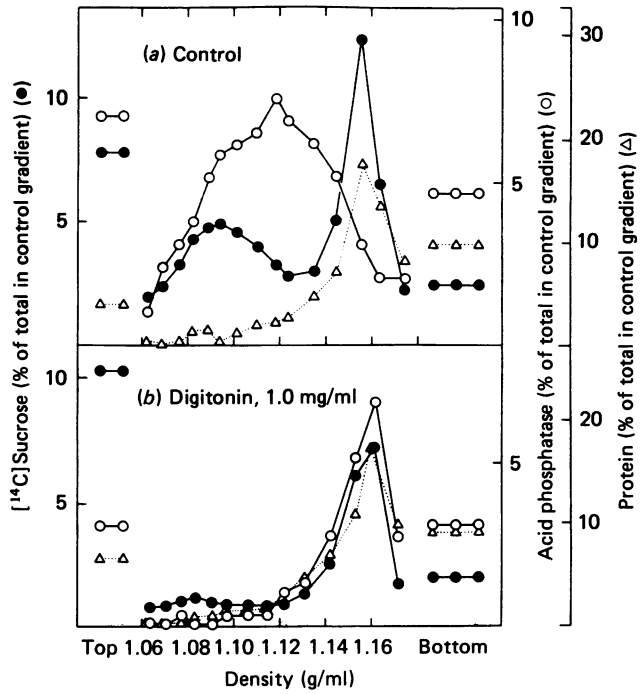

Fig. 1. Extraction of hepatocytic cell corpses with digitonin, 1.0 mg/ml

Cell corpses from hepatocytes which had been allowed to sequester [^{14}C]sucrose for 2.5 h at 37 °C were homogenized directly (*a*) or after a 10-min incubation at 0 °C with digitonin, 1.0 mg/ml (*b*). The homogenates were centrifuged on iso-osmotic 0–40% metrizamide/sucrose density gradients, and the fractions analysed for sequestered [^{14}C]sucrose (●), acid phosphatase (○) and protein (△).

which can be resolved on metrizamide/sucrose density gradients: a light fraction (1.08–1.10 g/ml), thought to represent lysosomes and autophagosomes, and a dense fraction (1.15–1.16 g/ml), thought to represent mitochondria (Tolleshaug *et al.*, 1984; Seglen & Solheim, 1985). The former shows up in gradients as a radioactive peak coincident with, or somewhat lighter than, lysosomal marker enzymes, whereas the latter coincides with mitochondrial marker enzymes and with the major (mitochondrial) peak of sedimentable cell protein (Fig. 1*a*).

The detergent digitonin has previously been shown to be capable of, at the appropriate concentration, solubilizing some cellular membranes without significantly disrupting mitochondrial structure (Zuurendonk & Tager, 1974). We therefore decided to investigate whether digitonin could be used to selectively extract autophagically sequestered [^{14}C]sucrose from hepatocytic cell corpses.

When cell corpses were briefly treated with digitonin (1 mg/ml) before homogenization, the light radioactivity peak virtually disappeared from the metrizamide gradient (Fig. 1*b*). The lysosomal marker enzyme, acid phosphatase, also disappeared from the light region of the gradient, suggesting that lysosomes had been disrupted by the detergent. A significant fraction of the enzymic activity was, however, now found to coincide with the dense radioactivity peak, possibly representing acid phosphatase bound to lysosomal membranes trapped by,

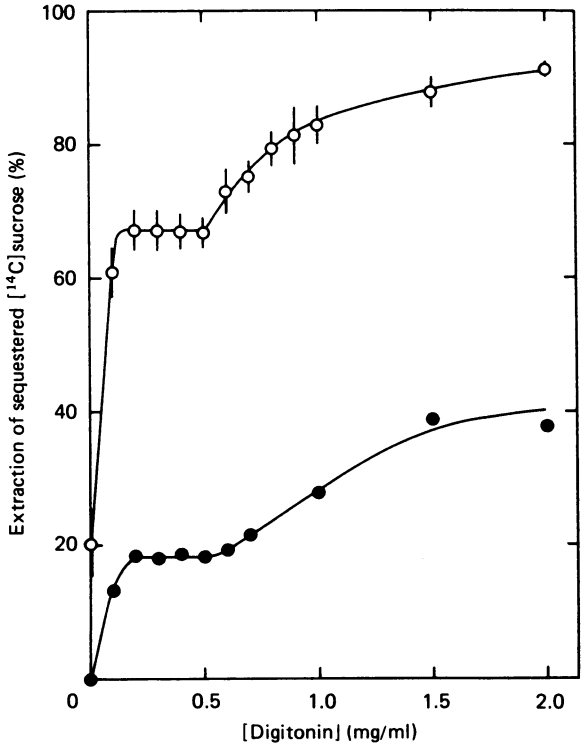

Fig. 2. Extraction of sequestered [^{14}C]sucrose as a function of digitonin concentration

Cell corpses were prepared from hepatocytes which had been allowed to sequester [^{14}C]sucrose for 1.5 h in the absence (○) or presence (●) of 3-methyladenine, 10 mM. The corpses were treated for 10 min at 0 °C with digitonin at the concentration indicated, and [^{14}C]sucrose radioactivity measured in the extract after sedimentation of the corpses. The upper curve is the mean ±S.E.M. for five experiments; the lower curve represents a single experiment. The [^{14}C]sucrose extracted below 0.5 mg of digitonin/ml in the lower curve largely reflects sequestration during the initial 30-min resealing period in the absence of 3-methyladenine.

or sticking to, the mitochondria. This makes it difficult to assess the effect of digitonin on the dense peak with any degree of precision, but in any case the net radioactivity decrease in this peak (approx. 30%) would suggest at least a moderate extraction of mitochondrial radioactivity.

To see if a more selective extraction could be obtained at lower digitonin concentrations, we made a series of experiments in the dose range 0.1–2.0 mg/ml. As shown in Fig. 2, the amount of [^{14}C]sucrose extracted from cell corpses was strikingly constant between 0.2 and 0.5 mg/ml. At higher digitonin concentrations, increasing additional quantities of sequestered radioactivity were extracted. The amount of [^{14}C]sucrose extractable at low digitonin concentrations was strongly reduced in cells which had been incubated in the presence of the autophagy inhibitor 3-methyladenine (Seglen & Gordon, 1982), suggesting the digitonin-sensitive fraction to be of autophagic origin, probably residing in autophagosomes and lysosomes. The additional radioactivity extracted above 0.5 mg of digitonin/ml was, on the other hand, not affected by 3-methyladenine (cf. the parallel course of the two dose–response curves), suggesting a non-autophagic origin consistent with a mitochondrial location.

The characteristic dose–response relationship displayed in Fig. 2 would thus suggest selective extraction of autophagically sequestered [^{14}C]sucrose by digitonin in the plateau range 0.2–0.5 mg/ml. Choosing 0.3 mg/ml as a standard concentration, another gradient analysis of digitonin-extracted, homogenized cell corpses was performed (Fig. 3). As can be seen, disappearance of the light peak was now completely selective, the dense (mitochondrial) peak being quantitatively (99%) recovered.

Sedimentation of lysosomal marker enzyme activity into the mitochondrial region is, however, still complicating the picture. Although the complete lack of effect of 3-methyladenine as well as of an autophagy-suppressive amino acid mixture on digitonin-resistant radioactivity (see below) would make the presence of autophagically sequestered [^{14}C]sucrose in the dense peak extremely unlikely, it would nevertheless be useful if the absence of intact lysosomes could be conclusively documented. We

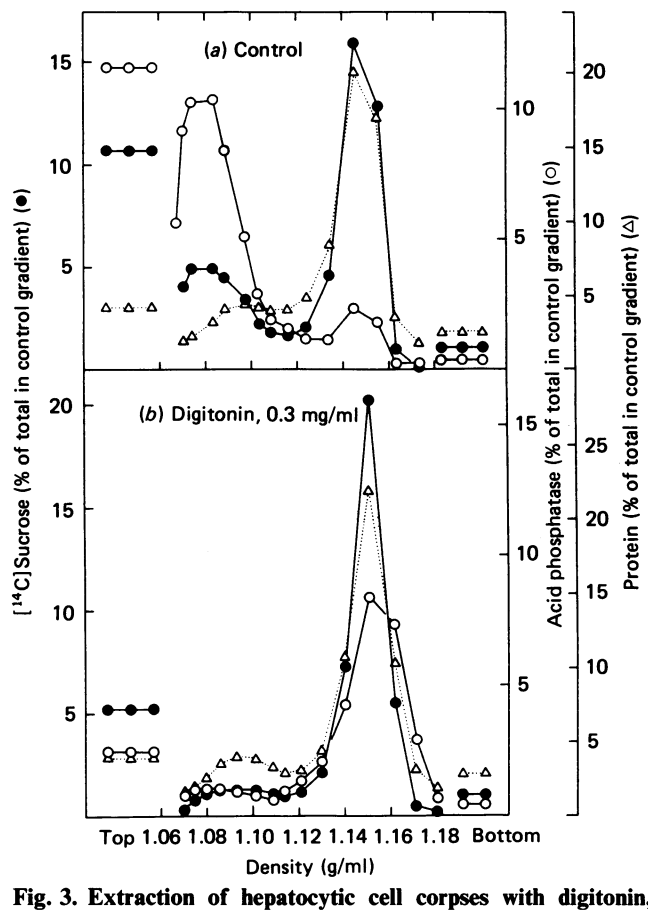

Fig. 3. Extraction of hepatocytic cell corpses with digitonin, 0.3 mg/ml

Cell corpses containing sequestered [^{14}C]sucrose (2.5 h sequestration) were homogenized directly (*a*) or after treatment with digitonin, 0.3 mg/ml (*b*). The homogenates were centrifuged on iso-osmotic 0–40% metrizamide/sucrose gradients, and the fractions analysed for [^{14}C]sucrose (●), acid phosphatase (○) and protein (△). The recovery of [^{14}C]radioactivity in the mitochondrial peak (1.14–1.16 g/ml) after digitonin extraction was 99%.

Table 1. Loss of acid phosphatase latency upon digitonin extraction

Cell corpses were treated with digitonin, 0.3 mg/ml, or buffer (as control), resedimented and separated into a post-treatment pellet and a post-treatment supernatant. Acid phosphatase was assayed (20 min incubation) with and without 0.5% Triton X-100, and latency expressed as the difference (in %). The recovery is given as a percentage of the total activity (i.e. with Triton) in whole cells. Notice that latency is lost during incubation for enzyme assay, the initial latency in cell corpses being 80–90% (Seglen & Gordon, 1984).

	Acid phosphatase recovery (%)		Acid phosphatase latency (%)	
	−Digitonin	+Digitonin	−Digitonin	+Digitonin
Cell corpses	93	95	62	0
Post-treatment pellet	96	46	61	1
Post-treatment supernatant	3	52	0	0

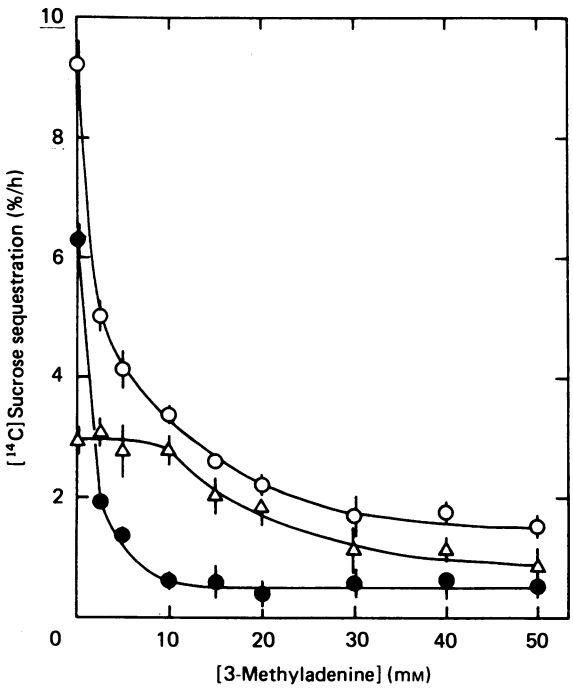

Fig. 4. Effect of 3-methyladenine concentration on sequestration of [^{14}C]sucrose into digitonin-extractable and digitonin-resistant cell compartments

Cell corpses were prepared from hepatocytes which had been sequestering [^{14}C]sucrose at 37 °C in the presence of the concentration of 3-methyladenine indicated. The corpses were extracted with digitonin (0.3 mg/ml), and the total radioactivity in the corpses (○), in the extract (●) and in the sedimented pellet (i.e. non-extracted radioactivity, △) was measured. Sequestration rates between 30 and 90 min (subsequent to the initial 30 min resealing period) are given, each value being the mean ±S.E.M. for three experiments.

therefore investigated whether the acid phosphatase that remained sedimented after digitonin extraction was latent (i.e. residing in intact lysosomes) or non-latent. As shown in Table 1, acid phosphatase was 60% latent in untreated cell corpses even after resedimentation. Since latency is rapidly lost during the acid phosphatase assay, this value is representative of lysosomes which are essentially intact at the beginning of the assay (Seglen & Gordon, 1984). After digitonin extraction, however, although one-half of the enzyme activity was still sedimentable, there was no detectable latency in either the sediment or the supernatant. The digitonin-treated material analysed on metrizamide gradients therefore does not contain any intact lysosomes, i.e. the acid phosphatase associated with the dense peak most probably represents enzyme or lysosome remnants adsorbed to mitochondria. Since, in contrast, [^{14}C]sucrose does not detectably adsorb to cellular constituents (Gordon & Seglen, 1982), it is most likely that the dense peak radioactivity is of exclusively mitochondrial origin.

Effect of 3-methyladenine on digitonin-sensitive and digitonin-resistant sequestration

The experiments shown in Fig. 2 would seem to indicate that the autophagy inhibitor 3-methyladenine at 10 mM selectively inhibits sequestration into the compartment extracted by 0.2–0.5 mg of digitonin/ml, since the additional amount of radioactivity extracted at higher digitonin concentrations was independent of whether the cells have been pretreated with 3-methyladenine or not (the terms 'digitonin-sensitive' and 'digitonin-resistant' will henceforth be applied to sequestered radioactivity which is or is not extracted by 0.2–0.5 mg of digitonin/ml). To further investigate the specificity of 3-methyladenine, its effect on digitonin-sensitive, digitonin-resistant and total sequestration was tested throughout the dose range 0.2–50 mM (Fig. 4). It can be seen that whereas the digitonin-sensitive sequestration was maximally and nearly completely (> 90%) inhibited by 3-methyladenine at 10 mM, the digitonin-resistant sequestration was not significantly effected at this concentration. At higher concentrations of 3-methyladenine, the digitonin-resistant sequestration was gradually suppressed as well, yielding a progressive dose-dependent inhibition of the total sequestration throughout the dose range, as previously observed (Seglen & Gordon, 1984).

These results suggest that the effect of 3-methyladenine at 10 mM is maximal as well as entirely specific for the digitonin-sensitive sequestration believed to represent autophagic–lysosomal activity. The latter process is clearly more sensitive to 3-methyladenine than previously assumed (Seglen & Gordon, 1984), inhibition above 10 mM being exclusively an effect, probably unspecific, on the mitochondrial (digitonin-resistant) [^{14}C]sucrose uptake.

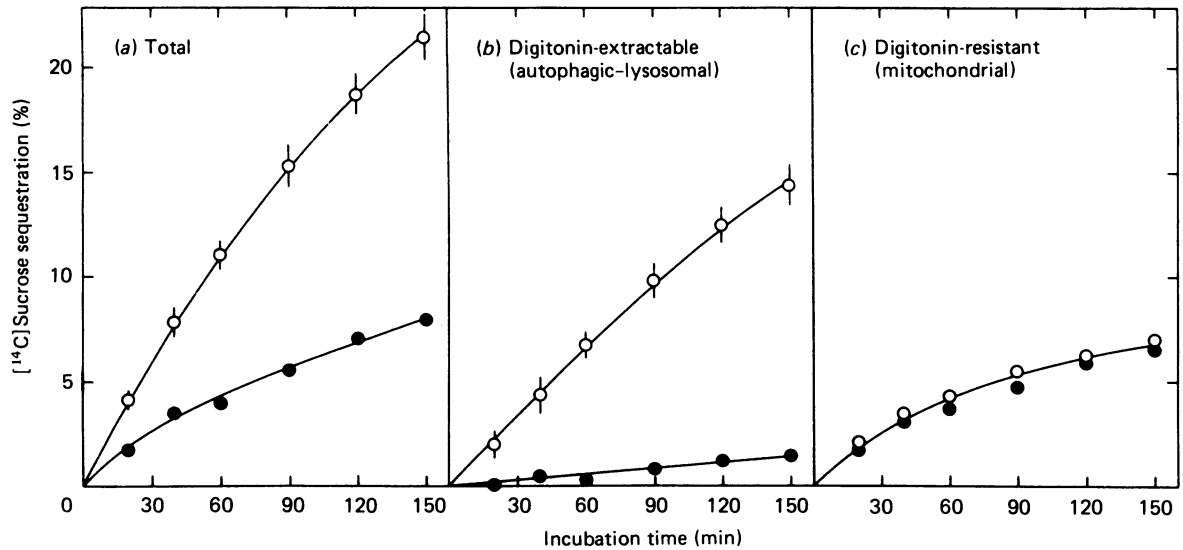

Fig. 5. Time course of [^{14}C]sucrose sequestration into digitonin-extractable and digitonin-resistant cell compartments: effect of 3-methyladenine

Hepatocytes were loaded with [^{14}C]sucrose and incubated (following a 30-min resealing period) at 37 °C for the length of time indicated, in the absence (○) or presence (●) of 10 mM-3-methyladenine. The net amount of [^{14}C]sucrose sequestered into cell corpses (*a*), and into the separated digitonin (0.3 mg/ml) extract (*b*) and the digitonin-resistant corpse pellet (*c*) was measured. Control values (○) are the means ±S.E.M. for three experiments (the S.E.M. values in *c* are so small as to be hidden by the symbols); values with 3-methyladenine (●) represent a single experiment.

Fig. 5 shows the time course of [^{14}C]sucrose sequestration in the presence and absence of 10 mM-3-methyladenine. It is evident that the total intracellular sequestration (Fig. 5*a*) represents the sum of two distinct processes, one which is virtually completely inhibited by 10 mM-3-methyladenine (Fig. 5*b*) and one which is completely unaffected (Fig. 5*c*). The small extent of 3-methyladenine-resistant sequestration observed in the digitonin-sensitive fraction (Fig. 5*b*) can probably be ascribed to some radioactivity being extracted from the mitochondria as well (i.e. their digitonin-resistance being somewhat less than absolute).

3-Methyladenine inhibits autophagic sequestration of [^{14}C]sucrose without a lag (Fig. 5*b*), i.e. the 20 min delay seen when protein degradation is measured (Seglen & Gordon, 1982) apparently represents the minimum time elapsing between the beginning of autophagic sequestration and the beginning of intralysosomal degradation. The immediacy of the effect of 3-methyladenine on sequestration is consistent with its easy penetration into intact hepatocytes, which is demonstrable even at 0 °C (K. Toft & P. O. Seglen, unpublished results).

The mitochondrial [^{14}C]sucrose uptake proceeded at a rapidly declining rate (Fig. 5*c*), suggesting that within a few more hours an equilibrium might have been reached. With the 0 min value included (representing uptake during the 30-min resealing period, subtracted from Fig. 5), intramitochondrial [^{14}C]sucrose would equilibrate around 10–15% of the total intracellular radioactivity, i.e. within the range of values determined for the intramitochondrial volume fraction in hepatocytes (Quinlan *et al.*, 1983). It is therefore possible that the mitochondrial uptake is non-concentrative, representing some kind of facilitated diffusion [since the process is strongly temperature-sensitive (P. B. Gordon & P. O. Seglen, unpublished work), a simple physical diffusion would seem unlikely].

The progressively declining mitochondrial uptake rate can in large measure account for the curvilinear shape of the time curve for total sequestration (Fig. 5*a*). The autophagic sequestration rate (Fig. 5*b*) declines only slowly, and is very nearly constant during the first 1 h. The rate is as high as 6–7%/h, i.e. nearly twice the rate of autophagic protein degradation to be estimated on the basis of 3-methyladenine sensitivity (Seglen & Gordon, 1982). No explanation for this discrepancy is available at present, but it remains possible that the 'fluid-phase' autophagy measured by the sucrose sequestration technique proceeds at a higher rate than the overall sequestration of protein.

Amino acids specifically inhibit digitonin-sensitive sequestration

Amino acids, the major physiological regulators of hepatocytic protein degradation (Schworer & Mortimore, 1979; Seglen *et al.*, 1980) have been found to inhibit markedly overall [^{14}C]sucrose sequestration in isolated hepatocytes (Seglen & Gordon, 1984). As shown in Fig. 6, amino acids suppressed uptake of [^{14}C]sucrose into the light (autophagosomal/lysosomal) metrizamide gradient fraction, but had no effect on the dense (mitochondrial) fraction. Amino acids would therefore seem to be specific inhibitors of that part of the overall sequestration attributable to autophagy, setting the mitochondrial [^{14}C]sucrose uptake aside as a distinct, unrelated process.

Fig. 6 also shows that the lysosome population (as identified by the marker enzyme, acid phosphatase) undergoes a dramatic shift in average density during 2 h of incubation in an amino acid-free medium (Figs. 6*a* and 6*b*). This shift was at least partially prevented by amino

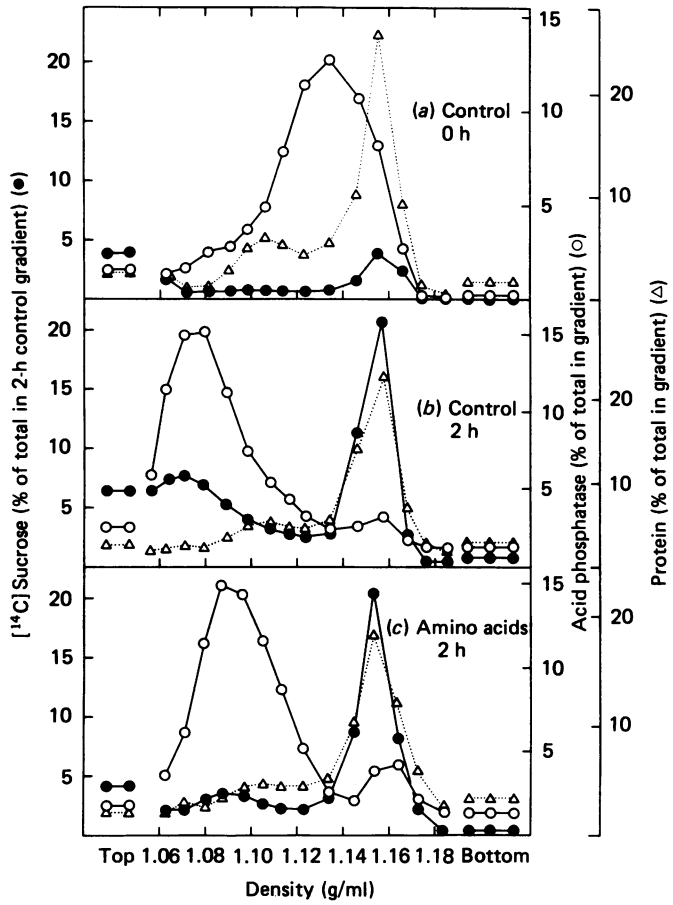

Fig. 6. Amino acid suppression of [^{14}C]sucrose sequestration into the autophagic–lysosomal compartment

Hepatocytes were loaded with [^{14}C]sucrose and preincubated 30 min at 37 °C for resealing (time zero, *a*), then another 2 h at 37 °C in the absence (*b*) or presence of an amino acid mixture (*c*). Cell corpses were homogenized and centrifuged on 0–40% metrizamide gradients, and the fractions analysed for sequestered [^{14}C]sucrose (●), acid phosphatase (○) and protein (△). The amino acid mixture (Kovács *et al.*, 1981) was used at single-strength concentration (approx. 25 mM).

acids (Fig. 6*c*), suggesting autophagic activity as a contributory factor. Suppression of the density shift has been found to be even more marked with 3-methyladenine (probably because 3-methyladenine is not metabolized; cf. the rapid subsidence of the amino acid effect described in Fig. 8), prompting the idea of dense, resting lysosomes becoming light when activated upon fusion with autophagosomes or other substrate-filled vesicles (Seglen & Solheim, 1985). The decreased density could result from a combination of the fusing vesicle being of lower density than the resting lysosome, and of osmotic swelling in the active lysosome due to the formation of low-M_r hydrolytic products.

Since the amino acid effect is exerted exclusively upon the light gradient fraction, it might be expected to be specifically digitonin-sensitive as well. As shown in Fig. 7, the amino acid mixture indeed inhibited sequestration of [^{14}C]sucrose into the digitonin-sensitive compartment in a dose-dependent manner, while leaving the digitonin-resistant cell corpse remnant unaffected. The mitochondrial sugar uptake thus seemed to be completely refractory to amino acids even at 50 mM.

Autophagic (digitonin-sensitive) sequestration of [^{14}C]sucrose was inhibited > 80% by amino acids at the highest concentration tested in Fig. 7. The inhibition was clearly submaximal at 25 mM under the conditions used (sequestration period 30–90 min), possibly because very high concentrations are required for a sustained effect throughout the measurement period.

The latter point is brought out more clearly by the time curves displayed in Fig. 8. The near-linear time course of total [^{14}C]sucrose sequestration in the presence of 25 mM amino acids (Fig. 8*a*) can be shown to be the composite result of a gradually declining control rate mainly in the mitochondrial sugar uptake (Fig. 8*c*), coincidentally balanced by a gradually subsiding amino acid inhibition of autophagy (Fig. 8*b*), as previously postulated (Seglen & Gordon, 1984). The time curves for autophagic (digitonin-sensitive) sequestration shown in Fig. 8(*b*) are strikingly similar to time curves for protein degradation published previously (Seglen *et al.*, 1980), which were interpreted as reflecting rapid consumption of amino acids at high cell concentrations. Taking this factor into consideration, it is conceivable that amino acids may suppress autophagic [^{14}C]sucrose sequestration by more than 80% even within a physiological concentration range, provided cell concentrations are kept low.

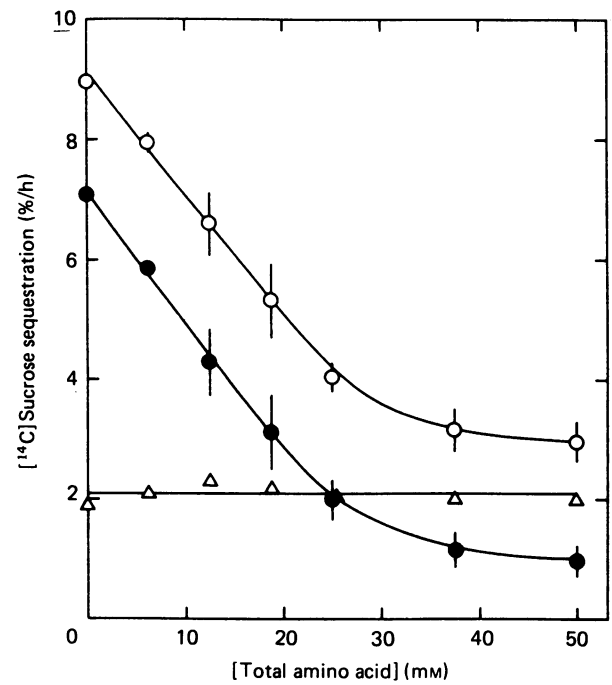

Fig. 7. Effect of amino acid concentration on [^{14}C]sucrose sequestration into digitonin-extractable and digitonin-resistant cell compartments

Hepatocytes were allowed to sequester [^{14}C]sucrose at 37 °C in the presence of various concentrations of amino acids (25 mM corresponds to the single-strength amino acid mixture used, e.g. in Figs. 6 and 8). The rate of sequestration (30–90 min after resealing) into cell corpses (○), digitonin (0.3 mg/ml) extracts (●) and digitonin-resistant corpse pellets (△) was measured. Each value is the mean ± range of two experiments; some of the range indicators are concealed by the symbols.

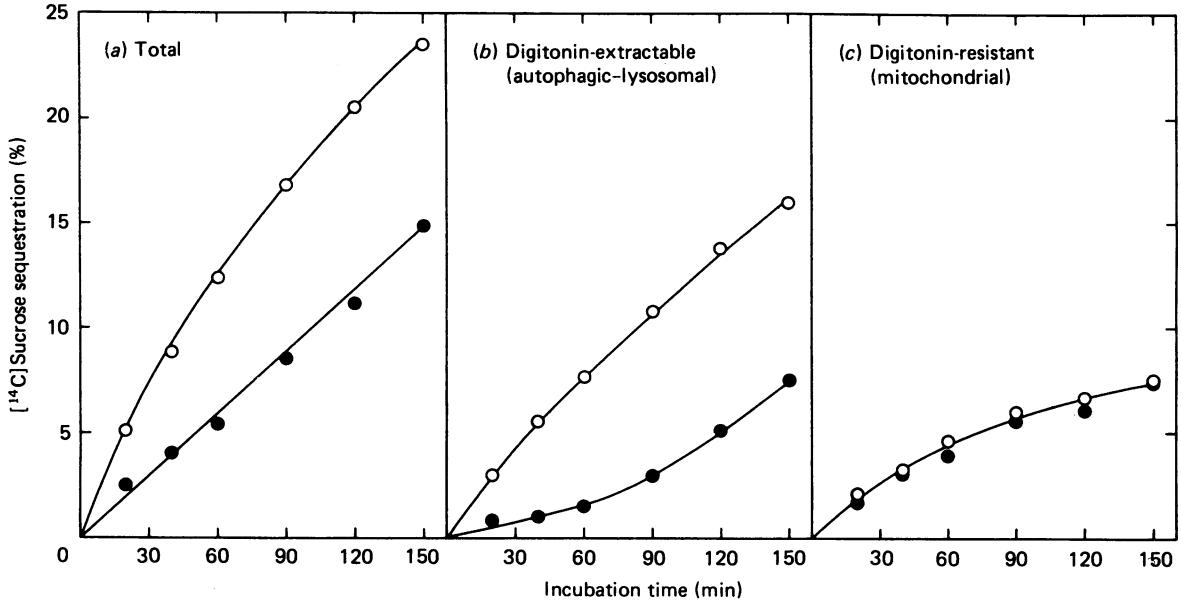

Fig. 8. Time course of [^{14}C]sucrose sequestration into digitonin-extractable and digitonin-resistant cell compartments: effect of amino acids

[^{14}]Sucrose-loaded hepatocytes were incubated at 37 °C for the length of time indicated (following the 30 min resealing period) in the absence (○) or presence of an amino acid mixture (●). The net amount of [^{14}C]sucrose sequestered was measured in cell corpses (*a*), digitonin (0.3 mg/ml) extracts (*b*) and digitonin-resistant corpse pellets (*c*).

DISCUSSION

The ability of digitonin to solubilize biological membranes is dependent on the membrane content of cholesterol (Zuurendonk & Tager, 1974). At the appropriate concentration, digitonin is clearly capable of distinguishing sharply between the relatively cholesterol-rich lysosomes and the relatively cholesterol-poor mitochondria. The membrane composition and digitonin sensitivity of autophagosomes is not known, but several lines of evidence indicate that they are completely extracted along with the lysosomes under the conditions used in our experiments. Firstly, the residual, non-extracted [^{14}C]sucrose has been sequestered by a process which is completely 3-methyladenine-resistant and which therefore cannot involve autophagosome formation to any significant degree. Secondly, digitonin extracts all of the extra sequestered [^{14}C]lactose that accumulates as a result of vinblastine treatment (H. Høyvik, P. B. Gordon & P. O. Seglen, unpublished work), believed to be located in autophagosomes prevented from fusing with lysosomes (Gordon *et al.*, 1985). Digitonin would therefore appear to allow a specific and quantitative estimation of sugar sequestered into lysosomes and autophagosomes.

The rate of autophagic (i.e. digitonin-sensitive) [^{14}C]sucrose sequestration is relatively high (6–7%/h). Autophagic–lysosomal protein degradation, as estimated on the basis of either amine-sensitivity (Seglen *et al.*, 1979) or 3-methyladenine-sensitivity (Seglen & Gordon, 1982), proceeds at only one-half this rate (3–3.5%/h). If sucrose is accepted as a valid fluid-phase marker, our data may suggest that intracellular fluid is sequestered autophagically at a higher rate than intracellular protein. While electron-microscopic evidence tends to indicate that the selectivity of hepatic autophagy is rather limited (Deter, 1971; Holtzman, 1976; Pfeifer, 1976), Dice and co-workers have found that fibroblasts preferentially degrade small and basic proteins (typified by ribonuclease) by a process which seems to be lysosomal (Backer *et al.*, 1983; McElligott & Dice, 1985). It should be noted, however, that our [^{14}C]sucrose technique specifically measures fluid-phase autophagy, and selective sequestration based on adsorption, as in the studies of Dice and coworkers, would therefore not be detected. [^{14}C]Sucrose does not adsorb to cell corpses to any measurable extent (Gordon & Seglen, 1982), and the similarity in the sequestration rates for [^{14}C]sucrose, [^{14}C]lactose (in the presence of vinblastine) and [^{3}H]raffinose (a trisaccharide) is counter-indicative of adsorptive sequestration of any of the sugar probes. Direct penetration of sugars through the membranes of autophagosomes or lysosomes is not very likely in view of the strong sensitivity of the uptake process towards amino acids and 3-methyladenine. Selective fluid sequestration would therefore seem to be a more plausible hypothesis at the moment. Since the fluid, by definition, is sequestered in excess relative to protein, it is obviously not equivalent to the protein-rich cytosol, but it is conceivable that it may contain those cytosolic components which are not organized in fixed, macromolecular assemblies. Alternatively, part of the sequestered protein (approximately one-half) may recycle back to the cytoplasm without being degraded, while sucrose is retained inside the lysosomes – which seems rather unlikely.

The possibility should be considered that the excess fluid-phase autophagy may represent a process distinct from bulk-phase 'macroautophagy', such as the direct invagination of the lysosome membrane known as 'microautophagy' or 'lysophagy' which has been postulated mainly on the basis of electron-microscopic evidence (de Duve & Wattiaux, 1966; Pfeifer, 1976;

Glaumann *et al.*, 1981). If so, both processes would have to be essentially completely suppressible both by amino acids and by 3-methyladenine, which would be highly surprising in view of the very different mechanisms involved. It seems more reasonable to assume a single autophagic sequestration process, the working mechanics of which are such as to include a larger proportion of intracellular fluid than of protein.

The virtually total inhibition of autophagic sequestration by 3-methyladenine has important implications for our understanding of the mechanisms and pathways of protein degradation. Since 3-methyladenine inhibits the overall degradation of (long-lived) protein to almost the same extent as do the lysosomotropic amines (Seglen & Gordon, 1982; Seglen, 1983), it follows that the lysosomal pathway of endogenous protein degradation is, in a quantitative sense, almost exclusively autophagic. The residual, inhibitor-resistant degradation, on the other hand, cannot involve sequestrational mechanisms (there is essentially no 3-methyladenine-resistant sequestration), supporting the notion that this degradation represents a distinctly non-lysosomal pathway (Knowles & Ballard, 1976; Seglen *et al.*, 1979).

The degradation of short-lived protein, a quantitatively minor protein subclass, is somewhat more complicated. The lysosomal (amine-sensitive) degradation of these proteins is only partially blocked by 3-methyladenine, and even less by amino acids (Seglen & Gordon, 1984), suggesting the existence of a non-sequestrational, lysosomal pathway. There are at least two ways by which endogenous proteins can enter lysosomes without being sequestered: as part of the membranes of endocytic vacuoles, or as part of secretory vesicles, both of which fuse directly with lysosomes. Newly synthesized secretory protein, degraded by the morphologically well-characterized process of crinophagy (Holtzman, 1976; Glaumann *et al.*, 1981), is perhaps the most likely candidate for a pre-sequestered, short-lived, lysosomally degraded protein class.

By including a digitonin extraction step in our sequestration assay (Gordon & Seglen, 1982; Seglen & Gordon, 1984) it would now seem possible to study autophagy undisturbed by other processes. Preliminary investigations have indicated that the autophagic sequestration step is both energy-dependent and temperature-sensitive (Seglen *et al.*, 1985), emphasizing its importance as the essential control point in the lysosomal pathway for degradation of endogenous macromolecules.

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