Degradation of transplanted mitochondrial proteins by hepatocyte monolayers

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Reductively [^3H]methylated rat mitochondria and mitochondrial-outer-membrane vesicles and mitochondrial-outer-membrane vesicles where monoamine oxidase is irreversibly labelled by [^3H]pargyline have been transplanted into hepatocytes by poly(ethylene glycol)-mediated organelle or organelle-vesicle cell fusion. During subsequent culture of hepatocyte monolayers for 4–5 days, under conditions which mimic endogenous catabolic rates *in vivo* the transplanted organelle proteins retain their degradation characteristics observed *in vivo* (e.g. mitochondria: average $t_{\frac{1}{2}}$ 72.5 h; monoamine oxidase: $t_{\frac{1}{2}}$ 55 h). In all cases protein degradation with first-order kinetics is only observed after an initial lag period (i.e. 24–30 h after fusion). Transplantation of fluorescein-conjugated organelles showed that the fluorescent material is rapidly internalized (average $t_{\frac{1}{2}}$ 1–6 h) and uniformly distributed in the cytoplasm. During a subsequent 18–24 h period (which corresponds to the lag period for intracellular destruction of transplanted mitochondrial material) the transplanted material is translocated to assume a perinuclear distribution. The destruction of transplanted mitochondrial proteins is compared with endogenous mitoribosomally synthesized proteins (average $t_{\frac{1}{2}}$ 52.5 h). Percoll fractionation of cell homogenates containing transplanted mitochondrial outer membranes where the enzyme monoamine oxidase is irreversibly labelled with [^3H]pargyline shows a distribution of enzyme similar to lysosomal acid phosphatase. After transplantation of reductively methylated ^3H -labelled mitochondrial-outer-membrane vesicles the cells were treated with leupeptin to alter lysosomal density. This treatment leads to the predominant association of acid phosphatase with dense structures, whereas the ^3H -labelled transplanted material predominantly does not change density. Therefore transplanted mitochondrial-outer-membrane proteins are found in intracellular vesicular structures from which the proteins are donated for destruction, at least in part, by a lysosomal mechanism.

The mechanism(s) and regulation of intracellular proteolysis is not understood, although the process(es) is extensive in almost all cell types (Mayer *et al.*, 1980). The advent of erythrocyte and erythrocyte-ghost-mediated microinjection of radio-labelled proteins into unlabelled cells (Loyer *et al.*, 1975; Schlegel & Rechsteiner, 1975; Kaltoft & Celis, 1978; Neff *et al.*, 1981) offers a new method to study the degradation of heterologous and homologous soluble (including cytosolic) proteins.

Abbreviations used: FITC, fluorescein isothiocyanate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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However, this method is not applicable to the majority of intracellular proteins, which function in defined extracytosolic cytomorphological (often membranous) sites. The degradation of these proteins can be studied after organelle transplantation into appropriate target cells.

We have previously reported (Evans & Mayer, 1982) the development of a technique for sub-organelle vesicle transplantation involving the poly(ethylene glycol)-mediated transfer of reductively methylated ^3H -labelled outer mitochondrial membrane to freshly isolated rat hepatocytes. The subsequent destruction of this deliberately mis-compartmentalized material was determined in hepatocyte monolayers maintained for several days

under culture conditions that preserved protein metabolic rates observed *in vitro*.

We have now extended the work to include mitochondrial transplantation, so that the destruction of proteins in different mitochondrial compartments can be studied. Biochemical measurements and fluorescent microscopy have been employed to assess the role of cytomorphological location in determining the degradation of transplanted mitochondrial proteins and the mitochondrial-outer-membrane enzyme, monoamine oxidase. The studies were complemented by investigating the destruction of mitoribosomally synthesized mitochondrial proteins.

Materials and methods

Collagenase was obtained from Boehringer-Mannheim, insulin from Boots, Nottingham, U.K., and Fisofluor from Fisons, Loughborough, Leics., U.K. Leibovitz L-15 culture medium was supplied by Flow Laboratories, Irving, Ayrshire, Scotland, U.K., newborn-calf serum and antibiotics were from Gibco, Paisley, Renfrewshire, Scotland, U.K., and L-[4,5-³H]leucine and NaB³H₄ were from Amersham International, Amersham, Bucks., U.K. [³H]-Pargyline was supplied by New England Nuclear Corp., Dreieich, Germany. Poly(ethylene glycol) 1500 was from BDH, Poole, Dorset, U.K. All other chemicals were obtained from Sigma, Poole, Dorset, U.K. Cells were examined by using a Leitz-Wetzlar fluorescent microscope; photographs were taken on Kodak Ektachrome 400 (daylight) EL135-36 film.

Preparation of hepatocytes

Hepatocytes were prepared as previously described (Evans, 1981). For further details, see the legends to the Figures.

Preparation and labelling of mitochondria and mitochondrial-outer-membrane vesicles

Rat livers were perfused *in situ* (Evans, 1981) with 150 ml of Ca²⁺-free Krebs & Henseleit (1932) bicarbonate buffer. Mitochondria were prepared from the blood-free livers (Bustamante *et al.*, 1977). Mitochondrial-outer-membrane vesicles were prepared by the method of Martinez & McCauley (1977). Reductive methylation of both preparations was carried out as described by Evans & Mayer (1982).

A solution of FITC in Dulbecco's phosphate-buffered saline, pH 9.5 (Dulbecco & Vogt, 1954), was prepared by shaking FITC (1 mg/ml) for 1 h. The suspension was centrifuged (3000 *g*_{av} for 30 min) and the clear supernatant used for fluorescent labelling of mitochondria. Each mitochondrial preparation (4 mg of protein) was resuspended in Dulbecco's phosphate-buffered saline, pH 7.4, and

mixed with an equal volume of the FITC solution. The mixture was protected from light and intermittently shaken for 20 min at room temperature. The preparation was then dialysed against fresh changes (3 × 1 litre) of Dulbecco's phosphate-buffered saline, pH 7.4, supplemented with newborn-calf serum (4%, v/v) for 15 h.

Labelling of mitochondrial-outer-membrane vesicles with [³H]pargyline

Monoamine oxidase was labelled in mitochondrial-outer-membrane vesicles with [³H]pargyline (Pintar *et al.*, 1979). Each preparation was dialysed against fresh changes (2 × 1 litre) of Dulbecco's phosphate-buffered saline, pH 7.4, supplemented with pargyline (0.1 mM) and bovine serum albumin (1 mg/ml) and then against one change (1 litre) of Dulbecco's phosphate-buffered saline, pH 7.4.

Hepatocyte-mitochondria / mitochondrial - outer - membrane-vesicle fusions

Fusions were carried out essentially as described previously (Evans & Mayer, 1982). Hepatocytes (3.5 × 10⁷) were pelleted by low-speed centrifugation (100 *g* for 3 min). Mitochondrial-outer-membrane protein (1 mg) or mitochondrial protein (2 mg) was added in 200 μ l of Dulbecco's phosphate-buffered saline, pH 7.4, and the tube (Sterilin Universal container, 30 ml) gently rotated to ensure thorough mixing. A 500 μ l portion of poly(ethylene glycol) (50%, v/v) in serum and antibiotic-free Leibovitz L-15 medium, supplemented with glucose (8.3 mM) and Hepes (25 mM), pH 7.4, was added and the tube gently rotated for 90 s. A 10 ml portion of poly(ethylene glycol)-free supplemented medium was then slowly added. The cells were pelleted (100 *g* for 3 min) and washed three times in culture medium, which consisted of 10 ml of the L-15 medium, pH 7.4, supplemented with glucose (8.3 mM), Hepes (25 mM), heat-inactivated newborn-calf serum (10%) containing streptomycin and penicillin (100 units of each/ml). At each wash the hepatocytes were redispersed by passage through a 10 ml pipette. Finally the cells were suspended in 10 ml of the culture medium. Preliminary experiments showed that, in the absence of poly(ethylene glycol), less than 10% of the radioactivity found in the presence of poly(ethylene glycol) was associated with the cell pellet. Hepatocytes were cultured on an adsorbed collagen support (type III) in 60 mm-diameter tissue-culture dishes. Coated dishes were prepared by covering the bottom of each dish with sterile collagen (2 mg/ml) in 0.1% acetic acid. The dishes were left for 1 h, washed three times with sterile water and placed in a 37°C incubator for 48 h before use. Approx. 1.2 × 10⁶ cells were added to 3 ml of culture medium, which contained insulin

(0.8 µg/ml) and dexamethasone (1 µM). The medium was changed after 2.5 h and then at 24 h intervals, unless otherwise indicated. Inhibitors, where appropriate, were added at the first change of medium.

Labelling of endogenous mitoribosomally synthesized hepatocyte proteins

Freshly isolated hepatocytes (2.5×10^6) were plated out in leucine-free culture medium supplemented with insulin (0.8 µg/ml), dexamethasone (1 µM), cycloheximide (50 µg/ml) ± chloramphenicol (100 µg/ml). The medium was changed after 2.5 h. Cells were then cultured in the same medium but containing 3 µCi/ml of L-[4,5-³H]leucine. After 14 h the cells were washed twice with culture medium containing leucine (10 mM). Each washing entailed a 45 min period at 37°C. Subsequent experiments on the effects of inhibitors of protein degradation on the endogenously labelled proteins were carried out in culture medium supplemented with insulin (0.8 µg/ml), dexamethasone (1 µM) and leucine (10 mM). Preliminary studies showed that, under these labelling conditions, cycloheximide inhibited protein synthesis by 98%. The combined presence of cycloheximide (50 µg/ml) and chloramphenicol (100 µg/ml) inhibited protein synthesis by 99.4%. Hepatocytes labelled in the presence of cycloheximide and subfractionated on a Percoll gradient (30%, v/v) showed a distribution of labelled material coincident with succinate dehydrogenase, in contrast with control cultures, where a general labelling of cell protein was observed.

Cell harvesting

At the indicated times the medium was removed and frozen. Cells were harvested by treatment with trypsin (Evans & Mayer, 1982), washed twice in culture medium (1.5 ml), pelleted and frozen.

Assay of succinate dehydrogenase activity

The enzyme-locating solution contained: (a) a dye mixture containing 1 vol. of Nitroblue Tetrazolium (10 mg/ml in Dulbecco's phosphate-buffered saline, pH 7.4), 1 vol. of phenazine methosulphate (0.4 mg/ml in phosphate-buffered saline, pH 7.4) and 2.5 vol. of phosphate-buffered saline, pH 7.4; (b) the enzyme substrate, sodium succinate (20 mM) was dissolved in phosphate-buffered saline, pH 7.4.

The enzyme-activity-locating mixture consisted of 1 vol. of the dye mix (a) and 5 vol. of the substrate solution (b). Medium was aspirated from each fused hepatocyte-mitochondria monolayer and 3 ml of the locating mixture added to each dish. The dishes were incubated at 37°C.

Cytochemical procedures

(a) *Fluorescence microscopy*. Glass coverslips (16 mm diameter) were cleaned aseptically by

successive washes (5 min each) in ethanol (98%), HCl (1 M) and sterile water. Coverslips (three) were placed in 60 mm-diameter tissue-culture dishes. Finally coverslips were rinsed with ethanol (98%) before coating the coverslips with collagen. Hepatocytes were cultured on these supports as described above. At the times indicated the culture medium was removed and the cells washed with phosphate-buffered saline, pH 7.4. The hepatocytes were fixed in 4% (v/v) formaldehyde in phosphate-buffered saline, pH 7.4, for 30 min. The coverslips were then mounted in a mixed glycerol/p-phenylenediamine solution, pH 8.0, to reduce photo-bleaching (Johnston & de C. Nogueira Aranjó, 1981).

(b) *Optical microscopy*. Hepatocytes were cultured as above, fixed in glutaraldehyde (3%, v/v, in phosphate-buffered saline) and stained with Toluidine Blue (1% in 50 mM-sodium phosphate buffer, pH 7.4). Excess stain was removed from the cells with distilled water and finally with ethanol (98%).

Cell homogenization and Percoll gradient centrifugation

Hepatocytes were harvested by treatment with trypsin, washed and resuspended in 0.25 M-sucrose (1 ml). The cells were sonicated (one 10 s burst at amplitude 4 µm peak-to-peak with an MSE sonicator) and layered on 10 ml of Percoll (30%, v/v) in 0.25 M-sucrose in 14 ml centrifuge tubes. Cell homogenates were centrifuged at 33 000 rev./min for 30 min in rotor 43114-121 (MSE Prepspin 75). Fractions (0.5 ml) were collected from the gradients.

Analyses

Protein and the activities of tyrosine aminotransferase (EC 2.6.1.5) and lactate dehydrogenase (EC 1.1.1.27) were determined as described previously (Evans, 1981). Subcellular fractions were identified by measuring plasma-membrane alkaline phosphodiesterase I (EC 3.1.4.1; Touster *et al.*, 1970), lysosomal acid phosphatase (EC 3.1.3.2; Nelson, 1966) and mitochondrial succinate dehydrogenase (EC 1.3.99.1; Pennington, 1961). Cell pellets were resuspended in 0.1 M-potassium phosphate, pH 7.6, and broken by sonication (two 10 s bursts at an amplitude setting of 6 µm peak-to-peak in an MSE sonicator). The containing tube was cooled in ice. Radioactivity in cell proteins was determined after precipitation with trichloroacetic acid (10%, w/v, final concn.) containing 5 mM-leucine or 1 mM-pargyline where appropriate. The precipitates were redissolved and reprecipitated once before being finally dissolved in formic acid. Radioactivity was measured with Fiso fluor as scintillant with a Packard CD 460 liquid-scintillation counter.

Fig. 1. Destruction of transplanted ^3H -labelled mitochondrial proteins in hepatocyte monolayers

The efficiency of the fusion process [(d.p.m. transplanted/d.p.m. added) $\times$ 100] was 11%. The trichloroacetic acid-insoluble radioactivity in the cell pellet is shown (●). The values are the means $\pm$ S.D. of measurements on a minimum of four separate plates.

Results

The average rate of destruction of reductively methylated ^3H -labelled mitochondrial proteins, in rat liver mitochondrial preparations transplanted by means of poly(ethylene glycol)-mediated fusion to hepatocytes, which are subsequently maintained in monolayers under conditions that mimic protein turnover rates *in vivo* (Evans & Mayer, 1982), is shown in Fig. 1. After an initial lag period the average first-order rate of destruction of mitochondrial proteins is $72.5 \pm 7.8\text{ h}$ (S.D.) ($n = 5$). Transplanted radiolabelled material was resistant to trypsin (within 7 h). Furthermore, after fusion and attachment of the cells to the substratum (7 h), a succinate-dehydrogenase-activity-locating mixture failed to detect any mitochondrial succinate dehydrogenase activity adventitiously attached to the

hepatocyte surface or collagen support; formazan colour developed intracellularly only after eventual cell death in the locating mixture. Viable hepatocytes are normally impermeable to succinate (Mapes & Harris, 1975). The slow initial rate of degradation of transplanted mitochondrial proteins occurred in every experiment and was also observed for mitochondrial-outer-membrane proteins after fusion of mitochondrial-outer-membrane vesicles with hepatocytes (see below).

Mitochondrial preparations were fluorescently labelled by FITC before hepatocyte fusion. Subsequently the fate of transplanted material was examined by fluorescent microscopy. Immediately after fusion the rounded cells in suspension exhibited a surface fluorescence (results not shown). Subsequently after plating the cells exhibited a generalized distribution of fluorescence (Fig. 2a). After 6 h (Fig. 2b) the cells had flattened and showed a more punctate arrangement of fluorescent label in the cytoplasm. By 24 h after fusion (Fig. 2c) there was a pronounced perinuclear orientation of fluorescent material. Optical-microscopic examination, after cell staining with Toluidine Blue, failed to show any vacuoles in a similar juxtannuclear position (Fig. 2d). By 96 h after fusion, fluorescence was associated with large vacuolar structures occupying a perinuclear location (Fig. 2e). These vacuoles were now also visible by optical-microscopical observation of stained cells (Fig. 2f). Vacuolation is not cytopathological, in that it does not affect the first-order degradation of transplanted mitochondrial proteins (Fig. 1). Vacuolation is not observed when mitochondrial-outer-membrane vesicles are transplanted in hepatocytes; the punctate vesicular fluorescence fades gradually in the cells (results not shown).

The consistent observation of this initial re-distribution of transplanted fluorescent material in all microscopic fields of view of recipient cells is worthy of special comment. The initial 're-orientation-translocation' of transplanted material seen by fluorescence microscopy (Fig. 2) occurs over a time period that essentially corresponds to the lag period during which apparent first-order destruction of mitochondrial proteins or monoamine oxidase does not occur (Fig. 1, and Figs. 5 and 6 below).

It is well established that proteins synthesized in the presence of cycloheximide are mitoribosomally synthesized (Knecht *et al.*, 1980), and we have conclusively demonstrated this for hepatocyte monolayers (see the Materials and methods section). Therefore a comparison between the rates of degradation of transplanted mitochondrial proteins and endogenous mitoribosomally synthesized mitochondrial proteins can be made routinely without problems of controlled cell disruption and sub-cellular fractionation to prepare mitochondrial fractions.

Fig. 2. *Fate of fluorescein-conjugated mitochondrial material in hepatocyte monolayers*
 (a) At 1 h after fusion; (b) 6 h after fusion; (c) 24 h after fusion; (d) Toluidine Blue-stained cells 24 h after fusion; (e) 96 h after fusion; (f) Toluidine Blue-stained cells 96 h after fusion. Magnifications: fluorescence microscopy, $\times 320$; optical microscopy, $\times 640$. The three rounded fluorescent bodies (arrow) represent dead hepatocytes above the monolayer.

Endogenous mitoribosomally synthesized proteins were pulse-radiolabelled by incubation of hepatocytes for 14 h in the presence of cycloheximide ($50 \mu\text{g/ml}$). Control hepatocytes were also cultured with [^3H]leucine in the combined presence of cycloheximide ($50 \mu\text{g/ml}$) and chloramphenicol

($100 \mu\text{g/ml}$) for 14 h to inhibit protein synthesis (99.4%). Hepatocytes were washed twice before protein-degradation measurements commenced. Complete inhibition of protein synthesis with two inhibitors is necessary so that the release of trichloroacetic acid-soluble radioactivity from hepato-

Fig. 3. Degradation of mitoribosomally synthesized mitochondrial proteins in hepatocyte monolayers

Hepatocytes were labelled with L-[4,5-³H]leucine in the presence of cycloheximide as described in the Materials and methods section. The medium was not changed after the washing procedure. □, Trichloroacetic acid-insoluble radioactivity in the cell pellet; ■, trichloroacetic acid-soluble radioactivity in the medium. The values are means ± s.d. of measurements on a minimum of four separate plates.

cytes derived from degradation of mitoribosomally synthesized proteins can be corrected for the leakage of [³H]leucine from a residual post-wash intracellular [³H]leucine pool (Fig. 3). Since cycloheximide is an inhibitor of hepatocyte protein degradation (Ballard, 1977), it is necessary to show that it is completely removed from the cells after the radiolabelling period. This is demonstrated, since there is a rapid initial increase in the activity of the inducible enzyme, tyrosine aminotransferase, after washing hepatocytes to remove cycloheximide and [³H]leucine (results not shown).

The degradation of mitoribosomally synthesized mitochondrial proteins in hepatocyte monolayers is shown in Fig. 3. The average rate of destruction of the proteins corresponds to a $t_{1/2}$ of 52.5 h. Chloroquine (0.2 mM), leupeptin (0.5 mM) and chymostatin (0.2 mM) inhibited the degradation of mitoribosomally synthesized proteins by 81, 43 and 29% respectively (Fig. 4). Leupeptin is the least cytotoxic compound in long-term cultures (Tanaka *et al.*, 1981). Interestingly, this compound also inhibited the de-

Fig. 4. Effect of inhibitors on the degradation of mitoribosomally synthesized mitochondrial proteins in hepatocyte monolayers

Experiments were conducted as described in Fig. 3. After the washing procedure fresh medium was added with or without inhibitors. Cells labelled in presence of cycloheximide: trichloroacetic acid-soluble radioactivity in medium (○) and trichloroacetic acid-soluble radioactivity in medium with 0.2 mM-chymostatin (■), with 0.5 mM-leupeptin (□) and with 0.2 mM-chloroquine (△) is shown. Cells labelled in the combined presence of cycloheximide and chloramphenicol: ●, trichloroacetic acid-soluble radioactivity in medium. Values are means ± s.d. of measurements on a minimum of four separate plates.

gradation of proteins in a mixture (1:1) of transplanted reductively methylated ³H-labelled and fluorescent mitochondria by approx. 40–50% (Fig. 5).

Measurements on the average rate of destruction of both transplanted reductively methylated mitochondrial proteins (Figs. 1 and 5) and mitochondrial-outer-membrane proteins (Evans & Mayer, 1982) have been made in hepatocyte monolayers. In contrast, the results in Fig. 6 show the rate of destruction of a single enzyme in transplanted mitochondrial-outer-membrane

Fig. 5. Effect of leupeptin (0.5 mM) on the degradation of a mixture (1:1) of transplanted ^3H -labelled mitochondria and fluorescein-conjugated mitochondria in hepatocyte monolayers

Trichloroacetic acid-soluble radioactivity in medium in the absence of leupeptin (□) and in the presence of leupeptin (■) is shown. The values are the means $\pm$ S.D. of measurements on a minimum of four separate plates.

vesicles. Monoamine oxidase was irreversibly derivatized by the suicide inhibitor [^3H]pargyline (Pintar *et al.*, 1979). The enzyme, in the transplanted mitochondrial-outer-membrane material was, after a lag period (cf. Figs. 1 and 5), degraded at a rate that corresponds to a t_4 of 55 h.

Fluorescence observation (see above) indicates a vesicular intracellular distribution of transplanted mitochondrial and mitochondrial-outer-membrane material (results not shown). The nature of internalized mitochondrial-outer-membrane material was further examined by Percoll gradient fractionation of homogenized cells. Fig. 7 shows the distribution of transplanted mitochondrial-outer-membrane vesicles with monoamine oxidase derivatized with [^3H]pargyline. The radiolabel profile corresponds to the lysosomal acid phosphatase.

Several particulate vesicular species are coincident with the more buoyant lysosomal peak (Merion & Sly, 1983; Fig. 7c). However, leupeptin can dramatically increase lysosomal density (Furuno *et al.*, 1982) and therefore can be used to identify

Fig. 6. Destruction of [^3H]pargyline-labelled monoamine oxidase in transplanted mitochondrial outer membranes in hepatocyte monolayers

The efficiency (see Fig. 1) of the fusion process was 6.5%. Trichloroacetic acid-insoluble radioactivity in cell pellet (□) and trichloroacetic acid-soluble radioactivity in medium (■) is shown. Values are means $\pm$ S.D. of measurements on a minimum of four separate plates.

lysosomal components or content. Fig. 8 shows that leupeptin treatment of hepatocytes (5 h) dramatically increases the density of vesicles containing lysosomal acid phosphatase without producing a corresponding alteration in the density of intracellular structures containing transplanted reductively methylated mitochondrial-outer-membrane proteins. Therefore, at a time when transplanted mitochondrial outer membrane has been internalized (cf. Fig. 2), it is localized in extralysosomal vesicular structures.

Discussion

In the present study we have successfully transplanted mitochondrial material to hepatocytes by a poly(ethylene glycol)-mediated process. The intracellular location of the transplanted mitochondrial material is shown by its inaccessibility to the mitochondrial-succinate-dehydrogenase-locating mixture, its resistance to proteolytic digestion and direct observation of intracellular material by fluorescence microscopy. Fluorescent mitochondrial material is ubiquitously distributed amongst the hepatocytes.

The destruction of transplanted mitochondrial proteins with apparent first-order kinetics is initially preceded by a lag period corresponding to a phase of slower degradation (Figs. 1, 5 and 6).

The initial slow destruction of transplanted proteins corresponds to the period (Fig. 2) when

Fig. 7. Percoll-gradient fractionation of sonicated hepatocytes after transplantation of [^3H]pargyline-derivatized mitochondrial-outer-membrane vesicles

Hepatocyte monolayers (32h after fusion) were harvested and fractionated as described in the text. Gradient fractions were analysed for the activities of acid phosphatase (a), lactate dehydrogenase (b), alkaline phosphodiesterase (c), succinate dehydrogenase (d) and trichloroacetic acid-insoluble ^3H radioactivity (e).

fluorescence microscopy reveals a redistribution of the transplanted material to assume a perinuclear distribution, i.e. cytomorphological location may be of fundamental importance for mitochondrial (organelle) protein degradation.

The observed 'reorientation-translocation' of mitochondrial material may really just represent the distribution of transplanted material in the cytoplasm, i.e. the distribution may simply reflect the cytoplasmic arrangement of hepatocytes in cultured monolayer. However, a 'perinuclear' distribution of lysosomes (Stacey & Allfrey, 1977), mitochondria (Couchman & Rees, 1982) and Golgi elements (Pastan & Willingham, 1981) has been described in cultured cells. We have observed the distribution of fluorescent material in hepatocytes cultured on gelatin and polylysine, which prevent and retard cell shape changes and cell spreading respectively (Gjessing & Seglen, 1980). In both cases the redistribution of fluorescent material was the same as for cells on collagen supports, indicating that the redistribution of fluorescent material to a perinuclear position is a real hepatocyte phenomenon.

Perinuclear distribution of heterologously microinjected fluorescein-conjugated soluble protein has been previously reported (Stacey & Allfrey, 1977). Such material may be destroyed faster than unconjugated [^{125}I]iodinated microinjected soluble protein (Zavortink *et al.*, 1979). However, reductively methylated mitochondria (Fig. 5) in a mixture (1:1) of methylated and fluorescein-conjugated mitochondria are destroyed at the same rate as in the absence of fluorescein-conjugated mitochondria. More importantly (results not shown) the rate of destruction of monoamine oxidase (labelled with [^3H]pargyline) is the same in hepatocytes after transplantation of fluorescein-conjugated or unconjugated mitochondrial-outer-membrane vesicles. We therefore have no evidence for faster degradation of transplanted fluorescein-conjugated mitochondrial proteins in hepatocytes.

Translocation of mitochondrial material to give a perinuclear distribution before degradation of mitochondrial proteins (at rates comparable with those observed *in vivo*) occurs, should involve cytoskeletal elements. We have demonstrated in preliminary

Fig. 8. *Effect of leupeptin on the distribution of acid phosphatase and transplanted reductively methylated mitochondrial outer membrane on Percoll-gradient fractionation*

The medium above hepatocyte monolayers (7 h after fusion) was supplemented with leupeptin (0.5 mM). After a further 5 h incubation the cells were harvested and fractionated as described in Fig. 7. The distribution of trichloroacetic acid-insoluble radioactivity and acid phosphatase was compared in control (a and b) and leupeptin-treated (c and d) cells.

experiments (results not shown) that colchicine (2 μM) but not cytochalasin B (2 μM) disrupts the translocation process and consequently inhibits (60%) degradation of monoamine oxidase in transplanted mitochondrial-outer-membrane vesicles.

The initial slowed rate of destruction of transplanted proteins could be a response to overloading of the intracellular proteolytic system(s) with transplanted material. However, continuous saturation of the destructive system with transplanted protein material could not give rise to the measured apparent first-order rate of protein destruction (Figs. 1, 5 and 6).

We do not currently know the intracellular nature of the transplanted mitochondria, but since introduction of material into recipient cells is poly-

(ethylene glycol)-dependent, we would surmise that mitochondrial outer membrane may fuse with recipient-hepatocyte plasma membrane and therefore could inject the mitoplast core of each mitochondrion into the target cell. Alternatively, it has recently been suggested that whole mitochondria may be internalized by some endocytic process (see below), but only after prolonged exposure (12 h) of mitochondrial preparations to recipient cells (Clark & Shay, 1982). In this way resistance to chloramphenicol can be stably transferred to sensitive murine adrenal-tumour cells by culture of the cells with mitochondrial preparations from resistant murine tumour cells.

The nature of the internalized mitochondria after transplantation does not preclude the subsequent

redistribution of fluorescently labelled mitochondrial material to assume a perinuclear orientation (Fig. 2) similar to that seen in some cell types for endogenous mitochondria which have been fluorescently labelled with organelle-specific dyes (Erbrich *et al.*, 1982; Chen *et al.*, 1981).

We have compared the destructive fate of transplanted exogenous proteins with endogenous mitoribosomally synthesized proteins. Interestingly, the average rate of destruction (Fig. 3) of mitoribosomally synthesized proteins is relatively slow ($t_{\frac{1}{2}}$ 52.5 h) and similar to the average destructive rate of transplanted mitochondrial proteins. Chloroquine, leupeptin and chymostatin inhibit the breakdown of mitoribosomally synthesized proteins by 80, 43 and 29% respectively (Fig. 4). Leupeptin (the least cytotoxic inhibitor when assessed by cell detachment from the culture dishes) inhibited the destruction of transplanted mitochondrial proteins by approx. 40–50% (Fig. 5). This suggests that mitoribosomally synthesized proteins and transplanted mitochondrial proteins may be degraded by a similar mechanism.

In the majority of these studies we have measured the average degradation rates for a mixture of mitochondrial or mitochondrial-outer-membrane (Evans & Mayer, 1982) proteins. Therefore we have also measured the rate of destruction in transplanted mitochondrial-outer-membrane vesicles of monoamine oxidase after irreversible derivatization via its FAD prosthetic group with [^3H]pargyline (Fig. 6). The measured destruction rate of the enzyme corresponds to a $t_{\frac{1}{2}}$ of 55 h, which is the same as the rate in liver *in vivo* when measured by a double-isotope technique (Russell *et al.*, 1980) or by the restoration of enzyme concentration after irreversible inhibition of enzyme activity (Tipton & Della Corte, 1979).

Percoll fractionation (Fig. 7) suggests that transplanted mitochondrial outer membrane containing [^3H]pargyline/monoamine oxidase co-distributes with the lysosomal acid phosphatase after mild sonication of hepatocytes. Mild sonication of hepatocytes causes a bimodal distribution of lysosomal acid phosphatase in a similar manner to the effects of nitrogen cavitation (Rome *et al.*, 1979) or Polytron homogenization (Merion & Sly, 1983) on fibroblasts. The more buoyant acid phosphatase marker may be found in vesicular structures derived from the collapse of lysosomal vesicles on sonication or represent a special intracellular organelle, e.g. Golgi–endoplasmic-reticulum–lysosome complex (GERL; Rome *et al.*, 1979). It is noteworthy that transplanted fluorescent mitochondrial material (Fig. 2c) and fluorescent mitochondrial outer membrane (results not shown) often assume a pronounced sidedness after translocation to a perinuclear site, which is similar to that shown by endogenous Golgi

apparatus in tissue-culture cells (Couchman & Rees, 1982).

Vesicular structures derived from several intracellular organelles, i.e. lysosomes (Fig. 7a), plasma membrane (Fig. 7c), endoplasmic reticulum and Golgi apparatus (Rome *et al.*, 1979; Merion & Sly, 1983) are all found at the same low buoyant density. The proportion of transplanted mitochondrial outer membrane in the lysosomal compartment cannot therefore be estimated directly.

However, a large increase in the density of lysosomes can be achieved by administration *in vivo* of the proteolytic inhibitor leupeptin (Furuno *et al.*, 1982), which induces lysosomal constipation.

The distribution of acid phosphatase and reductively methylated ^3H -labelled mitochondrial-outer-membrane proteins on Percoll gradients after treatment (5 h) of hepatocytes with leupeptin (0.5 mM) is shown in Fig. 8. Leupeptin clearly causes a dramatic increase in the density of most acid phosphatase-containing vesicular structures (cf. Figs. 8b and 8d) in a manner similar to that observed for liver lysosomes after administration of leupeptin *in vivo* (Furuno *et al.*, 1982). However, most of the transplanted ^3H -labelled mitochondrial-outer-membrane material does not undergo the change in density (cf. Figs. 8a and 8c). A small proportion of the ^3H -labelled mitochondrial-outer-membrane material does undergo a density change (Fig. 8c), indicating its presence in the leupeptin-sensitive lysosomal compartment.

Mitochondrial-outer-membrane material is clearly intracellular (fluorescence microscopy; results not shown) 7 h after transplantation when the leupeptin treatment was commenced. Therefore the use of leupeptin to change lysosomal density clearly shows that mitochondrial-outer-membrane proteins are found in some intracellular non-lysosomal vesicular structure from where they may be donated, at least in part, to lysosomes for destruction.

The data on the average rates of degradation of transplanted mitochondrial proteins, mitochondrial-outer-membrane proteins (Evans & Mayer, 1982) and monoamine oxidase suggest that some feature(s) of transplanted internalized mitochondrial membranes and/or their proteins survive the experimental insults and still retain the information to be destroyed at rates comparable with those observed *in vivo*. These results provide some mechanistic basis for the populations of mitochondrial degradation rates observed for mitochondria *in vivo* (Russell *et al.*, 1982).

Together these observations may mean that a rate-limiting step in the degradation of some mitochondrial proteins is vesicle–lysosome fusion or vesicle–lysosome protein exchange.

The mechanism by which transplanted material is internalized after poly(ethylene glycol)-dependent

fusion is not known. However, the destructive fate of internalized proteins is not the same as proteins internalized into hepatocytes (Berg & Tolleshaug, 1980) or other cells (Steinman *et al.*, 1974) by receptor-mediated endocytosis or pinocytosis, where proteins are destroyed extremely quickly (i.e. $t_{1/2}$ = 30–60 min and 7.5 h respectively). Endocytosed organelles are destroyed very quickly ($t_{1/2}$ 3–6 h) in Kupffer cells in liver *in vivo* when undetectable endocytic uptake into hepatocytes occurs (Glaumann & Marzella, 1981).

Material transplanted into the hepatocyte plasma membrane may be internalized by some continuous surface-surveillance mechanism (Widnell *et al.*, 1982) or by a surveillance mechanism invoked by the fusion insult. Such alternative mechanisms have been recruited to explain the lymphocytic capping phenomenon (Corps *et al.*, 1982).

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