Cellular and lysosomal uptake of methylamine in isolated rat hepatocytes

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Upon addition of methylamine to intact cells, this lysosomotropic weak base accumulates intracellularly as the result of at least two different mechanisms: (1) facilitated diffusion across the plasma membrane, i.e. a process which is carrier-mediated and subject to both *trans*-stimulation (accelerative exchange) and *cis*-inhibition (competition) by other amines (e.g. ammonia, methylamine and triethylamine); this transport process is furthermore non-concentrative, energy-independent, and (although moderately temperature-sensitive) operative even at 0°C; (2) active uptake, i.e. an energy-dependent concentrative process which is inhibited by anoxia and energy inhibitors. With time, methylamine accumulates in lysosomes and gives rise to a lysosomal swelling which is easily visible by optical microscopy, and which causes the cells to appear coarsely granular. After a 1 h incubation with 10 mM-methylamine, the total cell volume is increased by about 12%. Under anoxic conditions or in the presence of energy inhibitors, lysosomal swelling is abolished regardless of there being a high concentration of methylamine intracellularly (taken up by facilitated diffusion). The continuous accumulation of methylamine in lysosomes therefore seems to depend on an energy-requiring process (such as continuous proton pumping), and not only on trapping by Donnan-equilibrium-generated protons.

The interior of lysosomes is acidic, with a pH of 4.5–5.0 according to measurements made on intact cells (Reijngoud & Tager, 1977; Ohkuma & Poole, 1978; Jacques & Bainton, 1978; Geisow *et al.*, 1981). A low pH appears to be a prerequisite for maximal activity of the lysosomal hydrolases, most of which have an acidic pH optimum (Barrett, 1972). Two different mechanisms appear to contribute to this low pH: (1) a Donnan equilibrium (Reijngoud & Tager, 1977; Reijngoud, 1978), established by an excess of negatively charged sialate moieties intralysosomally (Adhikari & Vakil, 1980), capable of maintaining a pH difference of 1.2 units across the lysosomal membrane even in isolated lysosomes (Henning, 1975; Reijngoud *et al.*, 1976; Hollemans *et al.*, 1979); and (2) an ATP-dependent proton pump in the lysosomal membrane which injects protons into the lysosome at the expense of energy (Mego, 1979; Dell'Antone, 1979; Schneider, 1981; Ohkuma *et al.*, 1982). If the pump is blocked by treating cells with metabolic inhibitors, the intralysosomal pH increases (Ohkuma & Poole, 1978).

The addition of ammonia, methylamine and various other weakly basic alkylamines to intact

cells results in the depression of a number of lysosomal functions, such as protein degradation (Seglen, 1975; Carpenter & Cohen, 1976; Knowles & Ballard, 1976; Tolleshaug *et al.*, 1977; Amenta *et al.*, 1978; Seglen *et al.*, 1979; Livesey *et al.*, 1980; Seglen & Gordon, 1980), receptor recycling (Tolleshaug & Berg, 1979; Van Leuven *et al.*, 1980; Kaplan & Keogh, 1981; Basu *et al.*, 1981), intralysosomal deposition of iron (Morgan, 1981; Karin & Mintz, 1981) or hydrolytic enzymes (Fischer *et al.*, 1980; Hasilik & Neufeld, 1980), and of the fusion of lysosomes with endosomes (Berg & Tolleshaug, 1980), phagosomes (Gordon *et al.*, 1980) or autophagosomes (Kovács *et al.*, 1982). The lysosome-inhibitory effect of amines is now believed to be due to an elevation of the lysosomal pH towards neutrality (Seglen & Reith, 1976; Ohkuma & Poole, 1978, 1981) rather than to an inhibition of lysosomal enzymes as previously assumed (de Duve *et al.*, 1974). The ability of the amines to dissipate pH gradients may result in functional disturbance of other acidic organelles as well, such as secretory vesicles (Seglen & Reith, 1977), storage granules (Johnson *et al.*, 1978) and endosomes (Tycko & Maxfield, 1982).

Entry of weak-base amines into lysosomes and elevation of the lysosomal pH occurs rapidly, i.e. in a matter of seconds or minutes (Ohkuma & Poole, 1978). However, amines continue to accumulate in the cell (by an energy-dependent process) after the establishment of a constant, near-neutral, lysosomal pH value (Ohkuma & Poole, 1978), and the visible swelling of the lysosomes (Seglen & Reith, 1976; Seglen *et al.*, 1979) suggests that at least part of this accumulation is intralysosomal. The continued cellular amine accumulation has been ascribed to lysosomal proton pumping, which would cause trapping of the amine as NH_4^+ ion intralysosomally (de Duve *et al.*, 1974; Ohkuma & Poole, 1978), but the contribution, e.g. by plasma-membrane transport, has never been assessed. Energy-dependent concentrative amine transport has been demonstrated in several lower eukaryotes (Kleiner, 1981), but not in mammalian cells. In the present work we have investigated whether plasma-membrane transport of a weak-base amine, methylamine, can be demonstrated in isolated rat hepatocytes, and sought to address the question of whether intralysosomal methylamine accumulation is the result of energy-dependent plasma-membrane uptake (active transport) or an energy-linked lysosomal process.

Materials and methods

Chemicals

[^{14}C]Methylamine hydrochloride (40–60 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Luciferin/luciferase reagent for the ATP assay was obtained from LKB-Wallac, Turku, Finland; other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Hepatocyte preparation and incubation

Hepatocytes were prepared from the liver of 16h-starved male Wistar rats (250–300g body wt.) by collagenase perfusion (Seglen, 1976). Cells in suspension buffer supplemented with Mg^{2+} (Poli *et al.*, 1981) were incubated at 37°C as 0.4 ml portions in shaking centrifuge tubes at a cell concentration of 50–100 mg wet wt./ml. The wet weight of packed cells was measured before incubation and used as a basis for the expression of other parameters. Intracellular water was assumed to represent 50% of the wet weight (Krebs *et al.*, 1974).

[^{14}C]Methylamine uptake

Hepatocytes were incubated with [^{14}C]methylamine (usually 10 mM) for various lengths of time, rapidly cooled to 0°C and washed three times in ice-cold wash buffer (Seglen, 1976). The cells were then precipitated with 2% (w/v) HClO_4 , and the radioactivity in the supernatant was measured by liquid-scintillation counting (Seglen, 1978). Intra-

cellular methylamine concentrations were calculated on the basis of the intracellular water content, and expressed relative to the initial extracellular concentration.

ATP measurement

ATP was measured in HClO_4 extracts of the cells by means of a bioluminescence monitor (LKB-Wallac), by using the luciferin/luciferase reagent accompanying the instrument.

Isopycnic banding of hepatocytes in metrizamide gradients

Linear 10–25% (w/v) metrizamide gradients were generated by allowing spontaneous diffusion to occur between four layers (10, 15, 20 and 25%) of iso-osmotically buffered metrizamide in horizontally positioned centrifuge tubes at 0°C (Munthe-Kaas & Seglen, 1974). Cell suspension (0.2 ml) was layered on top of an 8 ml gradient and centrifuged for 15 min at 7000g.

Hepatocyte volume determination

This was done with a Coulter counter as described by Steen & Nielsen (1979).

Results and discussion

Cellular uptake of [^{14}C]methylamine by facilitated diffusion (exchange transport)

At a very low concentration (tracer dose), [^{14}C]methylamine was taken up by hepatocytes according to biphasic kinetics: a rapid initial uptake followed by a slower accumulation (Fig. 1a). At 10 mM, [^{14}C]methylamine accumulated at a gradually diminishing rate for at least 2 h (Fig. 1b).

The rapid initial uptake of [^{14}C]methylamine was inhibited in the presence of unlabelled methylamine (Fig. 1a). The observation might be explained on the basis that labelled and unlabelled molecules are competing for a methylamine-transport site on the plasma membrane. When cells were preloaded with unlabelled methylamine, by incubation in the presence of 10 mM-methylamine for 1 h, and re-incubated in the presence of labelled methylamine, the rate of uptake of label was greater than that observed in cells that were not preloaded and was again most prominent during the initial phase of uptake (Fig. 1b). Both *cis*-inhibition (competition) and *trans*-stimulation (exchange) were observed even when ammonia or triethylamine was used instead of unlabelled methylamine (Table 1), suggesting a common transport system (carrier) for ammonia and the alkylamines. The *trans*-stimulatory effect would suggest transport of the facilitated-diffusion type, i.e. non-concentrative bidirectional transport with accelerative exchange properties. Analogous plasma-membrane-located trans-

Fig. 1. Uptake of methylamine into hepatocytes

The amount of [¹⁴C]methylamine taken into cells is given relative to the initial extracellular concentration of [¹⁴C]methylamine. (a) Uptake of a tracer dose without (○) or with the addition of 10mM-unlabelled methylamine (●); (b) uptake of 10mM-[¹⁴C]methylamine into cells that had been (●) or not been (○) preincubated with 10mM-methylamine. Each point is the mean for three samples.

Table 1. Effects of amines on the cellular uptake of [¹⁴C]methylamine

Competition experiments (A) were performed by measuring the uptake (after 10 min at 37°C) of a tracer dose of [¹⁴C]methylamine into cells in the presence of 0.9% NaCl (control), 10mM-methylamine, 10mM-triethylamine or 10mM-ammonia. Exchange experiments (B) were performed by pre-loading cells for 1 h with 0.9% NaCl, 10mM-methylamine, 10mM-triethylamine or 10mM-ammonia, and then measuring the 10 min uptake of a tracer dose of [¹⁴C]methylamine. Each value is the mean ± s.e.m. for three samples.

	Intracellular [¹⁴ C]methylamine at 10 min (relative to initial extracellular concn.)	
	(A) Competition	(B) Exchange
Control	1.95 ± 0.03	1.01 ± 0.04
Methylamine	0.73 ± 0.02	3.68 ± 0.08
Trimethylamine	0.70 ± 0.01	4.47 ± 0.08
Ammonia	0.90 ± 0.03	3.02 ± 0.01

port systems have previously been found to be responsible for the uptake of glucose (Baur & Heldt, 1976) and valine (Seglen & Solheim, 1978) in isolated hepatocytes.

In the exchange experiments depicted in Fig. 1 and Table 1, the hepatocytes were preloaded for 1 h

with ammonia or alkylamines. Since such treatment has been shown to induce a considerable osmotic swelling of the lysosomal system (Seglen & Reith, 1976; Seglen *et al.*, 1979; Seglen & Gordon, 1980), the possibility that general cell swelling might increase the available water space and thus account for the increased [¹⁴C]methylamine uptake ascribed to *trans*-stimulation had to be considered. However, both cell density measurements (by means of isopycnic banding in metrizamide gradients; Seglen, 1979) and cell volume determinations (by means of Coulter counting/multi-channel analysis; Steen & Nielsen, 1979) indicated an increase in cellular water content (relative to control cells) of only 12% after a 1 h incubation with 10mM-methylamine. This value was in close agreement with that measured for the swelling of the lysosomal compartment of cells incubated for 1 h with 10mM-NH₄Cl, which corresponded to 12% of the cytoplasmic volume (Seglen & Reith, 1976). Thus all of the increase in cell volume can be accounted for by lysosomal swelling, which, although appreciable, is quantitatively insufficient to explain the several-fold increase in the initial [¹⁴C]methylamine uptake observed after preloading cells with unlabelled amine. The fact that *trans*-stimulation can be observed even for [¹⁴C]methylamine efflux (see below) further suggests that it is an inherent property of the transport system and not an experimental artifact.

Additional characteristics of the plasma-

membrane-located exchange transport system for methylamine are shown in Figs. 2 and 3. The transport was temperature-sensitive, as would be expected of a carrier-mediated process, but significant uptake was observed even at 0°C (Fig. 2). This residual uptake might easily have been considered to represent carrier-independent (physical) diffusion. However, as shown in Fig. 3, even at 0°C the

Fig. 2. Temperature-dependence of cellular methylamine uptake

The amount of 10 mM- $[^{14}\text{C}]$ methylamine taken into cells at 37°C (●) and 0°C (○) is given relative to the initial extracellular concentration of $[^{14}\text{C}]$ -methylamine. Each point is the mean for three samples.

transport of methylamine was *trans*-stimulated and could therefore be ascribed to the exchange transport system. In the latter experiment, efflux of $[^{14}\text{C}]$ methylamine from preloaded cells was measured, and the result further documents the bidirectional mode of operation of this transport system.

Energy-dependent cellular uptake of $[^{14}\text{C}]$ methylamine

The fact that exchange transport operates even at 0°C would indicate that it is an energy-independent and therefore non-concentrative process. Nevertheless the overall uptake of methylamine by the hepatocytes was concentrative, and the biphasic kinetics observed (cf. Fig. 1a) suggest the existence of an additional transport process that is responsible for the slow, continuous accumulation after the rapid initial uptake. To see if the latter uptake might be energy-dependent, hepatocytes were incubated under hypoxic conditions by replacing the usual air atmosphere with nitrogen. Under these conditions cellular ATP decreased gradually during the first ½ h of incubation (indicating that the conditions were not yet completely anoxic), and remained at a low concentration during the last ½ h (Fig. 4a). The rate of accumulation of $[^{14}\text{C}]$ methylamine declined rapidly between 20 and 30 min of incubation, and there was no significant net accumulation during the last ½ h. Thus the second phase of $[^{14}\text{C}]$ methylamine uptake was clearly energy-dependent.

To achieve a more rapid and complete depletion of ATP, cells were treated simultaneously with an inhibitor of mitochondrial energy production (NaN_3) and an inhibitor of glycolysis (2-deoxy-

Fig. 3. *trans*-Stimulated efflux of methylamine

The efflux of $[^{14}\text{C}]$ methylamine from cells preloaded for 1 h with 10 mM- $[^{14}\text{C}]$ methylamine is measured at 37°C (a) and 0°C (b). ●, Extracellular medium supplied with 10 mM-methylamine; ○, medium free of methylamine. Each point is the mean for three samples.

glucose). This treatment produced a strong and immediate depression of the cellular ATP content (Fig. 4b), but the rapid initial uptake of [14 C]-methylamine was not inhibited (substantiating the energy-independence of the exchange transport system); in fact it appeared to be slightly stimulated. However, results from these experiments were complicated by the relatively rapid onset of cell death in the energy-depleted state; fewer than half of the cells were viable at 1 h. The paradoxical stimulation of [14 C]methylamine uptake observed during the first 10 min might therefore reflect an increase in the volume of accessible cellular water in dying cells owing to blebbing and swelling. As shown in Fig. 6(c), hepatocytes incubated simultaneously with energy inhibitors and methylamine displayed very extensive surface blebbing.

The equilibrium intracellular content of [14 C]-methylamine established under hypoxic conditions (Fig. 4a) was only moderately above the extracellular concentration (ratio 1.4:1). Since the uptake of methylamine was appreciable even at 0°C, no correction for background adsorption of radioactivity could be made. Allowing for some background radioactivity, the real distribution ratio would be even lower, and therefore the energy-independent uptake of methylamine should be regarded as largely non-concentrative.

In the presence of energy inhibitors after 30 min of incubation the intracellular content of methylamine was directly proportional to the extracellular concentration (Fig. 5). Such non-saturable uptake would be consistent with a process of passive equilibration, i.e. facilitated diffusion, across the plasma membrane. In the absence of energy inhibitors, the

concentration-dependence of the methylamine uptake could no longer be expressed by a straight line, the uptake at low concentrations being much larger than in the energy-depleted state. The nearly parallel

Fig. 5. Dose-response curves for total and energy-independent uptake of [14 C]methylamine

Cells were incubated with the indicated doses of methylamine for 30 min at 37°C in the absence (O) or presence (●) of 10 mM-2-deoxyglucose and 7.5 mM- NaN_3 . Intracellular methylamine content is given as c.p.m./mg of cells. Each point is the mean for three samples.

Fig. 4. Energy-dependence of cellular methylamine uptake

The uptake of 10 mM- $[^{14}\text{C}]$ methylamine into cells incubated under nitrogen atmosphere (a, ●) or in the presence of 7.5 mM- NaN_3 and 10 mM-2-deoxyglucose (b, ●) is recorded and compared with the uptake under normal conditions (a, b, O). The cellular content of ATP under normal (Δ) and hypoxic ($\blacktriangle$) conditions and the cell viability under normal ($\square$) and hypoxic ($\blacksquare$) conditions are also indicated. Each point is the mean for three samples.

Fig. 6. *Vacuolization of cells induced by methylamine alone or in combination with inhibitors of energy metabolism* Cells were incubated for 30 (a–e) or 60 (f) min at 37°C. (a), Control (no additions); b, methylamine (10 mM); (c), NaN_3 (7.5 mM) plus 2-deoxyglucose (10 mM); (d), methylamine (25 mM); (e) and (f), methylamine (25 mM) plus NaN_3 and deoxyglucose. Magnification $\times 400$.

course of the two curves at higher concentrations (Fig. 5) would suggest that the energy-dependent uptake is saturated at low methylamine concentrations.

Relationship between cellular methylamine uptake and lysosome swelling

Although the results presented so far provide evidence for the existence of two distinct processes

involved in cellular methylamine uptake, one energy-independent and one energy-dependent, they yield no information about the subcellular location of these uptake systems. Whereas the energy-independent, non-saturable, uptake most probably represents facilitated diffusion across the plasma membrane, the energy-dependent uptake could be due either to active plasma-membrane transport or to specific uptake into the lysosomes, e.g. uptake coupled to energy-dependent proton pumping (Mego, 1979; Schneider, 1981).

The entry of methylamine into lysosomes could be confirmed by optical microscopy (Seglen *et al.*, 1979; Seglen & Gordon, 1980) as cytoplasmic vacuolation, i.e. the appearance throughout the cytoplasm of large vacuoles, found in the electron microscope to represent secondary lysosomes (Seglen & Reith, 1976). When cells were incubated with 10 mM-methylamine, vacuolation was visible as early as at 30 min (Figs. 6a and 6b).

In the presence of energy inhibitors (NaN_3 and deoxyglucose), the typical extensive surface blebbing made appraisal of the cells difficult (Fig. 6b), but by applying slight pressure to the cover-glass cells could be flattened sufficiently to allow examination of their cytoplasm (Fig. 6c). No vacuolation was visible in the energy-depleted cells, not even after incubation with a concentration of methylamine (25 mM) that was certain to give a higher intracellular methylamine content than was needed to produce vacuolation in the absence of the inhibitors (cf. Fig. 5). The observations on cells incubated under an atmosphere of N_2 were similar. Examination of the occasional non-blebbing cells furthermore failed to give any evidence of methylamine inducing cytoplasmic vacuolation in cells incubated in the presence of energy inhibitors. Cells incubated with energy inhibitors alone displayed a degree of blebbing far less extensive than that in the presence of methylamine (Figs. 6e and 6f).

These morphological observations indicate that the lysosomal uptake of methylamine, at least to the extent needed to produce visible vacuolation, is energy-dependent. Although some of the uptake probably represents trapping (methylammonium formation) by the protons present as a result of Donnan equilibration, this process would be insufficient to cause lysosomal swelling, even when the cytoplasmic methylamine concentration is very high. It therefore seems reasonable to ascribe at least part of the cellular energy-dependent methylamine uptake to lysosomal accumulation, possibly reflecting the continuous activity of the proton pump in the lysosome membrane. Whether an energy-dependent uptake across the plasma membrane also contributes cannot be determined on the basis of the present data.

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