Betaine is an osmolyte in RAW 264.7 mouse macrophages

Ulrich Warskulat, Matthias Wettstein, Dieter Häussinger*

Medizinische Universitätsklinik, Klinik für Gastroenterologie, Hepatologie und Infektiologie, Heinrich-Heine-Universität, Moorenstrasse 5, D-40225 Düsseldorf, Germany

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Abstract Hyperosmotic (405 mosmol/l) exposure of RAW 264.7 mouse macrophages led to a stimulation of betaine uptake and an increase in betaine transporter (BGT-1) mRNA levels. Conversely, hypoosmotic (205 mosmol/l) exposure decreased betaine uptake and diminished BGT-1 mRNA levels. Betaine uptake was Na^+-dependent and was inhibited by about 90% by GABA, whereas inhibition by methylaminoisobutyrate and myo-inositol was less than 15%. Addition of betaine strongly diminished BGT-1 mRNA levels in cells exposed to normoosmotic or hyperosmotic media. When mouse macrophages were preloaded with betaine, lowering of the extracellular osmolarity was followed by a rapid betaine efflux from the cells. This study identifies a constitutively expressed and osmosensitive betaine transporter in RAW 264.7 macrophages and the use of betaine as an osmolyte in these cells.

Key words: Macrophage; Betaine transport; Osmolyte; Cell volume; BGT-1; Anisoosmolarity

1. Introduction

Macrophages belong to the mononuclear phagocyte system and play an important role in the body's defense machinery (for review see [1]). Upon stimulation with endotoxin they produce a variety of cytokines, lipid mediators and radicals. In rat liver macrophages, lipopolysaccharide (LPS)-induced prostaglandin E and thromboxane B formation and cyclooxygenase-2-expression is stimulated about 7-fold when ambient osmolarity increases from 300 to 350 mosmol/l [2]. This sensitive and potent osmoregulation of prostanoid synthesis suggests that cell volume homeostasis may be a critical factor for macrophage function. We therefore investigated whether organic osmolytes participate in the cell volume homeostasis of macrophages. Organic osmolytes are compounds which are specifically accumulated or released by the cells in response to hyperosmotic cell shrinkage or hypoosmotic cell swelling, respectively, in order to maintain cell volume homeostasis. In mammals, osmolytes have been identified in astrocytes, renal medulla cells and lens epithelia [3–8]. The need for osmolytes in renal medulla cells is obvious, because ambient medullary osmolarity varies strongly between antidiuresis and diuresis [8]. In the antidiuretic state (high extracellular osmolarity), intracellular osmolarity increases in renal medullary cells as the result of the intracellular accumulation of inositol and betaine, which are taken up via Na^+-dependent transporters. These Na^+-dependent transporters are induced upon hyperosmotic exposure in renal cells and astrocytes [4,6,7,9,10] and recent studies with Madine–Darby canine kidney (MDCK) cells have identified a hypertonic stress-responsive element in the 5′-flanking region of the mammalian BGT-1 gene (betaine transporter) [10]. This report identifies betaine as an osmolyte in RAW 264.7 mouse macrophages.

*Corresponding author. Fax: (49) (211) 3118 838.

Abbreviations: BGT-1, betaine gamma-aminobutyric acid transporter; GABA, gamma-aminobutyrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; MeAIB, methylaminoisobutyrate.

2. Materials and methods

2.1. Materials

Cell culture medium and fetal bovine serum (FBS) were from Gibco (Eggenstein, Germany) and oligonucleotide-labelling kit was from Pharmacia (Freiburg, Germany). Guanidine thiocyanate and sodium lauroylsarcosinate were from Fluka (Karlsruhe, Germany). [α-^{32}P]dCTP (3000 Ci/mmol) and Hybond-N nylon membranes were purchased from Amersham Buchler (Braunschweig, Germany). [^{14}C]Betaine (48.1 mCi/mmol) was from New England Nuclear DuPont (Bad Homburg, Germany). All other chemicals were from Sigma (Munich, Germany). A plasmid containing full-length BGT-1 cDNA [11] was kindly provided by Dr. H. Moo Kwon (Division of Nephrology, The Johns Hopkins University School of Medicine, Baltimore, MD, USA). The 1.0 kb cDNA fragment for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used for standardization was from Clontech (Palo Alto, USA).

2.2. Culture of RAW 264.7 cells

RAW 264.7 cells (A.T.C.C. TIB 71) were grown to near-confluency in DME medium, 37°C, 5% CO_2, pH 7.4, supplemented with 10% fetal bovine serum (FBS) in Cluster 6 dishes (Costar). If not indicated otherwise, the osmolarity was varied by changing the NaCl concentration in the medium. For identification of BGT-1 mRNA levels, cells were maintained under various osmotic test conditions for 12 h, unless indicated otherwise. In some experiments, cells were treated with 1 μg/ml LPS and betaine as indicated. In Na^+-free incubations, Na^+ in the medium was replaced by equimolar Li^+ or choline and Cl^--free conditions were achieved by substituting gluconate for Cl^-.

2.3. Betaine transport

RAW 264.7 cells had been grown for 24 h in serum-free DME medium with an osmolarity as indicated. Then, the cells were rinsed twice and incubated for 15 min at 37°C with 1 ml incubation buffer, containing 10 mmol/l Tris-HEPES pH 7.4, 5 mmol/l KCl, 2 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$ and NaCl to get the same osmolarity like the media before. This solution was then changed for an identical buffer containing [^{14}C]betaine (100 μmol/l, 0.5 μCi/ml) and incubated for various time periods (0–60 min) at 37°C. Thereafter, radioactivity of ^{14}C in the supernatant was measured by scintillation counting. The cells were rinsed four times with 1 ml ice-cold stop solution, containing 10 mmol/l Tris/HEPES pH 7.4, 300 mmol/l mannitol and 300 mmol/l NaCl and harvested with 1 ml 0.25 N NaOH for liquid scintillation counting and protein determination (Bio-Rad protein assay).

For studies on betaine efflux, RAW 264.7 cells were preincubated for 24 h in hyper- (405 mosmol/l) or in normoosmotic (305 mosmol/l) DME medium. Then the medium was changed for an identical medium containing [^{14}C]betaine (100 μmol/l, 0.5 μCi/ml) in order to load the cells with radioisotope. After a loading period of 2 h at 37°C/5% CO_2, the cells were rinsed thrice with hyper- or normoosmotic incubation buffer, containing 10 mmol/l Tris-HEPES pH 7.4, 5 mmol/l KCl, 2 mmol/l

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$CaCl_2$, 1.2 mmol/l $MgCl_2$ and 193 mmol/l or 143 mosmol/l NaCl. Then the cells were incubated for 0–30 min in betaine-free hyper- (405 mosmol/l), normo- (305 mosmol/l) or hypoosmotic (205 mosmol/l) incubation buffer. Thereafter, the medium was collected and the cells were harvested with 1 ml 0.25 N NaOH. [^{14}C]betaine appearance in the supernatant was measured by scintillation counting and expressed as the percentage of total [^{14}C]betaine (contained in cells plus supernatant).

In order to prove the stability of [^{14}C]betaine during incubation, cell supernatant and cells were extracted and analysed by HPLC separation. At the beginning and the end of a 2 hour incubation period radioactivity was almost completely (>99%) recovered in the hydrophilic phase and single peaks coeluting with [^{14}C]betaine standards were obtained, thus demonstrating no further metabolism of betaine in RAW 264.7 cells. Accordingly, uptake or release of ^{14}C-radioactivity was seen to reflect betaine transport.

2.4. Northern blot analysis

Total RNA from near-confluent culture plates of RAW 264.7 cells was isolated by using guanidine thiocyanate solution as described in [12]. RNA samples were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted onto Hybond-N nylon membranes with 20× SSC (3 mol/l NaCl, 0.3 mol/l sodium citrate). After brief rinsing with water and UV-crosslinking (Hoefer UV-crosslinker 500), blots were subjected to a 3 h prehybridization at 43°C in 50% deionized formamide, in sodium phosphate buffer (0.25 mol/l, pH 7.2), containing 0.25 mol/l NaCl, 1 mmol/l EDTA, 100 mg/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with approx. 10^6 cpm/ml [α-^{32}P]dCTP-labeled random primed BGT-1 and GAPDH cDNA probes. Membranes were washed thrice in 2 × SSC/0.1% SDS for 10 min, twice in sodium phosphate buffer (25 mmol/l, pH 7.2)/EDTA (1 mmol/l)/0.1% SDS and twice in sodium phosphate buffer (25 mmol/l, pH 7.2)/EDTA (1 mmol/l)/1% SDS. Blots were then exposed to Kodak AR X-omat film at −70°C with intensifying screens.

2.5. Statistics

Values are expressed as means ± S.E.M. (n = number of cell preparations). Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered to be statistically significant.

3. Results

Betaine was transported rapidly into RAW 264.7 mouse macrophages when incubated in normoosmotic (305 mosmol/l) media (Fig. 1). Betaine uptake was significantly stimulated by 30%, when the cells were exposed to hyperosmotic (405 mosmol/l) medium for 24 h due to addition of NaCl (50 mmol/l). Stimulation of betaine uptake occurred regardless of whether hyperosmolarity was instituted by addition of NaCl (50 mmol/l) or by raffinose (100 mmol/l), indicating that the effect on

Table 1
Na^+ and Cl^--dependence of hyperosmolarity-induced betaine transport

	Betaine uptake (nmol/mg protein/15 min)	% inhibition of betaine uptake
Control	10.77 ± 0.88	0
Sodium replacement by		
lithium	0.20 ± 0.03	98
choline	0.06 ± 0.02	99
Chloride replacement by		
gluconate	1.93 ± 0.58	82

RAW 264.7 cells were preincubated for 24 h in hyperosmotic medium (405 mosmol/l, being prepared by addition of 50 mmol/l NaCl), in order to induce betaine transport activity. Then, the uptake of [^{14}C]betaine (10 μmol/l) was measured for 15 min in control hyperosmotic medium (405 mosmol/l), or hyperosmotic media in which Na^+ was replaced by choline or Li^+ or in which gluconate was substituted for Cl^-. Data are given as means ± S.E.M. (n = 3).

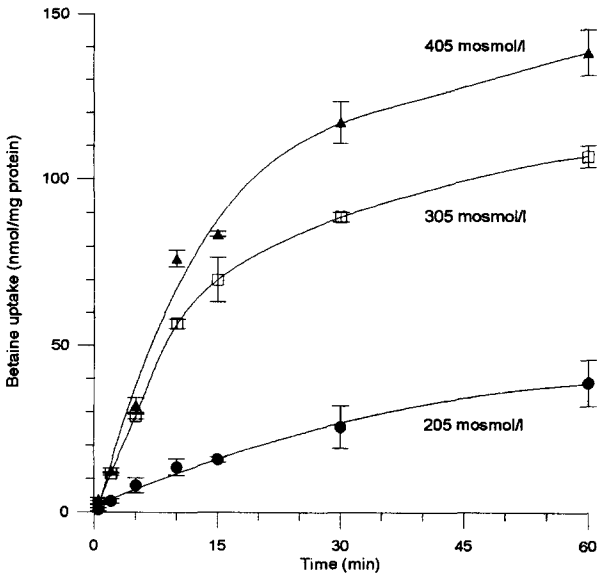

Fig. 1. Osmosesensitive betaine transport in RAW 264.7 cells. RAW 264.7 cells were preincubated for 24 h in hypo- (205 mosmol/l), normo- (305 mosmol/l) or hyperosmotic medium (405 mosmol/l). Osmolarity changes were performed by appropriate addition/removal of NaCl. Then, the uptake of [^{14}C]betaine (100 μmol/l) was measured in medium with the same osmolarity. Data are given as means ± S.E.M. and are from 4 separate experiments for each condition.

betaine transport was due to hyperosmolarity but not due to an increase in extracellular Na^+ or Cl^- activity (not shown). On the other hand, exposure of RAW 264.7 cells to hypoosmotic (205 mosmol/l) media for 24 h lowered betaine uptake about by 70% (Fig. 1). Irrespective of the medium osmolarity, betaine uptake in hyperosmotically preexposed cells was inhibited by more than 95% when Na^+ was replaced by either Li^+ or choline, indicating that betaine uptake occurred via a Na^+-dependent route. Substitution of Cl^- against gluconate also strongly inhibited betaine uptake (Table 1).

In MDCK cells, an osmoregulated Cl^- and Na^+-dependent betaine transporter has been identified, whose cDNA was cloned and termed BGT-1 [10,11,13]. As shown in Fig. 2A, hyperosmotic exposure of RAW 264.7 cells led to an increase in BGT-1 mRNA levels, whereas hypoosmotic exposure lowered BGT-1 mRNA levels. BGT-1 mRNA levels were similarly sensitive to small osmolarity changes when macrophages were simultaneously stimulated by addition of lipopolysaccharide (LPS; 1 μg/ml) for 12 h (Fig. 2B). The hyperosmolarity-induced increase of BGT-1 mRNA was time dependent and maximal BGT-1-mRNA levels were found after 6 h of hyperosmotic (405 mosmol/l) exposure (Fig. 3). As shown in Fig. 4, BGT-1 mRNA levels were diminished by betaine in a concentration-dependent manner in both, normoosmotic and hyperosmotic incubations.

As shown in Table 2, betaine (10 μmol/l) uptake in hyperosmotically preexposed RAW 264.7 macrophages was strongly inhibited by gamma-aminobutyrate (GABA; 5 mmol/l). This again indicates the involvement of the BGT-1, which is known to transport both GABA and betaine [14]. On the other hand, methylaminoisobutyrate, which is a model substrate for amino acid transport system A [15], inhibited betaine uptake by only 13% (Table 2), indicating that betaine transport occurred by only a negligible extent via system A. This is different to SV-3T3

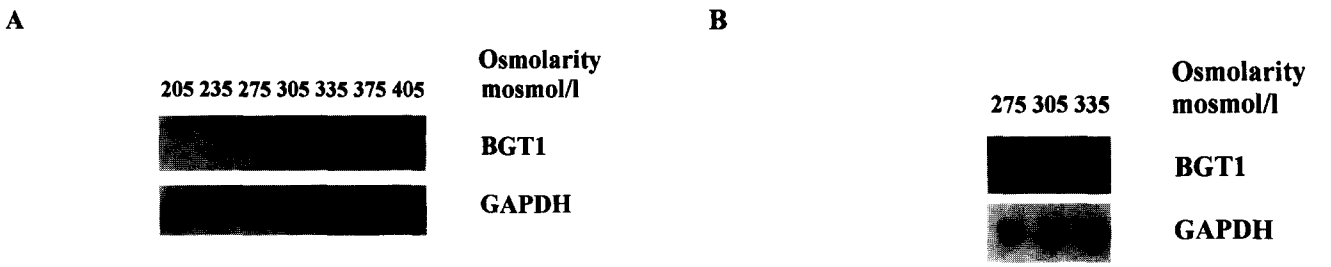

Fig. 2. Effect of medium osmolarity on the mRNA levels for BGT-1 and GAPDH in unstimulated (A) and LPS-stimulated (B) RAW 264.7 cells. RAW 264.7 cells were exposed for 12 h to media with the osmolarity indicated. Osmolarity changes were performed by appropriate addition/removal of NaCl. In Fig. 2B, cells were simultaneously stimulated by addition of lipopolysaccharide (LPS; 1 μg/ml). The mRNA levels for BGT-1 and glyceraldehydephosphate dehydrogenase (GAPDH) were determined by Northern blot analysis. This experiment is representative of three separate experiments.

cells, in which betaine is taken up via the amino transport system A [16]. Myo-inositol had no effect on betaine uptake (Table 2).

The osmosensitivity of betaine efflux from the cells was studied in a separate series of experiments. For this purpose, RAW 264.7 cells were incubated in normo- (305 mosmol/l) or hyperosmotic (405 mosmol/l) medium for 24 h and then preloaded with [^{14}C]betaine (100 μmol/l) for another 2 h in the same medium. After washing the cells, they were exposed to betaine-free media and the appearance of radioactivity in the supernatant was monitored. As shown in Fig. 5, following removal of extracellular betaine but maintaining the same medium osmolarity as during the loading period, less than 5% of the betaine accumulated inside the cells was released within 30 min. When however, the osmolarity was reduced by 100 mosmol/l, betaine efflux was significantly stimulated and occurred within 5 min of the onset of hypoosmotic stress.

4. Discussion

This study suggests that betaine serves as an osmolyte in RAW 264.7 mouse macrophages. This conclusion is based on the following findings: (i) a strong osmosensitivity of betaine transport and its induction by hyperosmotic stress, (ii) an increase (decrease) in mRNA levels for the betaine-transporting system BGT-1 in response to hyperosmolarity (hypoosmolarity), (iii) counteraction of this induction by betaine, and (iv) rapid betaine release from RAW 264.7 cells in response to hypoosmotic stress. It is likely that the osmoregulated betaine-transporting activity, is similar to or identical with the osmosensitive betaine transporter in kidney, i.e. BGT-1. Several lines of evidence indicate that osmosensitive betaine uptake in RAW 264.7 mouse macrophages is mediated by the BGT-1; i.e. the osmosensitivity of BGT-1 mRNA levels, the Na^+- and Cl^--dependence of betaine transport, its inhibition by GABA and the lack of effect of MeAIB, which is a model substrate for transport via system A. Thus, RAW 264.7 cells are another cell type using the so called osmolyte strategy in order to counteract osmotic stress and to maintain cell volume homeostasis. The functional relevance of this osmolyte strategy in the macrophage cell line, however, remains to be established, because the rationale for an osmolyte strategy in RAW 264.7 cells is less obvious than in cells from kidney or brain, i.e. organs facing either extreme osmotic stresses or being encapsulated by rigid bone. However, recent studies have indicated that eicosanoid formation by LPS-stimulated rat liver macrophages (Kupffer cells) is critically dependent upon the ambient osmolarity and probably cell volume within a narrow range [2]. Thus, betaine availability and/or regulation of betaine transporter expression by osmolarity and other factors could be a potential site for the regulation of macrophages cell function. In this respect it should be noted that betaine concentration used in the present study is well in the range of the physiological plasma concentration, which was reported to be 20–120 μmol/l [17,18]. Interestingly enough, the betaine transporting activity is already strongly expressed in RAW 264.7 mouse macrophages under normoosmotic conditions. Because betaine was found to be

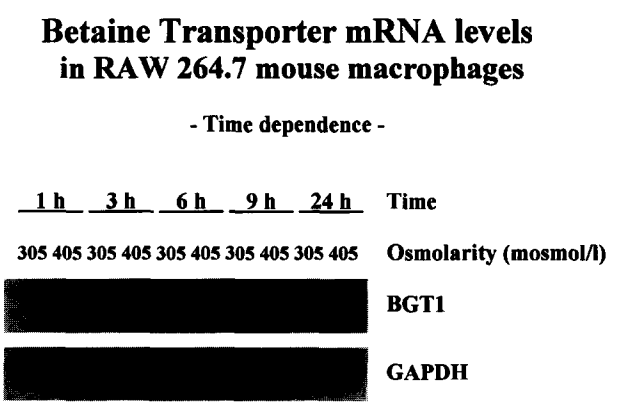

Fig. 3. Time-dependent induction of BGT-1 mRNA levels in RAW 264.7 cells during hyperosmolarity. RAW 264.7 cells were exposed in normoosmotic (305 mosmol/l) or hyperosmotic (405 mosmol/l) media for the time periods indicated and mRNA levels for BGT-1 and GAPDH were determined by Northern blot analysis. Similar results were seen in another experiment.

Table 2
Inhibition of hyperosmolarity-induced betaine uptake by various amino acids

	Betaine uptake (nmol/mg protein/15 min)	% inhibition of betaine uptake
Control	9.25 ± 0.39	0
GABA	0.72 ± 0.06*	92
MeAIB	8.02 ± 0.12*	13
myo-Inositol	8.01 ± 0.33	13

RAW 264.7 cells were exposed for 24 h to hyperosmotic medium (405 mosmol/l); thereafter [^{14}C]betaine (10 μmol/l) uptake was measured for 15 min in the absence (control) or presence of various effectors, which were added at a concentration of 5 mmol/l, each. Data are given as means ± S.E.M. (n = 3).
*Significantly different from the control ($P < 0.05$).

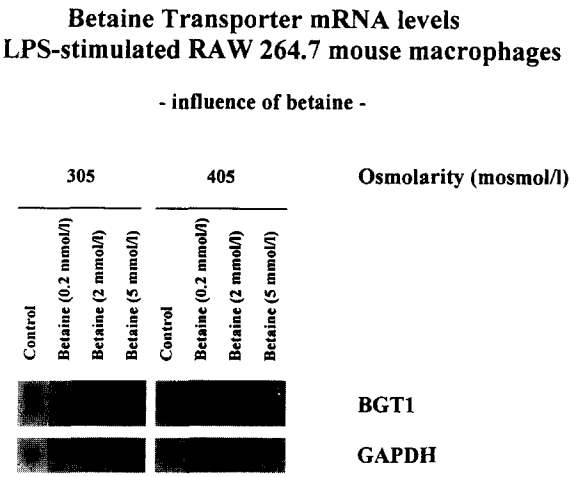

Fig. 4. Effect of betaine on BGT-1 mRNA levels in RAW 264.7 cells. RAW 264.7 cells were exposed to normoosmotic (305 mosmol/l) or hyperosmotic medium (405 mosmol/l) for 12 h. This medium contained no further additions (control) or betaine at the concentrations indicated. The mRNA levels for BGT-1 and GAPDH were determined by Northern blot analysis. This experiment is representative of three separate experiments.

metabolically inert in these cells, its primary function may reside in cell volume homeostasis. Previous studies have shown that cell swelling is one prerequisite for cell proliferation [19] and *ras*-oncogene expression in NIH-3T3 fibroblasts is accompanied by a 30% increase of cell volume [20]. Thus, one might speculate that the high constitutive expression of BGT-1 in the RAW 264.7 cell line, whose activity tends to swell the cells, is related to the malignant phenotype of these tumour cells.

In line with findings on osmolyte transport in the kidney, betaine efflux from the macrophages in response to hypoosmolarity was much faster than betaine uptake during hyperosmolarity and there is general agreement that osmolyte efflux occurs via routes distinct from uptake. Evidence has been given that the efflux of some osmolytes such as taurine may occur via Cl^- channels which are activated during regularoty volume decrease. The route of betaine efflux, however, remains to be established.

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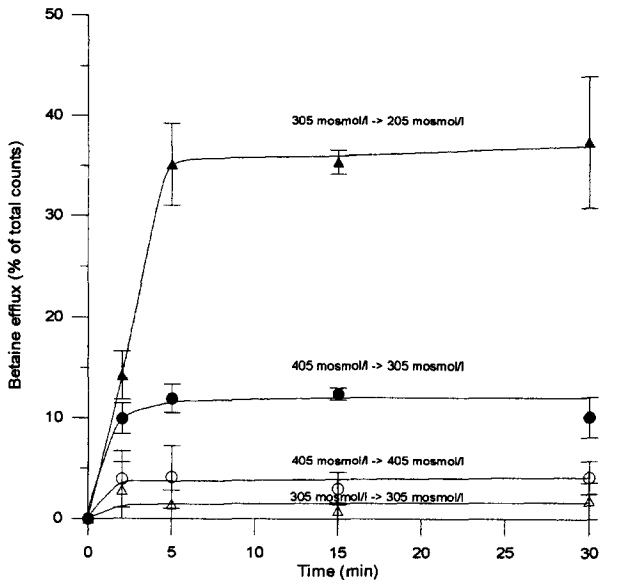

Fig. 5. Betaine efflux from RAW 264.7 cells following osmotic-reduced exposure. RAW 264.7 cells were preincubated for 24 h in normoosmotic (305 mosmol/l) (▲,△) or hyperosmotic medium (405 mosmol/l) (●,○). Then, cells were allowed to accumulate [^{14}C]betaine (added at a concentration of 100 μmol/l) for 2 h and washed thrice. Thereafter, cells preincubated with normoosmotic medium were exposed to betaine-free hypoosmotic (205 mosmol/l) (▲) or normoosmotic (305 mosmol/l) (△) medium. Cells preincubated with hypermotic medium were exposed to betaine-free normoosmotic (305 mosmol/l) (●) or hyperosmotic (405 mosmol/l) (○) medium. [^{14}C]Betaine appearance in the supernatant was measured and expressed as the percentage of total [^{14}C]betaine (contained in cells plus supernatant). Data are given as means ± S.E.M. and are from 3–4 separate experiments for each condition.

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