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Modulation by betaine of cellular responses to osmotic stress

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Various solutes were tested to see if they could modify the responses of SV-3T3 cells to hyperosmotic (0.5 osM) conditions, which cause an inhibition of general cell protein synthesis and of the rate of cell proliferation, coupled with an induction of amino acid transport activity. The added solutes were glycerol, proline, taurine, betaine, dimethylglycine and sarcosine. Of these, betaine produced the most dramatic and consistent effects. Addition of 10–25 mM-betaine to the hyperosmotic medium largely prevented the 90% inhibition of cell proliferation that occurred in its absence. Whether it was added initially or after the cells were exposed to hyperosmotic medium, 25 mM-betaine also converted a 50% recovery of the rate of protein synthesis into 100%. Similarly, the same concentrations of betaine prevented a 30% decrease in cell volume and decreased the induction of amino acid transport via system A by 73%. Lower concentrations of betaine produced smaller but still significant changes in these functional responses. With chick-embryo fibroblasts, under identical hyperosmotic conditions, 25 mM-betaine completely counteracted a 75% inhibition of the rate of protein synthesis. At present it is not clear how betaine modulates these effects of hyperosmolarity on cell functions.

INTRODUCTION

When some cell cultures are subjected to hyperosmotic stress, various marked changes in cell functions occur that appear to result from changes in gene expression. For example, in chick-embryo fibroblasts general protein synthesis decreases by 50–70% (Petronini *et al.*, 1987), but there are concomitant increases in amino acid transport activity (2.6-fold increase in $V_{max.}$) via system A (Tramacere *et al.*, 1984) and in the synthesis of various stress proteins (Petronini *et al.*, 1986). Similarly amino acid transport capacity is induced in SV-3T3 cells, accompanied by inhibition of protein synthesis and a dramatic decrease (75% or more) in their rate of proliferation (Silvotti *et al.*, 1991). The molecular signal that triggers these changes is still unknown, although it could be a sudden increase in intracellular ionic strength, because similar responses are induced when fibroblasts are made to shrink in iso-osmotic media (Petronini *et al.*, 1989, 1990). Nor is it known how the cells compensate, in terms of internal osmolytes, when they partially recover normal functions during prolonged exposure to the osmotic stress. Many cells, bacterial, plant and animal, have been shown to adapt to such an increase in external osmotic pressure by accumulating so-called 'compatible solutes' (Yancey *et al.*, 1982; Higgins *et al.*, 1987; Garcia-Perez & Burg, 1991). These solutes, which include neutral amino acids, polyols, sugar alcohols and methylamines, are termed 'compatible' because their presence generally does not perturb the structures of cell macromolecules, in contrast with the effects of high concentrations of common salts. Accumulation of such compatible solutes in response to osmotic stress may occur through an increase in either their synthesis or their uptake by the cells concerned (Garcia-Perez & Burg, 1991). Hence it seemed possible that neutral amino acids might be important as compatible solutes in these cultured cells, amino acid accumulation being enhanced under hyperosmotic conditions by the induction of transport system A. This possibility was particularly attractive because proline, which is transported predominantly via system A in these cells and is the amino acid that we use to monitor the activity of system A, is well established as a compatible solute in other cells (Yancey *et al.*, 1982). On the other hand, direct measurements of cellular pools of amino acids suggest that the increases due to the induction of system A are unlikely to be sufficient to provide complete compensation for the imposed osmolarity (Silvotti *et al.*, 1991). We have therefore tested the ability of proline and several other common compatible solutes to modulate the effects of hyperosmotic conditions on cultured cells. For most of the experiments reported here we used SV-3T3 cells, which, besides being more convenient to culture than chick-embryo fibroblasts, are particularly sensitive to osmotic stress (Silvotti *et al.*, 1991). We also concentrate on the effects of betaine because they were so remarkable.

EXPERIMENTAL

Materials

Common chemicals were obtained from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or B.D.H./Merck (Poole, Dorset, U.K.) and were of analytical grade where possible. Culture media, foetal-calf serum, chicken serum and antibiotics were purchased from GIBCO (Grand Island, NY, U.S.A.). Radioactively labelled compounds (L-[4,5-^{3}H]leucine, L-[5-^{3}H]proline and 3-*O*-methyl-D-[1-^{3}H]glucose) were supplied by Amersham International (Amersham, Bucks., U.K.).

Cell cultures

Simian-virus-40-transformed Balb/c 3T3 cells (clone SV-3T3) were kindly provided by Dr. Paul H. Black (Boston) and obtained through Dr. Salvatore Ruggieri (Florence), as described previously (Borghetti *et al.*, 1980). The cells were maintained in 'complete growth medium', which consisted of Dulbecco's modified Eagle medium containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% (v/v) foetal-calf serum. Chick-embryo fibroblasts were obtained from 9–11-day embryos by the method of Rubin (1973), as described in detail previously (Tramacere *et al.*, 1984). All experiments were performed with secondary cultures seeded at 2.5×10^4 cells/4 cm^2 on disposable plastic dishes (Costar) and grown in medium 199, containing penicillin (100 units/ml) and streptomycin (100 μg/ml) and supplemented with 2% chicken serum. All cultures were kept in an incubator at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. Measurements of cell volume and rates of pro-

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liferation, protein synthesis and amino acid transport were made on subcultures from cells plated on 9 cm^2 wells of disposable multiwell trays, whereas intracellular ion content was determined with cells plated on 25 cm^2 disposable plastic dishes. All subcultures were incubated for the desired period of time in 2 or 6 ml, respectively, of complete growth medium.

Cell density

Cells were detached from the substratum by treatment with trypsin, and appropriate dilutions of the resulting suspension were counted in a model ZM Coulter counter, as described in detail previously (Piedimonte *et al.*, 1982).

Culture media of altered osmolarity

Media were adjusted to the desired osmolarity by the addition of NaCl for most experiments, but sucrose and betaine were also used where indicated in the Results section. The final osmolarities of the modified media were checked with an osmometer (Roebling). Control medium contained 155 mM-Na^+, 110 mM from NaCl and the remainder derived from $NaHCO_3$ and sodium phosphate: its osmolarity was 0.340 osM. The usual hyperosmotic medium contained 250 mM-Na^+ and its osmolarity was 0.515 osM.

Rate of protein synthesis

This was measured in terms of the incorporation of labelled leucine during a 30 min incubation of the cell monolayers in complete culture medium containing 0.8 mM-leucine (2.5 Ci/mol). At the end of the incubation, the cell monolayers were washed 3 times with cold Earle's balanced salt solution containing 0.1 % glucose and then the protein was precipitated by addition of 10 % (w/v) trichloroacetic acid. The precipitate was dissolved in 0.2 M-NaOH, and the radioactivity in this acid-insoluble fraction was determined by liquid-scintillation counting. The concentration of protein was measured with the use of a dye-binding method (Bio-Rad), with bovine serum albumin as the standard (Bradford, 1976).

Determination of proline transport activity, cell volume and cellular cation content

The method of measurement of proline transport activity under conditions approaching initial rates of uptake has been described in detail previously (Tramacere *et al.*, 1984).

Cellular contents of Na^+ and K^+ were assayed by the method of Owen & Villereal (1983) with minor modifications (Petronini *et al.*, 1986). Briefly, at the end of the incubation cell monolayers were quickly washed five times with ice-cold 0.1 M-$MgCl_2$ and then extracted with 2 ml of 5 % trichloroacetic acid. The cell extract was centrifuged at 3000 *g* for 10 min and the clear supernatant analysed for its Na^+ and K^+ contents in a Varian atomic absorption spectrometer model A275.

Cell volume was estimated by the method of Kletzien *et al.* (1975) as described by Erlinger & Saier (1982), with the use of 3-*O*-methyl-D-[1-^{3}H]glucose. Preliminary experiments showed that the tracer reached an equilibrium distribution after 15–30 min incubation, so that a 30 min incubation was then used as a routine. After incubation in the control or osmotically modified medium, the cell monolayers were quickly washed with the appropriate medium containing 5 % foetal-calf serum and 2.5 mM-glucose, and then incubated in the same medium in the presence of 2 μCi of 3-*O*-methyl-D-[1-^{3}H]glucose/ml for 30 min at 37 °C. At the end of the incubation, cells were quickly washed three times with ice-cold Earle's balanced salt solution containing 0.1 mM-phloretin, and the macromolecular fraction was then precipatated by addition of ice-cold 10 % trichloroacetic acid. The radioactivity present in the acid-soluble fraction was determined by liquid-scintillation counting.

RESULTS

Cell proliferation

The effects on cell proliferation of including various potential compatible solutes in the culture media are shown in Table 1(*a*). In the absence of any such solute, cell growth rate was severely inhibited in the hyperosmotic medium. The addition of 10 mM-glycerol, -proline, -taurine or -betaine each produced some increase in the proliferation rate, betaine having the clearest effect. Under iso-osmotic conditions, however, taurine drastically inhibited proliferation, whereas betaine elicited a slight increase.

Table 1(*b*) shows the effects of a higher concentration (25 mM) of betaine and of physiological concentrations of non-essential amino acids. The latter had no effect under hyperosmotic conditions and only a small effect under control conditions. In contrast, betaine produced a marked increase in the rate of proliferation in the hyperosmotic medium, this time with no significant change under control conditions. The addition of proline at concentrations above 10 mM significantly inhibited the rate of proliferation of the control cells and did not enhance growth under hyperosmotic conditions (results not shown).

Cell protein synthesis

Cell protein synthesis, monitored as the rate of incorporation of [^{14}C]leucine into protein, was assayed in experiments paralleling those described in Table 1(*a*), except that the extra solutes were added to a final concentration of 25 mM. After the cells had been incubated in hyperosmotic medium for 6 h, their rate of protein synthesis was inhibited by about 50 % (Table 2). Addition of either glycerol or taurine had no significant effect under iso-osmotic or hyperosmotic conditions. Proline caused a significant inhibition in control cells, but had no significant effect under hyperosmotic conditions. In contrast, betaine afforded virtually complete protection against the inhibition of protein synthesis normally caused by incubation in hyperosmotic medium.

Since the findings described above indicated that, of the

Table 1. Effects of various solutes on the proliferation of SV-3T3 cells under hyperosmotic conditions

Cells were cultured for 3 days in either control medium (iso-osmotic, containing 155 mM-Na^+) or hyperosmotic medium (containing 250 mM-Na^+), and then cell density was measured as described in the text. In (*a*) the initial cell density was 3.8×10^4/4 cm^2 and the extra solutes were added to a final concentration of 10 mM. In (*b*) the initial cell density was 1×10^5/4 cm^2; betaine was added to a final concentration of 25 mM, and non-essential amino acids (NEAA: glycine, alanine, aspartate, asparagine, glutamate, proline and serine) were each present at 0.1 mM. Mean values (±S.D.) from three independent measurements are given. Significant differences from values for no additions, assessed by Student's *t* test: $*P < 0.05$, $**P < 0.01$.

		10^{-5} × Number of cells/4 cm^2		
	Addition	Control	Hyperosmotic	(% of control)
(*a*)	None	14.8±1.6	1.0±0.1	7
	Betaine	18.8±1.0*	5.9±0.1**	31
	Glycerol	12.1±0.8	2.2±0.2**	18
	Proline	13.9±1.2	2.8±0.4**	20
	Taurine	4.5±1.6**	4.0±0.3**	89
(*b*)	None	18.4±1.0	2.9±0.2	16
	Betaine	16.0±0.8	11.9±0.6**	74
	NEAA	16.0±0.1*	3.1±0.2	19
	Betaine + NEAA	18.1±0.8	12.9±0.5**	85

Table 2. Effects of various solutes on the rate of protein synthesis in SV-3T3 cells incubated under hyperosmotic conditions

Cells were incubated for 6 h in control medium (iso-osmotic, containing 155 mM-Na^+) or hyperosmotic medium (containing 250 mM-Na^+) and their rates of protein synthesis were assayed during the last 30 min of incubation, as described in the text. The extra solutes were added to a final concentration of 25 mM. Mean values (±S.D.) from three independent measurements are given. Significant differences from values for no additions, assessed by Student's t test: $*P < 0.05$, $**P < 0.01$.

	Addition	Rate of protein synthesis (nmol of leucine/30 min per mg of protein) Control	Hyperosmotic	(% of control)
(*a*)	None	19.4±1.6	9.9±0.7	48
	Betaine	19.4±0.8	18.5±1.1**	95
	Glycerol	16.8±0.9	8.5±1.3	51
	Proline	14.1±0.7**	11.4±1.1	81
	Taurine	19.3±0.7	9.7±0.3	50
(*b*)	None	13.5±1.7	6.3±0.7	47
	Glycine	13.5±0.6	8.7±0.5**	64
	Sarcosine	12.7±1.4	9.0±0.6**	71
	Dimethylglycine	15.2±3.0	8.2±0.8*	54
	Betaine	12.0±1.1	13.1±1.8**	109

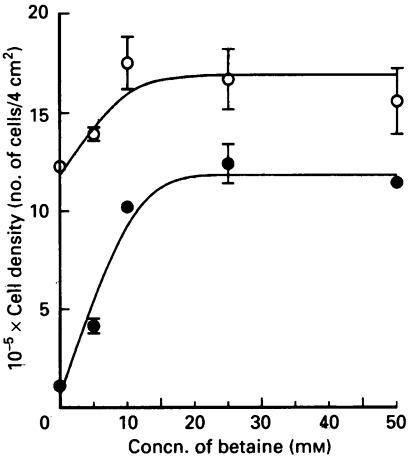

Fig. 1. Variation in cell proliferation as a function of betaine concentration

Experimental conditions were exactly as described in the legend to Table 1, the initial cell density being $2 \times 10^5/4$ cm^2. Mean values (±S.D.) from three independent measurements are given. Key: culture in: ○, iso-osmotic medium; ●, hyperosmotic medium.

solutes tested, only betaine caused marked changes under hyperosmotic conditions, coupled with little effect on control cells, we examined the action of betaine in more detail.

Concentration of betaine and length of incubation

Fig. 1 shows dose–response curves for the effect of betaine on the rate of cell proliferation. Again, added betaine slightly increased the proliferation rate of cells in the iso-osmotic medium; but its ability to counteract the normal inhibitory action of hyperosmotic medium is most striking and was clearly a saturable function of the concentration present. (Note that no osmotic compensation was used in these experiments, so that the added betaine slightly increased the osmolarity of both media in parallel.)

The effects on cell protein synthesis of 10 mM- and 25 mM-

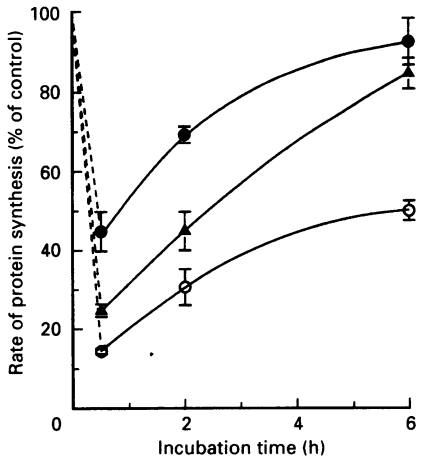

Fig. 2. Effect of betaine on cellular protein synthesis under hyperosmotic conditions

SV-3T3 cells were incubated with the indicated concentrations of betaine either in control medium (0.340 osM) or in hyperosmotic medium (0.515 osM) and their rate of protein synthesis was determined as described in the text. Rates in the hyperosmotic media are expressed as percentage of those in the corresponding control media, and mean values (±S.D.) from three independent measurements are given. The absolute values (in nmol of leucine/30 min per mg of protein) of the controls were: without betaine, 15.7±1.1; with 10 mM-betaine, 14.7±1.0; with 25 mM-betaine, 14.8±0.3. Key: betaine concentration: ○, 0; ▲, 10 mM; ●, 25 mM.

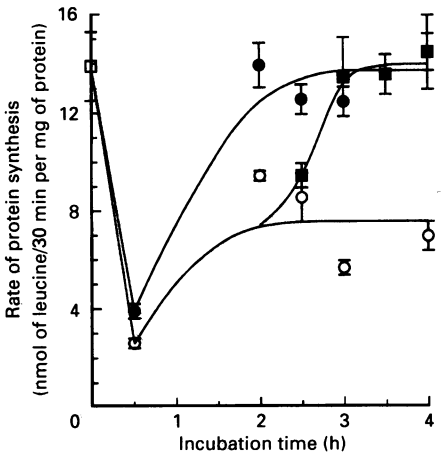

Fig. 3. Effect of addition of betaine after exposure of cells to hyperosmotic conditions

The experimental conditions and measurements made were the same as described in the legend to Fig. 2, except that some cells were incubated in hyperosmotic medium in the absence of betaine for the first 2 h and then transferred to medium containing betaine for the last 2 h. Mean values (±S.D.) from the independent measurements are given. Key: □, control; ○, hyperosmotic; ●, hyperosmotic plus 25 mM-betaine; ■, hyperosmotic without betaine for 2 h, followed by hyperosmotic with 25 mM-betaine for 2 h.

betaine, and of the time of incubation under iso-osmotic or hyperosmotic conditions, are shown in Fig. 2. Protein synthesis in control cells fell drastically during the first 30 min of incubation in hyperosmotic medium and then partly recovered during prolonged incubation. The presence of betaine diminished the extent of the initial fall and appeared to accelerate slightly the subsequent rate of recovery, both effects increasing with increase in betaine concentration. The effect on the rate of recovery was

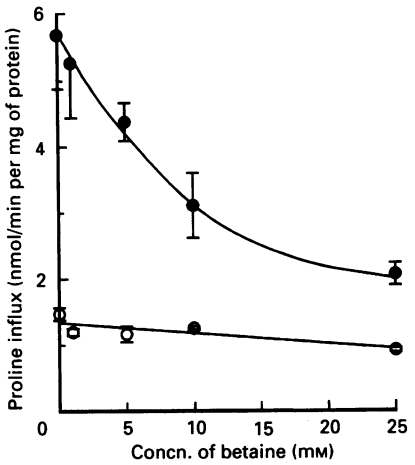

Fig. 4. Decrease in induction of amino acid transport activity in the presence of betaine

SV-3T3 cells were incubated for 6 h in either control medium (0.340 osM) or hyperosmotic medium (0.515 osM) with the addition of betaine as indicated, and their initial rates of uptake of proline were measured as described in the text. Mean values (±S.D.) from three separate measurements are given. Key: ○, control; ●, hyperosmotic.

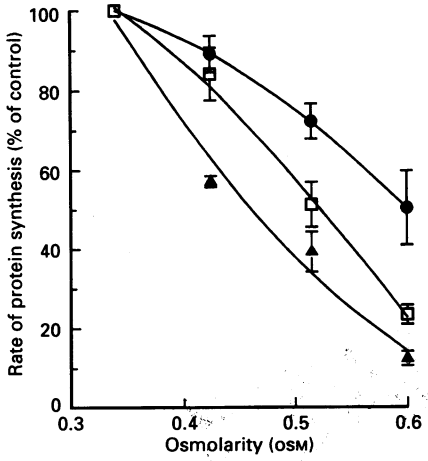

Fig. 5. Betaine as an osmolyte in hyperosmotic medium

SV-3T3 cells were incubated for 6 h in control medium (0.340 osM) or hyperosmotic media (containing appropriate concentrations of sucrose, betaine or more NaCl to give the indicated osmolarities), and rates of protein synthesis were assayed as described in the text. Results for the hyperosmotic conditions are expressed as percentages of the value in the control medium (14.3±2.4 nmol of leucine/30 min per mg of protein), and mean values (±S.D.) from three independent measurements are given. Key: ●, betaine; □, NaCl; ▲, sucrose.

confirmed by the results given in Fig. 3, which show that betaine was equally effective when added after the cells had been exposed to hyperosmotic conditions for 2 h.

Comparison of effect of betaine with that of related compounds

Table 2(*b*) shows the rates of cell protein synthesis under iso-osmotic and hyperosmotic conditions in the presence of 25 mM-glycine, -*N*-methylglycine (sarcosine), -*N*-dimethylglycine or -*N*-trimethylglycine (betaine). Although each compound produced a significant stimulation of protein synthesis under hyperosmotic conditions, that caused by betaine was most dramatic and completely countered the inhibitory action of the hyperosmotic medium.

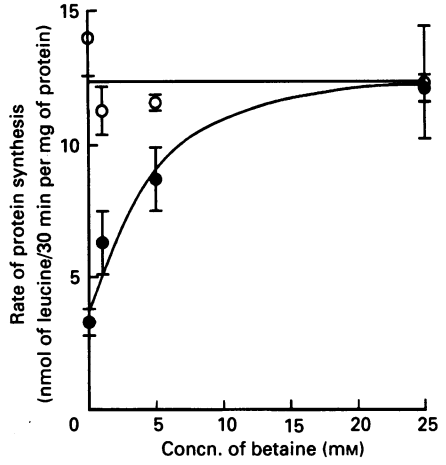

Fig. 6. Effect of betaine on the rate of protein synthesis in chick-embryo fibroblasts under hyperosmotic conditions

Chick-embryo fibroblasts were treated as described in the legend to Table 2, except that the total incubation period was only 4 h and betaine was added to the concentrations indicated. Mean values (±S.D.) from three separate measurements are given. Key: ○, control; ●, hyperosmotic.

Induction of amino acid transport capacity

The induction of amino acids transport capacity observed in cells incubated in hyperosmotic medium for 6 h was decreased when betaine was also present, apparently as a saturable function of betaine concentration (Fig. 4). With 25 mM-betaine the induced transport was decreased by about 73% and the non-induced transport by about 40%. The latter effect on control cells was observed consistently, suggesting that betaine might interact directly with the transport system. However, direct tests with betaine present from 1 to 25 mM during the measurement of proline uptake failed to reveal any significant inhibition (results not shown). Similarly, betaine present in the assay medium failed to inhibit the uptake of glycine.

Cell volume and cation content

Estimation of cell volume at the beginning of the incubation was not really satisfactory, because of the relatively long incubation needed. But measurements made at the end of 6 h incubations seemed to be more reliable and were reasonably reproducible. The volume (in μl/mg of protein) of cells incubated in hyperosmotic medium was 4.4±0.3 (9), some 30% smaller than the volume of 6.6±0.4 (9) recorded for cells incubated in the control medium (mean values±S.E.M.; $P < 0.05$). With 25 mM-betaine also present, however, there was no significant difference between the volumes of cells under control conditions [5.9±0.6 (8)] and hyperosmotic conditions [6.2±0.6 (8)].

The Na^+ content of the cells was about 80 nmol/mg of protein, and there was no significant change during a 6 h incubation in either control or hyperosmotic medium. The K^+ content was about 1100 nmol/mg of protein in cells incubated in control medium, but fell by about 24% in cells incubated in hyperosmotic medium for 6 h. Inclusion of 25 mM-betaine in the media had no significant effect on any of these values.

Use of betaine as an osmolyte

In view of the results described above, we also examined the effect of using betaine in place of NaCl or sucrose to form hyperosmotic medium. The results in Fig. 5 show that the extent of inhibition of cell protein synthesis varied considerably according to the osmolyte used, with betaine always causing less inhibition than either NaCl or sucrose.

Parallel response of chick-embryo fibroblasts

Fig. 6 shows that the inhibitory effect of hyperosmotic conditions on general protein synthesis in chick-embryo fibroblasts was similarly counteracted by the addition of betaine to the incubation medium.

DISCUSSION

The approach used for these studies is obviously limited in that a test solute could be expected to modulate the effects of hyperosmotic conditions on cell functions only if it readily enters the cells. Also, any such modulation would have to be large enough to be detectable when superimposed on the cells' normal adaptation to the osmotic stress. Hence little can be concluded from the negative and marginal observations described above. On the other hand, the large and very clear responses of the stressed cells to added betaine were quite remarkable. Addition of 10–25 mM-betaine to the hyperosmotic medium largely prevented the 90% inhibition of cell proliferation that occurred in its absence (Fig. 1). Whether it was added initially or after the cells were exposed to hyperosmotic medium, betaine converted recoveries of the rate of protein synthesis from 50% (Table 2 and Fig. 3) and 25% (Fig. 6) into 100%. Similarly 25 mM-betaine prevented a 30% decrease in cell volume and markedly decreased the induction of amino acid transport capacity (Fig. 4). Lower concentrations of betaine produced smaller but still significant effects. In comparison with betaine, the other solutes tested either produced considerably smaller effects or inhibited the function of control cells. The only significant effect of glycerol was a small stimulation of cell proliferation under hyperosmotic conditions. Proline behaved similarly when present at a concentration of 10 mM (Table 1), but at 25 mM it caused significant inhibition of both proliferation and protein synthesis (Table 2) in control cells. Taurine's actions were particularly variable, in that a concentration of 25 mM had no effect on protein synthesis under control or hyperosmotic conditions (Table 2), whereas 10 mM drastically inhibited cell proliferation under control conditions, but significantly stimulated it under hyperosmotic conditions (Table 1). The effects on protein synthesis of glycine, sarcosine and dimethylglycine were qualitatively similar to, but quantitatively smaller than, the effect of betaine (Table 2).

Betaine has received considerable attention as a compatible solute, and it is known to be an important osmolyte in vascular plants and various marine animals (Yancey *et al.*, 1982). Induction of betaine uptake in response to osmotic stress has also been demonstrated in a few systems, the clearest and best understood being that in prokaryotes, carefully analysed by Higgins and his colleagues. They have shown that in both *Escherichia coli* and *Salmonella typhimurium* the transcription of genes encoding high-affinity transporters for K^+ and betaine is regulated by external osmolarity, such that hyperosmotic conditions result in vastly increased uptake of both solutes (Higgins *et al.*, 1987). In addition, they have provided convincing evidence that this mechanism is triggered by high intracellular K^+ concentration via an effect on the supercoiling of DNA (Higgins *et al.*, 1988). More recently, hyperosmotic conditions have also been shown to increase an intrinsic betaine transport capacity in MDCK cells (Nakanishi *et al.*, 1990).

The uptake of betaine by MDCK cells is mediated by an active, Na^+-dependent, transport system (Nakanishi *et al.*, 1990), but at present the nature of the entry of betaine into chick-embryo fibroblasts and SV-3T3 cells is unknown. We have not yet been able to monitor the process directly, because radiolabelled betaine is not readily available. If uptake is mediated by a membrane transport system, it must be distinct from the amino acid transport system A (and probably ASC), because we could not detect any significant inhibition by betaine of the uptakes of either proline or glycine. Whether betaine uptake by these cells is changed under hyperosmotic conditions also remains to be discovered.

Although the latter questions must be answered in order to clarify our findings, and before any physiological significance can be attached to them, quantitative considerations indicate that the efficacy of betaine in reversing the effects of incubation in hyperosmotic medium cannot be explained simply in terms of diffusion. Equilibration of betaine at a concentration of 25 mM across the cell membrane, whether by simple or mediated diffusion, obviously could not compensate for the imposed high osmolarity. Hence it seems that betaine must either be accumulated by the cells via an unusually efficient process or have some action other than just an osmotic one. A metabolic role cannot be excluded; but betaine's acting as a donor of methyl groups seems unlikely, because added choline did not mimic the action of betaine (results not shown). In this context, it is interesting that the literature on compatible solutes provides some evidence for their exerting effects that cannot be explained simply in terms of osmotic action (Yancey *et al.*, 1982; Higgins *et al.*, 1987; Yancey & Burg, 1990; Garcia-Perez & Burg, 1991).

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REFERENCES

Borghetti, A. F., Piedimonte, G., Tramacere, M., Severini, A., Ghiringhelli, P. & Guidotti, G. G. (1980) J. Cell. Physiol. **105**, 39–40
Bradford, M. (1976) Anal. Biochem. **72**, 248–254
Erlinger, S. & Saier, M. H. (1982) In Vitro **18**, 196–202
Garcia-Perez, A. & Burg, M. B. (1991) J. Membr. Biol. **119**, 1–13
Higgins, C. F., Cairney, J., Stirling, D. A., Sutherland, L. & Booth, I. R. (1987) Trends Biochem. Sci. **12**, 339–344
Higgins, C. F., Dorman, C. J., Stirling, D. A., Waddell, L., Booth, I. R., May, G. & Brewer, E. (1988) Cell **52**, 569–584
Kletzien, R. F., Pariza, M. W., Becker, J. E. & Potter, V. R. (1975) Anal. Biochem. **68**, 537–544
Nakanishi, T., Turner, R. J. & Burg, M. B. (1990) Am. J. Physiol. **258**, F1061–F1067
Owen, N. E. & Villereal, M. L. (1983) Exp. Cell. Res. **143**, 37–46
Petronini, P. G., Tramacere, M., Kay, J. E. & Borghetti, A. F. (1986) Exp. Cell. Res. **165**, 180–190
Petronini, P. G., Tramacere, M., Mazzini, A., Piedimonte, G., Silvotti, L. & Borghetti, A. F. (1987) Exp. Cell Res. **172**, 450–462
Petronini, P. G., Tramacere, M., Mazzini, A., Kay, J. E. & Borghetti, A. F. (1989) J. Cell. Physiol. **140**, 202–211
Petronini, P. G., Tramacere, M., Wheeler, K. P. & Borghetti, A. F. (1990) Biochim. Biophys. Acta **1053**, 144–150
Piedimonte, G., Borghetti, A. F. & Guidotti, G. G. (1982) Cancer Res. **42**, 4690–4693
Rubin, H. (1973) in Tissue Culture Methods and Applications (Kruse, P. F., & Paterson, M. K., eds.), pp. 119–122, Academic Press, New York
Silvotti, L., Petronini, P. G., Mazzini, A., Piedimonte, G. & Borghetti, A. F. (1991) Exp. Cell. Res. **193**, 253–261
Tramacere, M., Petronini, P. G., Severini, A. & Borghetti, A. F. (1984) Exp. Cell Res. **151**, 70–79
Yancey, P. H. & Burg, M. B. (1990) Am. J. Physiol. **258**, R198–R204
Yancey, P. H., Clarke, M., Hand, S., Bowlus, R. & Somero, G. (1982) Science **217**, 1214–1222

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