Effects of osmolarity, ions and compatible osmolytes on cell-free protein synthesis

Maurizio BRIGOTTI*, Pier Giorgio PETRONINI†, Domenica CARNICELLI*, Roberta R. ALFIERI†, Mara A. BONELLI†, Angelo F. BORGHETTI† and Kenneth P. WHEELER‡¹

*Dipartimento di Patologia Sperimentale, Università degli Studi di Bologna, 40126 Bologna, Italy, †Dipartimento di Medicina Sperimentale, Sezione di Patologia Molecolare e Immunologia, Università degli Studi di Parma, 43100 Parma, Italy, and ‡School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K.

To mimic what might happen in cells exposed to hypertonicity, the effects of increased osmolarity and ionic strength on cell-free protein synthesis have been examined. Translation of globin mRNA by rabbit reticulocyte lysate decreased by 30–60% when osmolality was increased from 0.35 to 0.53 osmol/kg of water by the addition of NaCl, KCl, CH₃CO₂Na or CH₃CO₂K. In contrast, equivalent additions of the compatible osmolytes betaine or *myo*-inositol caused a 40–50% increase in the rate of translation, whereas amino acids (50–135 mM) that are transported via system A had no significant effect. Addition of 75 mM KCl caused a dramatic fall in the amount of the 43 S pre-initiation complex, whereas it was totally preserved when osmolarity was similarly increased by the addition of 150 mM betaine. The formation of a non-enzymic initiation complex between rabbit [³H]Phe-tRNA, poly(U) and the 80 S ribosomes was

unaffected by the addition of 75 mM NaCl or KCl, but translation of the complex decreased by 70%. Density-gradient centrifugation of reticulocyte extracts translating endogenous mRNA revealed that addition of 150 mM betaine had no effect, whereas addition of 75 mM KCl caused a marked decrease in the polysome peak, concomitant with an increase in the proportion of 80 S ribosomes and ribosomal subunits, even when elongation was inhibited with fragment A of diphtheria toxin. These results are consistent with the notion that both initiation and elongation are inhibited by unusually high concentrations of inorganic ions, but not by the compatible osmolytes betaine or *myo*-inositol.

Key words: betaine, hyperosmolarity, ionic strength, *myo*-inositol, translation.

INTRODUCTION

Animal cells exposed to hypertonic conditions undergo an immediate shrinkage that causes a marked increase in cellular concentrations of Na⁺ and K⁺. This is usually followed by a regulatory volume increase, mediated by the uptake of Na⁺ and K⁺ via Na⁺/K⁺/Cl[−] co-transport and the accompanying influx of water. The rise in cellular Na⁺ concentration presumably increases Na⁺/K⁺-ATPase activity, because the net result is a rise in the cell content and concentration of K⁺. Many mammalian cells in culture then adapt to the hyperosmolarity by accumulating ‘compatible osmolytes’, such as methylamines, polyols and some amino acids or amino acid derivatives [1,2]. This adaptation involves changes in specific gene expression that result in an increased synthesis of transporters for neutral amino acids (amino acid transporter ATA2) [3], betaine (betaine/γ-aminobutyric acid transporter BGT-1), *myo*-inositol (sodium/*myo*-inositol transporter, SMIT), taurine (taurine transporter, TauT) or of aldose reductase to catalyse the synthesis of sorbitol [2]. These changes are usually explained in terms of the need to replace the inorganic ions initially concentrated in the cells with compatible osmolytes that, unlike the inorganic ions, do not perturb macromolecular structures [4]. The main evidence supporting this latter contention comes from a number of demonstrations of protective effects of compatible osmolytes on enzyme structure and function. These include the counteraction of the inhibition of malate dehydrogenase by high concentrations of NaCl, the renaturation of acid-denatured lactate dehydrogenase, changes in the accessibility of interior thiol groups in glutamate dehydrogenase and in the quaternary structure of phosphofructokinase [4].

Although it seems reasonable to extrapolate from the latter findings to the situation inside osmotically shrunken cells, it

would be preferable to obtain some more direct evidence. The effects of inorganic ions and compatible osmolytes on cell protein synthesis are particularly important, because impairment of cell protein synthesis occurs immediately after cells shrink in hypertonic media. For example, Petronini et al. [5,6] showed that after a 30 min exposure of either chick embryo fibroblasts or porcine endothelial cells to hypertonic medium (0.5 osmol/kg of water), cell protein synthesis was inhibited by more than 40%. When hypertonicity was maintained, however, induced activity of amino acid transport system A was detectable after 2–3 h and restoration of the rate of cell protein synthesis occurred as the cells accumulated amino acids and lost K⁺. Induction of the other transport systems and marked accumulation of the other compatible osmolytes followed later, after 6–24 h.

Some correlations between changes in cell cation concentrations and rates of protein synthesis have been noted. For example, Carrasco and Smith [7] showed that inhibition of the initiation step of the translation of globin mRNA during viral infection of the host cell was accompanied by an increased concentration of cellular Na⁺. Similarly, impairment of the rate of protein synthesis in chick embryo fibroblasts occurred when there was any significant change, an increase or decrease, in intracellular Na⁺ concentration [8]. Also, the inhibition of protein synthesis caused by hypertonic culture of SV-3T3 cells or chick embryo fibroblasts was largely prevented by the addition of 10–25 mM betaine [9], which is in keeping with the general ideas about compatible osmolytes. We have now extended these findings by examining the effects of various concentrations of inorganic ions and compatible osmolytes on the cell-free translation of mRNA, endogenous and synthetic. The aim was to mimic with a simplified translation system *in vitro* what might happen in whole cells exposed to hypertonicity.

Abbreviation used: EF2, elongation factor 2.

¹ To whom correspondence should be addressed (e-mail K.P.Wheeler@sussex.ac.uk).

EXPERIMENTAL

Materials

[³H]Leucine (66 Ci/mmol), [methyl-³H]methionine (83 Ci/mmol), [³H]NAD⁺ (37 Ci/mmol) and [³H]phenylalanine (120 Ci/mmol) were obtained from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Micrococcal nuclease, creatine phosphokinase, spermidine and all the other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.).

Translation of endogenous mRNA by rabbit reticulocyte lysate

Rabbit reticulocyte lysate was prepared as described in [10]. Translation of endogenous mRNA *in vitro* by the unfractionated rabbit reticulocyte lysate was performed in reaction mixtures (125 µl) containing 30 mM Hepes/KOH, pH 7.5, 80 mM KCl, 1.8 mM magnesium acetate, 50 µM of each amino acid except leucine, 1 mM ATP, 0.25 mM GTP, 5 mM creatine phosphate, 0.18 mg/ml creatine phosphokinase, 0.5 mM dithiothreitol, 0.4 mM spermidine, 0.24 µM (2 µCi) [³H]leucine and 50 µl of lysate [11]. Where indicated, the osmolality of the reaction mixture was increased by addition of KCl, NaCl, CH₃CO₂K, CH₃CO₂Na, betaine, *myo*-inositol or amino acids. The complete mixture was incubated for 5 min at 28 °C and then a 62.5 µl sample was taken and added to 1 ml of 0.1 M KOH. The solution was decolourized with two drops of 35% (w/v) H₂O₂ and 1 ml of 20% (w/v) trichloroacetic acid was added. The precipitate was collected on a Whatman GF/C filter and its radioactivity measured by scintillation counting. The osmolality of the residual reaction mixture was measured with a vapour pressure osmometer (Wescor), the standard mixture being 0.352 osmol/kg of water. Under these standard conditions the [³H]leucine incorporated was 57 599 ± 7225 d.p.m. (mean ± S.D., *n* = 15).

Determination of the 43 S pre-initiation complex in unfractionated reticulocyte lysate

To measure the 43 S pre-initiation complex, translation of endogenous mRNA by unfractionated rabbit reticulocyte lysate was carried out as described above except that the solution contained 0.6 µM [³H]methionine as the labelled amino acid. After incubation for 2 min at 28 °C, the polysome profiles of the reaction mixtures were determined by centrifugation in a sucrose density gradient (see below) and 1 ml fractions were collected. The RNA, including aminoacyl tRNA, present in each fraction was precipitated by addition of 2 vol. of 2% cetyltrimethylammonium bromide and 2 vol. of 0.5 M sodium acetate (pH 5.2) containing 0.5 mg/ml carrier calf liver RNA [12]. The precipitate was collected on Whatman GF/C filters, washed extensively with water and its radioactivity measured by scintillation counting.

Micrococcal nuclease treatment of rabbit reticulocyte lysate

Rabbit reticulocyte lysate was digested with micrococcal nuclease, to destroy endogenous mRNA and tRNA, as described by Jackson and Hunt [13].

Fractionation of rabbit tRNA

Total tRNA was isolated from rabbit liver with the use of classic phenol extraction procedures and differential precipitation [14]. The tRNA was fractionated on benzoylated DEAE-cellulose and the pure tRNA^{Phe} obtained was charged with [³H]phenylalanine (41 250 d.p.m./pmol) as described by Hultin and Näslund [15].

A tRNA pool minus tRNA^{Phe} was reconstituted by mixing the separated tRNAs obtained from benzoylated DEAE-cellulose according to the relative amount of each tRNA. The amount of lysate assayed for protein synthesis contained approx. 4 µg of tRNA, corresponding to 0.2 µg (8 pmol) of tRNA^{Phe} [16,17].

Poly(U) translation by rabbit reticulocyte lysate treated with micrococcal nuclease

Translation of poly(U) *in vitro* by rabbit reticulocyte lysate treated with micrococcal nuclease was performed as described above for the translation of endogenous mRNA, with the following modifications. A non-enzymic initiation complex between [³H]Phe-tRNA (8 pmol), poly(U) (145 µg) and the 80 S ribosomes present in the lysate was obtained during a preliminary incubation in 7 mM (CH₃CO₂)₂Mg [18–20]. After 10 min at 28 °C, 3.8 µg of rabbit tRNAs (without tRNA^{Phe}) and the other components of the translation mixture (except [³H]leucine) were added. The Mg²⁺ concentration was then reduced to 2.5 mM by diluting the mixture, and after a further 5 min incubation at 28 °C the extent of phenylalanine polymerization and the osmolality of each sample were measured as described above. Under normal osmotic conditions (0.33 osmol/kg of water) the [³H]-phenylalanine incorporated was 68 550 ± 5976 d.p.m. (mean ± S.D., *n* = 8). The formation of the non-enzymic initiation complex was assessed in parallel tubes by collecting the ribosome-bound radioactivity on Millipore filters [21]. The synthesis of oligopeptides during the formation of the initiation complex is likely, since the pre-incubation mixture contained the whole lysate, in which elongation factors and small amounts of ATP and GTP were present. Protein synthesis measured with samples of the pre-incubation mixture, however, gave values similar to the background radioactivity values.

Determination of polysome profiles

Translation of endogenous mRNA by unfractionated rabbit reticulocyte lysate was carried out as described above, except that the solution contained 50 µM NAD⁺ and non-radioactive leucine. Where indicated, the mixtures were treated with 0.2 µM fragment A of diphtheria toxin (see below), or made hyperosmolar by the addition of 75 mM KCl, or both. The polysome profiles of the reaction mixtures were determined by centrifugation in a sucrose density gradient (40 ml, 15–30%, w/v) at 70 000 *g* for 15 h in a Beckman SW 28 rotor. Rabbit reticulocyte ribosomal subunits [22] and *Artemia salina* 80 S ribosomes [23] were used as standards.

ADP-ribosylation of elongation factor 2 (EF2)

Fragment A was prepared as described by Chung and Collier [24] from diphtheria toxin purified by the method of Pope and Stevens [25]. ADP-ribosylation of EF2 was measured with 0.2 µM diphtheria toxin fragment A and 5 µM [³H]NAD⁺ in the reaction mixtures described above for the translation of endogenous mRNA by unfractionated reticulocyte lysate, modified to contain non-radioactive leucine. After 5 min of incubation at 28 °C the acid-insoluble radioactivity of 20 µl samples was collected on Whatman GF/C filters and counted.

RESULTS AND DISCUSSION

Effects of solutes on cell-free protein synthesis

Figure 1 shows the effect of increased osmolality and ionic strength on the translation of globin mRNA by unfractionated

Figure 1 Effects of increased osmolarity and ionic strength on the translation of globin mRNA by rabbit reticulocyte lysate

Translation was measured in terms of the rate of incorporation of ^3H -labelled leucine into protein, as described in the Experimental section, and expressed as a percentage of the value obtained with the standard reaction mixture (0.352 osmol/kg of water). The latter was made hyperosmolar by addition of 10–75 mM KCl ($\blacktriangle$), NaCl ($\bullet$), $\text{CH}_3\text{CO}_2\text{K}$ ($\triangle$) or $\text{CH}_3\text{CO}_2\text{Na}$ ($\circ$), or of 20–150 mM betaine ($\blacksquare$) or *myo*-inositol ($\square$). Final osmolalities were measured as described in the Experimental section. Means $\pm$ S.E.M. from three measurements are given.

rabbit reticulocyte lysate. Increased osmolarity alone clearly did not inhibit translation, because high concentrations of either betaine or *myo*-inositol actually increased protein synthesis. When both osmolarity and ionic strength were increased by addition of NaCl or KCl, however, the rate of translation fell, with NaCl inhibiting more than equimolar additions of KCl. $\text{CH}_3\text{CO}_2\text{Na}$ mimicked the effect of NaCl, whereas only the highest concentration of $\text{CH}_3\text{CO}_2\text{K}$ was inhibitory.

Some of the inhibitory effects of these inorganic ions are in keeping with the findings of early studies that established optimal experimental conditions for translation in cell-free systems. For example, translation specifically requires the K^+ concentration to be within a relatively narrow range, but tolerates higher concentrations of $\text{CH}_3\text{CO}_2\text{K}$ than of KCl. The effects of salts on the conformation and function of macromolecules are usually related to the destabilizing or stabilizing properties of their anions and cations according to the Hofmeister series [26,27]. The Hofmeister series for cations indicates that both Na^+ and K^+ destabilize macromolecules, Na^+ being more perturbing than K^+ . The series for anions indicates that Cl^- also has a destabilizing effect, whereas CH_3CO_2^- has stabilizing properties. Since the actions of the different ions are additive, however, the overall effects of the salts are not simply predictable. The results in Figure 1 are as expected from the Hofmeister series for the cations (NaCl versus KCl, and $\text{CH}_3\text{CO}_2\text{Na}$ versus $\text{CH}_3\text{CO}_2\text{K}$), but the effects of the anions are not so clear cut. The different effects of KCl and $\text{CH}_3\text{CO}_2\text{K}$ can be explained in terms of the stabilizing CH_3CO_2^- counteracting the inhibition caused by the mildly destabilizing K^+ , whereas Cl^- increases destabilization. On the other hand, the perturbing effect of Na^+ appears to be unmodified by the nature

Figure 2 Effect on translation of hyperosmolarity produced with different ratios of KCl and betaine

The experiments were performed as described in the legend to Figure 1, except that hyperosmolarity was produced by the combined addition of KCl (0–75 mM) and betaine (150–0 mM), maintaining osmolality at about 0.5 osmol/kg of water. The rates of translation are expressed as percentages of the value obtained with the standard medium (0.352 osmol/kg of water). Means $\pm$ S.E.M. from three measurements are given.

of the accompanying anion (NaCl versus $\text{CH}_3\text{CO}_2\text{Na}$). However, the specific requirements for K^+ and Mg^{2+} make it difficult to distinguish between specific and general salt effects. For example, added Na^+ would be expected to inhibit by competing with K^+ . It is clear, however, that the increased concentrations of the common physiologically important ions, Na^+ , K^+ and Cl^- , inhibited cell-free translation, whereas equivalent concentrations of the typical compatible osmolytes betaine and *myo*-inositol did not. With NaCl or KCl at osmolality values close to 0.5 osmol/kg of water, the extent of inhibition in the cell-free system was almost 60%, well within the 40–75% range found when intact cells were exposed to the same osmolality [6,28].

To mimic cell shrinkage a little more closely, we checked the effect of simultaneously increasing the concentrations of everything in the reaction mixture by reducing its final volume to 63% of that normally used. This inhibited translation by about 18%, compared with the approx. 25% inhibition predicted by the curve in Figure 1 from the increased KCl concentration (80–127 mM).

In general, these results are in keeping with the notion that impairment of translation in whole cells exposed to hypertonicity is the consequence of the increase in the concentrations of inorganic ions, particularly Na^+ , K^+ and Cl^- . Such increases are unavoidable during the initial rapid shrinkage of the cells, and are only modified in detail, not dissipated, during regulatory volume increase brought about by $\text{Na}^+/\text{K}^+/\text{Cl}^-$ co-transport and Na^+/H^+ exchange. The latter processes are also likely to change the relative concentrations of the ions, which might also be inhibitory.

Figure 2 shows the result of performing cell-free protein synthesis in the presence of different combinations of KCl and betaine, such that the total osmolality remained constant at about 0.5 osmol/kg of water. Clearly, the gradual iso-osmotic

Table 1 Effect of high concentrations of amino acids on the translation of globin mRNA by rabbit reticulocyte lysate

Translation was measured in terms of the rate of incorporation of ^3H -labelled leucine into protein, as described in the Experimental section, and expressed as a percentage of the value obtained with the standard reaction mixture (0.352 osmol/kg of water). Means $\pm$ S.E.M. from four experiments are given. The standard medium was made hyperosmolar by addition of amino acids, as indicated, giving final osmolalities (means $\pm$ S.D.) of 0.383 ± 0.004 osmol/kg of water with 50 mM and 0.454 ± 0.019 osmol/kg of water with 135 mM.

Amino acid	Cell-free protein synthesis (%)	
	50 mM	135 mM
Alanine	107 ± 3	103 ± 1
Serine	138 ± 3	107 ± 4
Glutamine	117 ± 3	96 ± 2
Threonine	104 ± 10	99 ± 1
Glycine	136 ± 2	100 ± 5
Proline	117 ± 14	111 ± 10
Valine	81 ± 4	44 ± 3
Glutamic acid + lysine*	43 ± 1	4 ± 1

* An equimolar mixture of glutamic acid and lysine.

replacement of the inhibiting KCl with betaine restored protein synthesis to the control rate. We also checked the effects of high concentrations of amino acids on cell-free protein synthesis because, as explained in the Introduction, many intact cells respond to hypertonic incubation by increasing their uptake of amino acids via transport system A. With the exception of valine, which was inhibitory when present at 135 mM, the neutral amino acids tested caused no significant inhibition of the rate of translation (Table 1). In contrast, an equimolar mixture of lysine and glutamic acid strongly inhibited protein synthesis. Since system A transports small neutral amino acids, not the larger more hydrophobic ones or those bearing a net charge, such as lysine and glutamate [29], these findings support the concept that induction of system A provides cells with amino acids as compatible osmolytes. The finding that only certain amino acids behave as compatible solutes is in keeping with early observations of enzyme activity being unaffected by amino acids such as glycine, alanine or proline, but markedly impaired by lysine or arginine (see [4]).

Effect of osmolality and ionic strength on the initiation and elongation steps of translation

In the experiments illustrated in Figure 1, a marked inhibition of protein synthesis by NaCl and KCl was observed with the use of unfractionated rabbit reticulocyte lysate, a system consisting mainly of polysomes engaged in the elongation step of protein synthesis. Several reports, however, indicate that the initiation step of translation is inhibited in whole cells exposed to hypertonic conditions [7,30–32]. Lu et al. [31], for example, showed that osmotic stress induced the phosphorylation of eukaryotic initiation factor 2α (eIF2 α) in erythroid cells, thus blocking the assembly of the 43 S pre-initiation complex. The formation of the 43 S pre-initiation complex in unfractionated rabbit reticulocyte lysate can be easily detected by labelling the 40 S subunit with the initiator [^3H]Met-tRNA_i [12], so we examined the sensitivity of this process to increased osmolality. Figure 3 shows that addition of 75 mM KCl caused a dramatic fall in the amount of the 43 S pre-initiation complex, whereas it was totally preserved when osmolality was similarly increased by the addition of 150 mM betaine. On the other hand, the persistence of the first peak of radioactivity, corresponding to the [^3H]Met-

Figure 3 Formation of 43 S pre-initiation complex

Labelling of the 40 S subunit with [^3H]methionine was measured in unfractionated rabbit reticulocyte lysate as described in the Experimental section. $\circ$, Control reaction mixture; reaction mixtures were made hypertonic by the addition of 75 mM KCl ($\blacktriangle$) or 150 mM betaine ($\blacksquare$).

tRNAs, indicates that the aminoacylation step was not affected by the added KCl.

To test whether the elongation, not just the initiation, is affected by increased ionic strength, translation from a non-enzymic initiation complex was examined. A non-enzymic initiation complex between rabbit [^3H]Phe-tRNA, poly(U) and the 80 S ribosomes present in the micrococcal nuclease-treated lysate

Figure 4 Effect of increased ionic strength on the translation of poly(U) *in vitro*

Rabbit reticulocyte lysate was treated with micrococcal nuclease and then used to translate poly(U), as described in the Experimental section. The media were made hyperosmolar by addition of 10–75 mM KCl ($\blacktriangle$) or NaCl ($\bullet$), or of 20–150 mM betaine ($\blacksquare$) or *myo*-inositol ($\square$). The rates of translation are expressed as percentages of the value obtained with the standard medium (0.352 osmol/kg of water). Means $\pm$ S.E.M. from three measurements are given.

was formed first during a preliminary incubation. Then poly(U) translation was measured in the presence and absence of added salts or compatible osmolytes. Figure 4 shows that increased concentrations of NaCl and KCl caused an inhibition of poly-phenylalanine synthesis similar to that observed with globin synthesis (Figure 1). Checks with the highest concentration of the salts used in Figure 4 showed that formation of the non-enzymic initiation complex was not affected (results not shown), perhaps because poly(U) is a relatively robust molecule having less stringent folding requirements than endogenous mRNA. Thus it seems clear that the elongation step is also inhibited by increased ionic strength due to added KCl or NaCl. In contrast, neither the betaine nor the *myo*-inositol significantly affected the rate of elongation (Figure 4), suggesting that their stimulation of protein synthesis shown in Figure 1 resulted from an effect on initiation alone.

Site of action of destabilizing ions

We examined the effect of increased osmolarity and ionic strength on polysome structure by density-gradient centrifugation of rabbit reticulocyte extracts translating endogenous mRNA (Figure 5). The polysome profile of the standard reaction mixture (Figure 5A) was compared with those of mixtures made to approx. 0.5 osmol/kg of water by the addition of 150 mM betaine (Figure 5B) or 75 mM KCl (Figure 5C). Addition of betaine did not affect the polysome profile, whereas KCl caused a decrease in the polysome peak concomitant with an increase in the proportion of 80 S ribosomes and ribosomal subunits. Thus high concentrations of the salt, but not of betaine, markedly decreased the number of polysomes. Since this effect of KCl could be explained either by inhibition of polysome formation or by disaggregation of existing polysomes, we tested the effect of inhibiting elongation with fragment A of diphtheria toxin. This inhibits the elongation step of translation by ADP-ribosylation of EF2, with no obvious effect on ribosome structure. The activity of fragment A was evaluated directly by measuring the transfer of the [3 H]ADP-ribose moiety of NAD to the EF2 present in the translation mixture: it was not affected by the addition of 75 mM KCl (results not shown). This allowed us to check the effect of increased salt concentration on polysomes when nascent peptides could not be completed because EF2 was unavailable. Addition of fragment A of diphtheria toxin to the standard reaction mixture caused a strong inhibition (91 %) of protein synthesis, 'freezing' the proportion of polysomes carrying nascent peptides (Figure 5D). When 75 mM KCl was also added, however, the effect was very similar to that seen in the absence of the toxin inhibitor; a loss of polysomes accompanied by an increase in the proportion of 80 S ribosomes and ribosomal subunits (Figure 5E). This indicates that the high salt concentration does cause disaggregation of existing polysomes, though there are at least two possible interpretations of how this occurs. High ionic strength might simply cause the ribosome subunits to come off the mRNA and then form an increased pool of free 80 S ribosomes. Alternatively, the high salt concentration might perturb the polysome structure sufficiently to enable endogenous nucleases to attack the mRNA, splitting off ribosomes still attached to small remnants of the mRNA.

Effect of hypo-osmolarity

As expected from known properties of the reticulocyte lysate system, progressive decrease of the 80 mM KCl normally present in the assay buffer caused the rate of protein synthesis to fall. In a single experiment, it decreased almost linearly with the KCl

Figure 5 Effects of increased osmolarity and ionic strength on polysomes

Rabbit reticulocyte extracts translating endogenous mRNA were analysed by density-gradient centrifugation as described in the Experimental section. (A) Standard mixture (0.352 osmol/kg of water); (B) medium made to approx. 0.5 osmol/kg of water by addition of 150 mM betaine; (C) medium made to approx. 0.5 osmol/kg of water by addition of 75 mM KCl; (D) standard mixture treated with diphtheria toxin fragment A; (E) as in (D), but also made to approx. 0.5 osmol/kg of water by addition of 75 mM KCl.

concentration, to $35 \pm 2\%$ (mean $\pm$ S.D., $n = 3$) of the control when KCl was omitted from the assay buffer. The addition of betaine to maintain constant osmolarity afforded some protection, giving a value of $51 \pm 4\%$ under the same conditions. Hence, although the specific requirement for K^+ dominates, decreased osmolarity seems to be partly responsible for this effect. We did not pursue this aspect further, however, because there is no evidence for accumulation of compatible osmolytes following cell swelling, only the reverse.

Physiological implications

This simple cell-free system is limited by the obvious differences between it and cell cytosols, with their higher viscosity, macromolecules, cytoskeleton and organelles. Nevertheless, the results described above are consistent with the notion that both the initiation and elongation steps of protein synthesis are inhibited by unusually high concentrations of inorganic ions, but not when the osmolarity is similarly increased by the addition of a compatible osmolyte, such as betaine or *myo*-inositol. We have detected both increased Na^+ concentration (100%) and inhibition of protein synthesis (40%) as soon as 5 min after exposure of porcine endothelial cell to hypertonicity (0.5 osmol/kg of water; R. R. Alfieri, P. G. Petronini, M. Brigotti, M. A. Bonelli, A. F. Borghetti and K. P. Wheeler, unpublished work), suggesting that these are important early responses to cell shrinkage. These cells undergo apoptosis during prolonged culture in hypertonic media depleted of compatible osmolytes, whereas in their presence hypertonicity causes induction of the membrane transporters for these osmolytes, the cells accumulate them and survive [33]. Thus the results reported here provide new experimental evidence that supports the usual interpretation, outlined in the Introduction, of the responses many cells show to hypertonic stress. However, as discussed previously [33], it remains unclear why so many cells possess this ability to adapt to a circumstance that they apparently are unlikely to encounter.

This work was supported by grants from Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR), Ministero della Salute, Pallotti's Legacy for Cancer Research and University of Bologna (funds for selected research topics). We thank Professor Guy Dirheimer and Dr Gérard Keith (Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France) for the generous gift of rabbit tRNAs.

REFERENCES

- Burg, M. B. (1995) Molecular basis of osmotic regulation. *Am. J. Physiol.* **268**, F983–F996.
- Burg, M. B., Kwon, H. M. and Kultz, D. (1997) Regulation of gene expression by hypertonicity. *Annu. Rev. Physiol.* **59**, 437–455.
- Alfieri, R. R., Petronini, P. G., Bonelli, M. A., Caccamo, A. E., Cavazzoni, A., Borghetti, A. F. and Wheeler, K. P. (2001) Osmotic regulation of ATA2 mRNA expression and amino acid transport System A activity. *Biochem. Biophys. Res. Commun.* **283**, 174–178.
- Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982) Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214–1222.
- Petronini, P. G., Tramacere, M., Kay, J. E. and Borghetti, A. F. (1986) Adaptive response of cultured fibroblasts to hyperosmolarity. *Exp. Cell. Res.* **165**, 180–190.
- Petronini, P. G., Alfieri, R. R., Losio, M. N., Caccamo, A. E., Cavazzoni, A., Bonelli, M. A., Borghetti, A. F. and Wheeler, K. P. (2000) Induction of BGT-1 and amino acid system A transport activities in endothelial cells exposed to hyperosmolarity. *Am. J. Physiol.* **279**, R1580–R1589.
- Carrasco, L. and Smith, A. E. (1976) Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. *Nature (London)* **264**, 807–809.
- Petronini, P. G., Tramacere, M., Mazzini, A., Kay, J. E. and Borghetti, A. F. (1989) Control of protein synthesis by extracellular Na^+ in cultured fibroblasts. *J. Cell. Physiol.* **140**, 202–211.
- Petronini, P. G., De Angelis, E. M., Borghetti, P., Borghetti, A. F. and Wheeler, K. P. (1992) Modulation by betaine of cellular responses to osmotic stress. *Biochem. J.* **282**, 69–73.
- Allen, E. H. and Schweet, R. S. (1962) Synthesis of hemoglobin in a cell-free system. Properties of the complete system. *J. Biol. Chem.* **237**, 760–767.
- Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L. and Sperti, S. (1989) Effect of α -sarcosine and ribosome-inactivating proteins on the interaction of elongation factors with ribosomes. *Biochem. J.* **257**, 723–727.
- Ligon, S., Jackson, R. J. and Hunt, T. (1973) Control of protein synthesis in reticulocyte lysate by haemin. *Nat. New Biol.* **241**, 150–152.
- Jackson, R. J. and Hunt, T. (1983) Preparation and use of nuclease-treated rabbit reticulocyte lysates for the translation of eukaryotic messenger RNA. *Methods Enzymol.* **96**, 50–74.
- Fournier, M., Dorizzi, M., Darger, C. and Labouesse, J. (1976) Purification of tRNA^{Trp}, tRNA^{Val}, and partial purification of tRNA^{Ala} and tRNA^{Met} from beef liver. *Biochimie* **58**, 1159–1165.
- Hultin, T. and Näslund, P. H. (1978) Stimulation of enzymatic phe-tRNA binding to mammalian ribosomes by thallium ions at concentrations blocking other ribosomal functions. *Eur. J. Biochem.* **88**, 143–148.
- Holt, C. E., Joel, P. B. and Herbert, E. (1966) Turnover of terminal nucleotides of soluble ribonucleic acid in reticulocytes. *J. Biol. Chem.* **241**, 1819–1829.
- Smith, D. W. E. and McNamara, A. L. (1972) The transfer RNA content of rabbit reticulocytes: enumeration of the individual species per cell. *Biochim. Biophys. Acta* **269**, 67–77.
- Mosteller, R. D., Culp, W. J. and Hardesty, B. (1968) Deacylated transfer ribonucleic acid as a factor for peptide chain initiation in rabbit reticulocyte systems. *J. Biol. Chem.* **243**, 6343–6352.
- Konecki, D., Kramer, G., Pinphanichakarn, P. and Hardesty, B. (1975) Polyamines are necessary for maximum *in vitro* synthesis of globin peptides and play a role in chain initiation. *Arch. Biochem. Biophys.* **169**, 192–198.
- Sperti, S., Brigotti, M., Zamboni, M., Carnicelli, D. and Montanaro, L. (1991) Requirements for the inactivation of ribosomes by gelonin. *Biochem. J.* **277**, 281–284.
- Sperti, S., Montanaro, L., Mattioli, A. and Testoni, G. (1975) Relationship between elongation factor I- and elongation factor II-dependent guanosine triphosphatase activities of ribosomes. Inhibition of both activities by ricin. *Biochem. J.* **148**, 447–451.
- Schreier, M. H. and Staehelin, T. (1973) Initiation of mammalian protein synthesis: the importance of ribosome and initiation factor quality for the efficiency of *in vitro* systems. *J. Mol. Biol.* **73**, 329–349.
- Sierra, J. M., Meier, D. and Ochoa, S. (1974) Effect of development on the translation of messenger RNA in *Artemia salina* embryos. *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2693–2697.
- Chung, D. W. and Collier, R. J. (1977) The mechanism of ADP-ribosylation of elongation factor 2 catalyzed by fragment A from diphtheria toxin. *Biochim. Biophys. Acta* **483**, 248–257.
- Pope, C. G. and Stevens, M. F. (1958) The purification of diphtheria toxin and the isolation of crystalline toxin-protein. *Br. J. Exp. Pathol.* **39**, 139–149.
- Hofmeister, F. (1888) On the understanding of the effects of salts. *Arch. Exp. Pathol. Pharmacol.* **24**, 247–260.
- von Hippel, P. H. and Schleich, T. (1969) The effects of neutral salts on the structure and conformational stability of macromolecules in solution. In *Structure and Stability of Biological Macromolecules* (Timasheff, S. N. and Fasman, G. D., eds.), pp. 417–574. Dekker, New York.
- Petronini, P. G., Tramacere, M., Mazzini, A., Piedimonte, G., Silvotti, L. and Borghetti, A. F. (1987) Hyperosmolarity-induced stress proteins in chick embryo fibroblasts. *Exp. Cell. Res.* **172**, 450–462.
- Christensen, H. N., Liang, M. and Archer, E. G. (1967) A distinct Na^+ -requiring transport system for alanine, serine, cysteine, and similar amino acids. *J. Biol. Chem.* **242**, 5237–5246.
- Saborio, J. L., Pong, S.-S. and Koch, G. (1974) Selective and reversible inhibition of initiation of protein synthesis in mammalian cells. *J. Mol. Biol.* **85**, 195–211.
- Lu, L., Han, A.-P. and Chen, J.-J. (2001) Translation initiation control by heme-regulated eukaryotic initiation factor 2 kinase in erythroid cells under cytoplasmic stresses. *Mol. Cell. Biol.* **21**, 7971–7980.
- Uesono, Y. and Toh-e, A. (2002) Transient inhibition of translation initiation by osmotic stress. *J. Biol. Chem.* **277**, 13848–13855.
- Alfieri, R. R., Cavazzoni, A., Petronini, P. G., Bonelli, M. A., Caccamo, A. E., Borghetti, A. F. and Wheeler, K. P. (2002) Compatible osmolytes modulate the responses of porcine endothelial cells to hypertonicity and protect them from apoptosis. *J. Physiol.* **540**, 499–508.