Eur. J. Biochem. 215, 449–454 (1993)

Cell swelling and the sensitivity of autophagic proteolysis to inhibition by amino acids in isolated rat hepatocytes

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(Received March 10, 1993) – EJB 93 0345/6

In the isolated perfused rat liver, autophagic proteolysis is inhibited by hypo-osmotic perfusion media [Häussinger, D., Hallbrucker, C., vom Dahl, S., Lang, F. & Gerok, W. (1990) *Biochem. J. 272*, 239–242]. Here we report that in isolated hepatocytes, incubated in the absence of amino acids to ensure maximal proteolytic flux, proteolysis was not inhibited by hypo-osmolarity while the synthesis of glycogen from glucose, a process known to be very sensitive to changes in cell volume [Baquet, A., Hue, L., Meijer, A. J., van Woerkom, G. M. & Plomp, P. J. A. M. (1990) *J. Biol. Chem. 265*, 955–959], was stimulated under identical conditions. However, in isolated hepatocytes, hypo-osmolarity increased the sensitivity of autophagic proteolysis to inhibition by low concentrations of amino acids. The anti-proteolytic effect of hypo-osmolarity in our experiments was not due to stimulation of amino-acid transport into the hepatocytes: neither the consumption of most amino acids, nor the rate of urea synthesis was appreciably affected by hypo-osmotic incubation conditions. In the course of these studies we also found that hypo-osmolarity increased the affinity of protein synthesis for amino acids.

In the presence of amino acids the intracellular level of ATP was not much affected. However, because of cell swelling under these conditions the intracellular concentration of ATP decreased. It is proposed that a small part of the inhibition of proteolysis by amino acids may be due to this fall in ATP concentration.

Evidence is rapidly accumulating that an increase in the volume of hepatocytes, in response either to intracellular amino acid accumulation or to a decrease in the osmolarity of the extracellular fluid, has anabolic and anti-catabolic effects. Thus, an increase in cell volume results in increased glycogen synthesis [1, 2], increased lipogenesis [3, 4], increased polyamine synthesis [5], increased metabolism of some amino acids [6, 7], and in decreased glycogenolysis [8]. Häussinger and coworkers [9–11] demonstrated that in the isolated perfused rat liver an increase in cell volume also inhibited protein breakdown and that this phenomenon could account for, at least in part, the well known property of some amino acids to inhibit this process. However, Caro [12] showed that in isolated rat hepatocytes, an increase in cell volume did not inhibit proteolysis, or even slightly stimulated it, when flux through the autophagic pathway was maximal, i.e. in the absence of added amino acids. Intrigued by this puzzling difference in results, which was not discussed [11], we decided to investigate this property of isolated hepatocytes in more detail. We have now found in rat hepatocytes that, although a decrease in medium osmolarity *per se* does not appreciably affect proteolysis, it does increase the sensitivity of proteolysis to inhibition by amino acids.

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Abbreviation. AA, basal mixture of amino acids.

Enzyme. Glutaminase (EC 3.5.1.2).

MATERIALS AND METHODS

Rat hepatocytes were isolated from 20–24-h fasted male Wistar rats (200–250 g) as in [13].

Hepatocytes (5 mg dry cells/ml) were incubated in closed 25-ml plastic counting vials at 37°C in Krebs-Henseleit bicarbonate medium containing 1.3 mM Ca^{2+}, 10 mM sodium Hepes, 20 mM glucose, 25 μM cycloheximide (unless otherwise indicated), and the additions mentioned in the legends to the figures. The gas phase was 95% O_2/5% CO_2. The final pH of the incubation medium was 7.4. The osmolarity of the medium (305 mOsm under iso-osmotic conditions) was varied by changing the concentration of NaCl, the main component of the medium, from 120 mM (iso-osmotic) to lower (hypo-osmotic) or higher (hyper-osmotic) values, as indicated in the legends to the figures.

The final concentration of amino acids in the basal mixture of amino acids (1 × AA) used in our studies was as described previously [13] and approximated the concentration in the portal vein of a starved rat, except that arginine was replaced by ornithine to avoid overestimation of ornithine-cycle flux and that valine was omitted from the mixture, to allow accurate measurements of proteolytic flux [14].

For the determinations of ATP and lactate, incubations were terminated in the cold by addition of $HClO_4$ (final con-

centration 3%, mass/vol). After removal of the protein by centrifugation, the supernatants were neutralised to pH 7 with a mixture of 2 M KOH and 0.3 M Mops.

For the amino-acid analyses, incubations were terminated by addition of sulphosalicylic acid (final concentration 4%, mass/vol). After removal of the protein by centrifugation, the supernatants were brought to pH 2.2 with 1 M LiOH.

Proteolysis was measured as the production of either valine or leucine in the presence of cycloheximide to prevent simultaneous protein synthesis [15]. These amino acids are not catabolized in rat liver [16] and they are produced in proportion to their quantitative occurrence in liver protein [14, 17].

Cell volumes were measured using the hematocrit method [1, 18]. To obtain the true water volume equivalent to 1 g dry cells, 1 ml was subtracted from the packed hepatocyte volume (the density of the dry cells was assumed to be equal to 1). The packed hepatocyte volume/g dry cells obtained with the hematocrit method under iso-osmotic conditions, was similar to the wet mass/dry mass ratio determined with other techniques [19]. Protein synthesis was measured according to Hensgens and Meijer [20]. Glycogen [21, 22], ATP [23] and lactate [24] were measured according to standard procedures. Amino acids and urea were determined as described previously [14] with a Pharmacia-LKB alpha-plus amino acid analyser.

For the determinations of intracellular amino acids, 0.4 ml cell suspension was diluted with 1.6 ml ice-cold Krebs-Henseleit bicarbonate buffer without amino acids, 5 s prior to centrifugation of the cells (2 s in a microcentrifuge). After removal of the extracellular fluid, the cell pellet was extracted with 300 µl 5% (mass/vol) sulphosalicylic acid.

The statistical significance of the differences between groups of observations was tested with a one-way repeated measures analysis of variance (ANOVA) or a paired *t*-test where appropriate. When the ANOVA yielded a significant *F* ratio, the location was identified with the Tukey post-hoc analysis. Only values of $P<0.05$ were considered to be significant.

RESULTS

When hepatocytes are incubated in the absence of added amino acids, autophagic flux is maximal [25, 26]. As shown previously by Caro [12], an increase in hepatocyte volume by decreasing the NaCl content of the extracellular fluid did not appreciably inhibit the rate of protein degradation under these conditions, or even slightly stimulated it, while a decrease in hepatocyte volume in hyper-osmotic medium resulted in inhibition of protein breakdown.

The synthesis of glycogen from glucose and other precursors is very sensitive to small changes in cell volume, caused by either a change in the osmolarity of the incubation medium or a change in the intracellular amino-acid content, and increases with increasing cell volume [1, 2, 22]. We therefore decided to study the effect of variation in cell volume in the presence of glucose, so that the effects on both proteolysis and glycogen synthesis could be compared under identical conditions, i.e. in the same incubations. Variation in cell volume was brought about by variation in the concentration of NaCl in the incubation medium or by the addition of physiological mixtures of amino acids [1, 2, 11]. As expected, cell volume decreased with increasing NaCl concentration while addition of amino acids resulted in an increase in cell volume

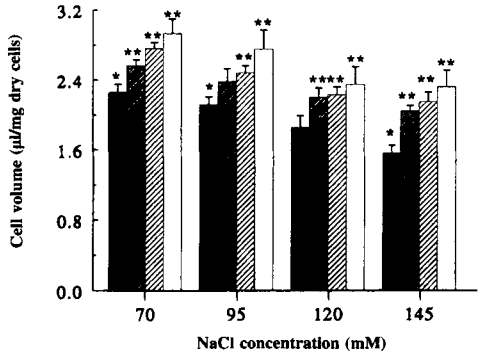

Fig. 1. Cell volume as a function of the osmolarity of the incubation medium. Hepatocytes were incubated for 45 min as described in Materials and Methods. The concentration of NaCl was as indicated in the figure. Amino acids were absent or added at 1×, 2× and 4× their basal concentrations (first, second, third and fourth bar at each NaCl concentration, respectively). Values are means ± SEM of four experiments performed with different hepatocyte preparations. (*) In the absence of added amino acids these values were significantly different ($P<0.05$; paired *t*-test) from the values obtained at 120 mM NaCl (iso-osmotic conditions). (**), Significantly different ($P<0.05$) from the corresponding control in the absence of added amino acids at the same NaCl concentration.

under all conditions (Fig. 1). In order to avoid direct inhibition of proteolysis by an increase in intralysosomal pH [27], ammonia was not added to activate glutamine degradation via glutaminase [28, 29]. In these experiments, we also included 25 µM cycloheximide to inhibit protein synthesis so that the production of valine was a true measure of protein breakdown. At this concentration, cycloheximide does not affect proteolysis. This is shown in the experiment detailed in Table 1. In this experiment, protein synthesis and degradation were measured simultaneously under iso-osmotic and hypo-osmotic conditions. A high concentration of valine (2.5 mM) was added to the cells to ensure constant specific radioactivity of intracellular valine. This concentration of valine had no effect on the proteolytic rate (compare Table 1 and Fig. 2). As expected, cycloheximide almost completely prevented protein synthesis, both in the absence and presence of added amino acids. In the absence of added amino acids, production of valine, which represents the difference between protein synthesis and protein degradation, increased in the presence of cycloheximide. The calculated proteolytic rate, i.e. the sum of valine incorporation into protein and valine release, was not significantly altered by cycloheximide and was similar under iso-osmotic and hypo-osmotic conditions. Interestingly, in the absence of added amino acids under hypo-osmotic conditions, protein synthesis was 60% higher than under iso-osmotic conditions. No such difference was observed when amino acids were present in excess (Table 1), which is in agreement with the data of Stoll et al. [30]. Apparently, hypo-osmolarity increases the affinity of protein synthesis for amino acids.

The effect of variation in the concentration of NaCl in the incubation medium on proteolytic flux was further analyzed in Fig. 2A. In the absence of added amino acids, proteolysis was maximal at 95 mM NaCl. As in Table 1, proteolytic rates at 120 mM (iso-osmotic) and 70 mM NaCl (hypo-osmotic) were about equal, while hyper-osmolarity (145 mM NaCl) proved to be inhibitory, in agreement with similar data by Caro [12]. Rates of proteolysis under all these conditions were constant with time and inhibition by hyperosmolarity was reversible (Table 2). When the osmolarity was further

Table 1. Effect of cycloheximide on protein turnover in hepatocytes. Hepatocytes were incubated for 90 min in the presence of 2.5 mM [3H]valine (specific activity, 0.5 μCi/μmol) as described in Materials and Methods under hypo-osmotic (70 mM NaCl) or iso-osmotic (120 mM NaCl) conditions, in the absence or presence of a complete mixture of amino acids at two-times their basal concentration (see Materials and Methods). Cycloheximide, if present, was added at 25 μM. Production of valine by proteolysis in the presence of 2.5 mM added valine was calculated from the production of leucine, allowing a conversion factor of 0.83 for the abundance of valine, relative to that of leucine, in liver protein [14, 17]. Protein degradation was calculated as the sum of protein synthesis and valine release. Values are means ± SEM from experiments performed with four different hepatocyte preparations. (*), Significantly different ($P<0.05$; paired t-test) from the corresponding control at the same osmolarity. (**), Significantly different ($P<0.05$) from the control under hypo-osmotic conditions.

Osmolarity	Addition	Protein synthesis	Valine release	Protein degradation
		μmol valine/g dry cells		
Hypo-osmotic	none	4.4 ± 0.1	11.3 ± 0.4	15.7 ± 0.4
	cycloheximide	0.3 ± 0.1*	14.4 ± 0.8*	14.7 ± 0.8
	2×AA	5.6 ± 0.5*	–	–
	cycloheximide/2×AA	0.1 ± 0.0*	–	–
Iso-osmotic	none	2.7 ± 0.2**	12.6 ± 0.7	15.3 ± 0.8
	cycloheximide	0.3 ± 0.1*	14.7 ± 1.1*	15.0 ± 1.1
	2×AA	5.1 ± 0.3*	–	–
	cycloheximide/2×AA	0.1 ± 0.0*	–	–

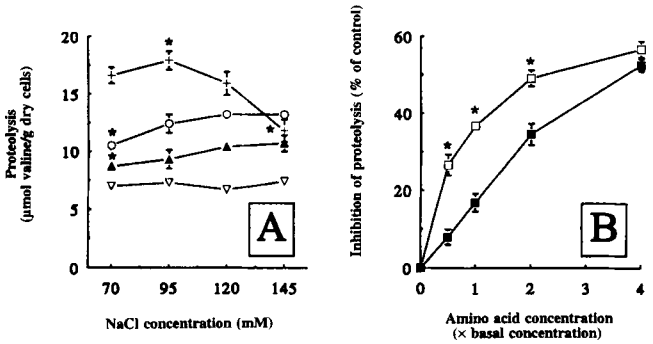

Fig. 2. Proteolysis as a function of the osmolarity of the incubation medium. Hepatocytes were incubated for 90 min as in Fig. 1, in the presence of the NaCl concentrations indicated in the figure. Amino acids were absent (+) or added at 1× (○), 2× (▲) and 4× (▽) their basal concentrations (A). Values are means ± SEM from experiments performed with 4–13 (0, 1× and 2×AA) different hepatocyte preparations. Data in the presence of 4×AA are the means of two hepatocyte preparations. When error bars are not indicated, errors fell within the symbols. (A) rates of proteolysis as a function of the NaCl concentration of the medium. (B) the percentage inhibition of proteolysis by amino acids under iso-osmotic conditions (■) (NaCl, 120 mM) and hypo-osmotic conditions (□) (NaCl, 70 mM) as a function of the added amino-acid concentration. (*), Significantly different ($P<0.05$) from the control under iso-osmotic conditions at the same amino-acid concentration. Values in the presence of 1×, 2× and 4× the basal concentration of amino acids at 70, 95 and 120 mM NaCl (A) were significantly different ($P<0.05$) from the corresponding controls in the absence of added amino acids; for the sake of clarity this is not indicated in the figure.

increased to 170 mM NaCl, inhibition was more than 50% but not completely reversible (data not shown). Due to this lack of reversibility, data at this high osmolarity were not included in this study.

Although the decrease in NaCl concentration from 120 mM to 70 mM did not appreciably affect the maximal proteolytic rate in the absence of amino acids, it increased the sensitivity of proteolysis to inhibition by amino acids (Figs 2A and B). Thus, a complete mixture of amino acids at their physiological concentration added under iso-osmotic

Table 2. Time dependence of proteolysis and reversibility of the inhibition of proteolysis by hyper-osmolarity. Hepatocytes were incubated under hypo-osmotic (70 mM NaCl), iso-osmotic (120 mM NaCl) or hyper-osmotic (145 mM NaCl) conditions for the times indicated, as described in Materials and Methods. In a fourth condition, hepatocytes were first incubated for 30 min under hyper-osmotic conditions; after this period, the osmolarity of the medium was brought back to iso-osmolarity and incubation was continued. Data are means ± SEM of experiments performed with different hepatocyte preparations, the number of which is given in parentheses. (*), Significantly different ($P<0.05$) from the values under iso-osmotic conditions; n.m., not measured.

Condition	Proteolysis at		
	30 min	60 min	90 min
	μmol valine/g dry cells		
Hypo-osmotic	5.3 ± 0.3 (4)	10.2 ± 0.8 (4)	15.5 ± 1.2 (4)
Iso-osmotic	5.1 ± 0.4 (8)	10.4 ± 0.6 (8)	15.5 ± 0.9 (8)
Hyper-osmotic	3.7 ± 0.2 (4)*	7.7 ± 0.7 (4)*	12.0 ± 1.0 (4)*
Hyper-osmotic→ iso-osmotic	n.m.	n.m.	15.4 ± 0.9 (3)

conditions inhibited proteolysis by 15% while inhibition was almost 40% under hypo-osmotic conditions (Fig. 2B). In other words, although hypo-osmolarity (i.e. cell swelling) had no effect on the rate of proteolysis in the absence of amino acids (or even slightly stimulated it at 95 mM NaCl, Fig. 2A), it did inhibit proteolysis in their presence.

In contrast to proteolysis (Fig. 2A), glycogen synthesis, a process that is very sensitive to changes in cell volume [1, 2], in the absence of added amino acids increased when changing from iso-osmotic to hypo-osmotic conditions, whereas inhibition was observed under hyper-osmotic conditions (Fig. 3A, compare [1]). Addition of amino acids resulted in stimulation of glycogen synthesis and this effect was additive to that of decreasing osmolarity (Fig. 3A).

To establish whether or not the potentiating effect of decreased osmolarity on the inhibition of proteolysis by amino acids was the result of increased amino-acid metabolism, we

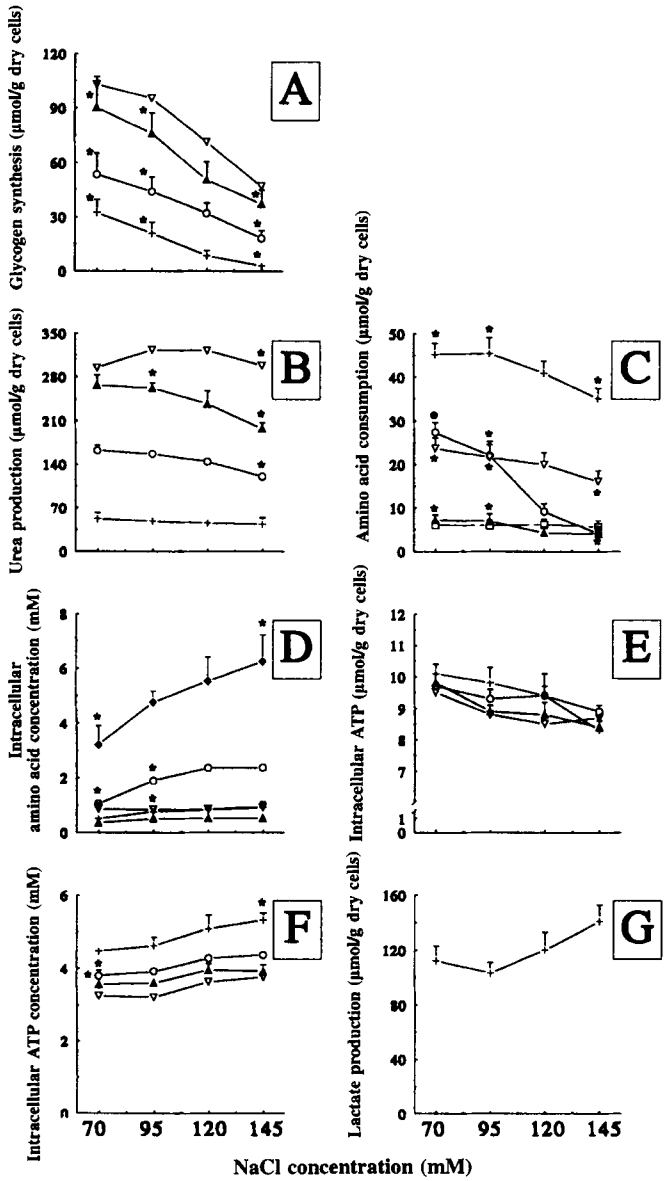

Fig. 3. Glycogen synthesis, nitrogen metabolism, ATP and glycolysis as functions of the osmolarity of the incubation medium. Experimental conditions were as in Fig. 1. The data are means ± SEM of experiments obtained with four (A, B, C, E, F) and three (D) different hepatocyte preparations. Data in the presence of 4×AA are the means of two hepatocyte preparations. (A) Glycogen synthesis; control, (+); 1×AA, (○); 2×AA, (▲); 4×AA, (▽). (B) Urea synthesis: symbols as in A. (C) Rate of amino acid consumption at 1×AA: alanine, (+); glycine, (○); serine, (▽); threonine, (▲); asparagine, (□). Consumption of other amino acids (data not shown) was relatively low and not affected by changes in osmolarity. (D) Intracellular amino-acid concentrations (intracellular amounts divided by cell volume, Fig. 1) at 1×AA: alanine, (+); glycine, (○); serine, (▽); threonine, (▲); glutamate, (◆). Intracellular asparagine was below the limit of detection. E, intracellular amount of ATP: symbols as in (A). (F) Intracellular ATP concentration (amount divided by volume, Fig. 1); symbols as in (A). (G) Lactate production; only the values in the absence of amino acids are shown because in their presence lactate is not only produced by glycolysis but also from amino acids. (*), Significantly different ($P<0.05$) from the values at 120 mM NaCl (iso-osmotic) at the same amino acid concentration. Values in the presence of 1×, 2× and 4× the basal concentration of amino acids at all NaCl concentrations were significantly different ($P<0.05$) from the corresponding controls in the absence of added amino acids (A, B, F); for the sake of clarity this is not indicated in (A), (B) and (F).

measured amino-acid-consumption rates, urea synthesis and the intracellular amino-acid concentrations. As shown in Fig. 3B, urea production in the absence or presence of amino acids was not very sensitive to changes in medium osmolarity, but the tendency was towards a slight increase in urea production with decreasing osmolarity of the medium. Consumption of the quantitatively most important amino acids followed a similar pattern (Fig. 3C) with the exception of glycine: consumption of this amino acid was clearly accelerated when the medium used was changed from iso-osmotic to hypo-osmotic, in agreement with the data of Häussinger and Lang [7]. It must be stressed that consumption of glutamine was negligible under our experimental conditions for the reasons indicated above and that catabolism of glutamine is known to be stimulated by increases in cell volume [31].

The intracellular concentration of amino acids, after correction for the changes in cell volume (Fig. 1), was largely independent of the medium osmolarity (serine, threonine) or slightly decreased with decreasing osmolarity (glutamate, glycine, alanine) (Fig. 3D).

Since autophagic proteolysis is dependent on ATP and sensitive to relatively small changes in intracellular ATP concentration [26], this parameter was also measured as a function of medium osmolarity. The intracellular level of ATP slightly increased with decreasing osmolarity and was unaffected by the presence of amino acids (Fig. 3E). However, the intracellular concentration of ATP, i.e. after correcting the level of ATP for the changes in cell volume, slightly decreased with decreasing osmolarity (Fig. 3F), although the effect was small (maximally 10%). In the presence of amino acids, the intracellular ATP concentration dropped significantly (15–25%, Fig. 3F), presumably because of the increased energy demands for the synthesis of glycogen and urea, combined with the further increase in cell volume (Fig. 1).

The fact that the intracellular ATP concentration was only slightly affected by medium osmolarity was reflected in the relative insensitivity of the production of lactate from glucose, which, in the absence of added amino acids, can be considered as an indicator of glycolytic flux (Fig. 3G). In the presence of amino acids, lactate is not only produced by glycolysis but also from amino acids. For that reason, production of lactate under these conditions is not depicted in Fig. 3G.

DISCUSSION

Although amino acids are known to be inhibitors of hepatic autophagic proteolysis, very little information is available regarding the mechanism by which they exert this effect [32, 33]. In their stimulating studies on the control of proteolysis in the isolated perfused liver, Häussinger and coworkers [7, 9–11] recently proposed that the increase in cell volume following amino-acid influx is in part responsible for the inhibition of proteolysis by amino acids. They not only demonstrated that perfusion with hypo-osmotic media was able to mimic the anti-proteolytic effect of amino acids, but also that the inhibitory effect of several amino acids (but not all of them) could be directly attributed to their ability to induce cell swelling.

Our experiments with isolated hepatocytes also indicate that changes in cell volume are important as a factor in the control of proteolysis. However, they differ from the perfusion experiments of Häussinger and colleagues in one impor-

tant aspect: with the isolated cell preparation, in the absence of added amino acids, hypo-osmolarity (as compared to iso-osmotic conditions) did not inhibit proteolysis or even slightly stimulated it ([12] and Fig. 2) under conditions where glycogen synthesis, another process known to be sensitive to cell-volume changes [1, 2] (Fig. 3A), was increased. However, in the presence of low, physiological, concentrations of amino acids, an increase in cell volume due to a decrease in the NaCl concentration of the medium did inhibit proteolysis and further stimulated glycogen synthesis. Thus, an increase in cell volume increased the sensitivity of proteolysis to inhibition by amino acids. This effect of an increased cell volume was unlikely to be due to an increase in the rate of amino-acid transport across the plasma membrane of the hepatocyte, since intracellular amino-acid concentrations had the tendency to fall rather than to rise with increasing cell volume (Fig. 3D).

Hyper-osmolarity (145 mM NaCl) in the absence of amino acids inhibited proteolysis by 25% (Fig. 2A, Table 2). When the osmolarity was further increased, inhibition of proteolysis also increased further. However, at this high concentration of NaCl, inhibition became irreversible even though intracellular ATP concentrations were about equal to those under iso-osmotic conditions (data not shown). At present, we have no explanation for this. Perhaps the cytoskeleton, which is involved in autophagy [34], becomes irreversibly damaged during extensive cell shrinkage.

The reason for the difference in results regarding the behaviour of proteolysis under hypo-osmotic conditions in the perfused liver [9–11] and in isolated hepatocytes may be related to the difference in experimental procedure to measure protein degradation, even though maximum rates of proteolysis were similar in both preparations. In the experiments of Häussinger et al. [9], cycloheximide was not used and rates of proteolysis were measured either as the production of leucine from non-labelled livers or as [^{3}H]leucine from previously labelled liver protein in the presence of non-labelled (0.1 mM) leucine in order to prevent reincorporation of the label into protein. In our experiments, we used cycloheximide to inhibit simultaneous protein synthesis. This was essential because in the absence of cycloheximide, accumulation of valine or leucine in the incubation medium was 15–20% lower than in its presence, indicating that proteolytically produced amino acids were reutilized for protein synthesis. It is important to stress that cycloheximide did not affect the proteolytic process itself (Table 1). Under conditions similar to ours, cycloheximide has been reported to inhibit proteolysis [35] but this occurred only after prolonged incubation at concentrations of cycloheximide 40-times higher than used in our studies. At these high concentrations, cycloheximide affects cellular ATP production (Meijer, A. J. and Seglen, P. O., unpublished results). One could argue that the difference in results of our study and those of Häussinger et al. [9] may find its explanation, at least in part, in our observation that in the absence of added amino acids, protein synthesis is increased by hypo-osmolarity (Table 1). Thus, according to this reasoning, the decrease in leucine-production rate by hypo-osmolarity [9] may have been due to increased protein synthesis. However, from the data of Häussinger et al. [9] it can be calculated that the concentration of proteolytic [^{3}H]leucine in the effluent of the perfused liver was 15–20 μM. This radiolabelled leucine derived from proteolysis was diluted by infused 100 μM non-labelled leucine. In these experiments of Häussinger et al. [9], hypotonicity decreased effluent radiolabel by about 25%. Assuming that the specific radioactivities of intracellular and extracellular leucine were equal, about 30 μM leucine (i.e. 25% of 100 μM+20 μM = 120 μM) should have been used for protein synthesis if increased protein synthesis would have been solely responsible for the decrease in [^{3}H]leucine release. This would mean that the rate of protein synthesis in their amino-acid-free open system of rat-liver perfusion was almost twice the rate of proteolysis. This is highly unlikely. Apart from these considerations, the difference in experimental conditions, i.e. perfusion of the liver with lactate, pyruvate and leucine [9] as opposed to isolated hepatocytes incubated with glucose in our experiments, remains as an explanation for the difference in results obtained in the two studies.

According to Stoll et al. [30], protein synthesis in the presence of high concentrations of added amino acids is the same under iso-osmotic and hypo-osmotic conditions (Table 1), but decreases under hyper-osmotic conditions. As shown by our experiments, in the absence of added amino acids, protein synthesis is higher under hypo-osmotic than under iso-osmotic conditions (Table 1). Thus, stimulation of protein synthesis and inhibition of protein degradation can take place in the same osmolarity range and the mechanism by which this occurs appears to be similar, i.e. both processes become more sensitive to amino acids when the cells are swollen.

The anabolic effects of cell swelling (see above) closely resemble those of insulin [7, 18]. Vom Dahl et al. [18] have suggested that the anti-proteolytic effect of insulin may, in fact, be related to its ability to increase cell volume. In our opinion, the analogy goes even further: as with an increase in cell volume (this study), insulin alone has little effect on hepatic proteolysis unless amino acids are also present [36, 37]. Synergistic effects of insulin and amino acids have also been reported with regard to muscle protein metabolism [38–40].

Under our experimental conditions, neither amino-acid consumption (among which that of alanine was quantitatively most important) nor urea synthesis were much affected by hypo-osmolarity, whereas hyper-osmolarity was slightly inhibitory. Only glycine consumption increased at low NaCl concentrations, in agreement with Häussinger et al. [7, 41]. This is presumably due to an increase in mitochondrial volume following hypo-osmotic cell swelling [7] resulting in activation of mitochondrial glycine oxidation [42]. It must be pointed out that glutamine degradation, which normally increases with increasing mitochondrial volume [43] and thus with cell volume [6, 31], was negligible, because under our conditions ammonia was not added to activate glutaminase, for the reasons outlined earlier.

An interesting observation was that the intracellular concentration of ATP, but not the amount of intracellular ATP, significantly decreased in the presence of amino acids (Fig. 3F). We have previously shown that proteolysis in hepatocytes is quite sensitive to inhibition by relatively small decreases in intracellular ATP concentration, a 25% fall in intracellular ATP resulting in 20% inhibition of proteolysis [26]. It can be concluded, therefore, that a small part of the inhibition of proteolysis by high concentrations of added amino acids under our experimental conditions was due to the fall in ATP concentration. Inhibition of proteolysis by hypo-osmolarity was not seen in the absence of amino acids even though the intracellular ATP concentration dropped by 10%. This should have resulted in an 8% decrease in proteolysis. That this was not observed may have been due to two opposing effects: a decrease due to the fall in ATP and a

slight increase in proteolysis by hypo-osmolarity *per se*. Indeed, as shown in Fig. 2A, transition from 120 mM to 95 mM NaCl increased proteolysis by 10%.

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