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THE RELATIONSHIP BETWEEN PLASMA UREA LEVELS AND SOME MUSCLE TRIMETHYLAMINE LEVELS IN *XENOPUS LAEVIS*: A ^{31}P AND ^{14}N NUCLEAR MAGNETIC RESONANCE STUDY

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Summary

Urea is known to be disruptive to proteins, yet high levels occur in a variety of tissues. It has been suggested that trimethylamines counteract the effects of urea on protein. We have, therefore, directly investigated whether elevating tissue urea levels produces an increase in trimethylamine concentrations by using ^{31}P and ^{14}N nuclear magnetic resonance (NMR) spectroscopy to detect two trimethylamines (glycine betaine and glycerylphosphorylcholine) in intact, living gastrocnemius muscle from *Xenopus laevis*. *Xenopus laevis* naturally elevates its tissue urea levels under dry conditions. This ability was used to obtain plasma urea levels ranging from 1 to 110 mmol l^{-1}. This procedure did not alter pH or levels of ATP or phosphocreatine in the muscles, but did raise trimethylamine levels. We find that there is a significant relationship between plasma urea concentrations and the trimethylamine levels studied. This relationship was, however, limited to the lower range of urea levels. We propose that other trimethylamines or mechanisms come into play at urea concentrations above 20 mmol l^{-1}.

Key words: trimethylamines, urea, NMR, glycine betaine, glycerylphosphorylcholine, *Xenopus laevis*.

Introduction

High urea concentrations (1–3 mol l^{-1}) are known to be disruptive to proteins and are used in biochemical preparations to denature them. Lower (below 1 mol l^{-1}) concentrations of urea have also been demonstrated to affect protein function (e.g. Yancey *et al.* 1982). It is therefore puzzling that enzymes and other proteins in tissues with high urea levels, for example the medulla of the mammalian kidney (0.3 mol l^{-1}) and the tissues of elasmobranch fishes (0.5 mol l^{-1}), maintain their functional integrity (Somero, 1986). It has been suggested that a group of compounds, the trimethylamines (TMAs) [glycine betaine, glycerylphosphorylcholine (GPC) and trimethylamine-*N*-oxide], which act as osmolytes, also counteract the effects of urea on proteins (Yancey *et al.* 1982; Somero, 1986, 1992; Yancey and Burg, 1990; Yancey, 1992). The perturbing effect of urea on the properties of a variety of functional and structural proteins from elasmobranchs has been shown to be counterbalanced by the TMAs. Together, urea and the TMAs have no *net* effect on the protein characteristics, although each individually does affect the protein. TMAs occur in a wide variety of species, but there have been few direct *in vivo* studies to test whether changes in tissue urea levels elicit changes in TMA concentration (Peterson *et al.* 1992; Nakanishi *et al.* 1993).

The African clawed toad *Xenopus laevis* adapts to life in dry or brackish conditions by elevating its urea levels (Balinsky *et al.* 1961). We have used this adaptive ability experimentally to manipulate urea levels in the toad. In this way, we have been able to determine *in vivo* whether there is a correlation between plasma urea levels and tissue TMA levels. To measure TMA levels in intact *Xenopus* gastrocnemius muscles we have used ^{31}P and ^{14}N NMR spectroscopy. ^{14}N NMR is a technique that so far has been little used for biological studies (Balaban and Knepper, 1983; Wray and Wilkie, 1992) and thus this paper also demonstrates its biological usefulness for such work.

We report here, for the first time, that there is a significant relationship *in vivo* between plasma urea levels and those of two trimethylamines, GPC and glycine betaine, but that the relationship is limited to the lower range of urea levels.

Some preliminary results have been previously reported to the Physiological Society (Wilkie and Wray, 1986).

Materials and methods

Animals

Mature, female *Xenopus laevis* were kept at room temperature (20 °C) in tanks, at a 12 h:12 h L:D photoperiod. One group of 14 control toads was kept in tap water for up to 21 days. A second group (of 20) was placed in tanks containing

300 mosmol l^{-1} NaCl for 3–21 days. This procedure resembles the natural process of aestivation, which occurs during dry periods, and these toads are subsequently referred to as 'salt-adapted'. For collection of blood samples, the toads were anaesthetized by immersion in 0.1 % tricaine (3-aminobenzoic acid, Sigma) and blood was withdrawn from the heart. The gastrocnemii were removed after the anaesthetized toads had been killed by decapitation.

Nuclear magnetic resonance spectroscopy

The gastrocnemii were transferred to 15 mm diameter NMR tubes containing Ringer's solution at 4 °C. The Ringer's solution was of the following composition (mmol l^{-1}): NaCl, 111; KCl, 2.5; $CaCl_2$, 2; Hepes buffer, 10; pH 7.4. A Brucker WM 200, 4.7 T spectrometer operating was used. For ^{31}P spectroscopy, radiofrequency pulses (30 μs) were applied every 2.2 s and each spectrum took 5–10 min to acquire. To correct for the effects of signal saturation, the peak areas were multiplied by saturation factors, obtained in separate experiments using a pulse rate of one 30 μs pulse per 12 s. The peaks were referenced to phosphocreatine (PCr) at 0 p.p.m. The ^{14}N spectroscopy was performed on the same spectrometer operating at 14.46 MHz. The radiofrequency pulses (55 μs) were applied every 100 ms. Owing to the brief relaxation times of the ^{14}N nucleus (Mason, 1981), this rapid pulsing was possible without signal saturation. The ^{14}N spectra were collected in 30 min and were referenced to NH_4^+ at 0 p.p.m.

To quantify metabolite concentrations, peak areas were measured by integration. The area of each peak was then compared with a standard in the Ringer's solution. In the case of ^{31}P this was 10 mmol l^{-1} inorganic phosphate and for ^{14}N it was 11.1 mmol l^{-1} sodium nitrate. The fraction of the NMR-sensitive volume (i.e. that contributing to the NMR signal) occupied by the tissue was determined by measuring the decrease in the size of the signal from the $NaNO_3$ standard, when tissue (which contains no nitrate) was added (Wray and Wilkie, 1992).

Intracellular pH was calculated from the resonance position of inorganic phosphate (P_i) using the following rearrangement of the Henderson–Hasselbalch equation:

$$\mathrm{pH} = \mathrm{pK} + \log\frac{\delta - \delta_1}{\delta_2 - \delta},$$

where δ is the observed chemical shift difference between P_i and phosphocreatine, and δ_1 and δ_2 are the chemical shifts of $H_2PO_4^-$ and HPO_4^{2-} respectively. A pK value of 6.71 and chemical shift values δ_1=3.19 and δ_2=5.49 were used.

Urea and osmolality determination

The plasma urea concentration was determined enzymatically by reaction with urease to convert it to NH_3, which was then reacted with oxoglutarate and NADH and the absorption measured at 340 nm. Osmolality was measured by depression of freezing point, using 100 μl samples and a Roebling osmometer.

Statistics

The figures given are means ± S.E.M. Significant differences were tested using unpaired *t*-tests, at $P<0.05$.

Results

The procedures used here to obtain elevated urea levels in the toads were successful, with plasma levels of up to 120 mmol l^{-1} being obtained after several weeks in the hypertonic saline. Different urea concentrations could be obtained by varying the period spent in hypertonic saline. Plasma urea levels in control animals (tap water) ranged from 0.6 to 7.4 mmol l^{-1} (mean 2.6±0.5 mmol l^{-1}, N=14). The normal range for human plasma is similar, 3–8 mmol l^{-1} (Ganong, 1983). Plasma osmolality was significantly increased ($P<0.01$) during salt adaptation, from 242±5 mmol kg^{-1} water (N=14) in control animals to 352±12 mmol kg^{-1} water (N=19) in salt-adapted animals. GPC concentration was positively correlated with osmolality (Fig. 1). Plasma sodium levels also rose slightly, from 145±5 to 164±5 mmol l^{-1}.

^{31}P nuclear magnetic resonance

Fig. 2 shows typical ^{31}P spectra from control and salt-adapted *Xenopus* gastrocnemius muscle. The phosphorus peaks seen in the control spectrum are typical for skeletal muscle, with large resonances from ATP and phosphocreatine (PCr) and a small peak from inorganic phosphate (P_i). Two small peaks occur in the phosphodiester region of the spectrum (3–4 p.p.m.). They arise from glycerylphosphorylcholine (GPC, at 3.1 p.p.m.) and glycerylphosphoryl serine (GPS, at 3.6 p.p.m.). These peaks have been previously reported in toad muscle (Burt *et al.* 1976; Dawson *et al.* 1977). The spectrum from the salt-adapted toad shows the typical peaks seen in the control spectrum, but the peak from GPC is very much larger.

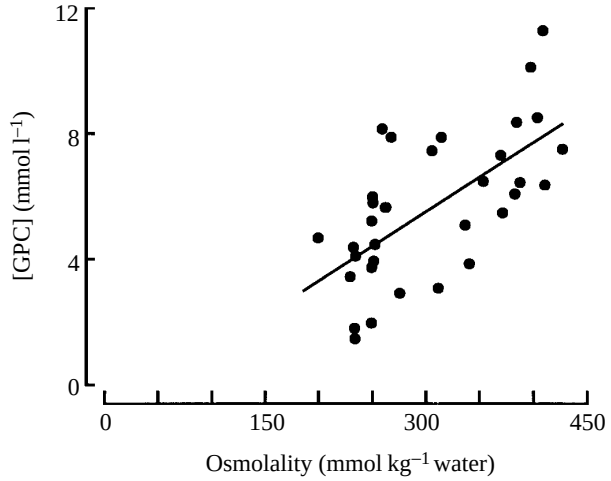

Fig. 1. The relationship between plasma osmolality (mmol kg^{-1} water) and tissue glycerylphosphorylcholine levels (GPC, mmol l^{-1}) in *Xenopus laevis*. The regression line fitted to the data had an r^2 value of 0.42.

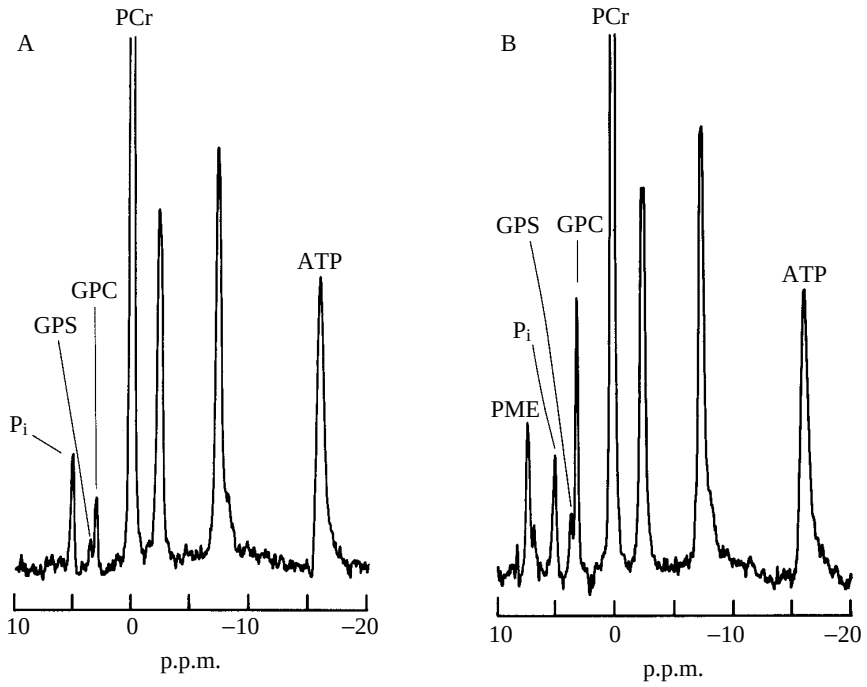

Fig. 2. ^{31}P NMR spectra of *Xenopus laevis* gastrocnemii from a control (A) and a salt-adapted (B) animal. The phosphocreatine (PCr) peaks have been truncated. P_i, inorganic phosphate; GPC, glycerylphosphorylcholine; GPS, glycerylphosphorylserine; PME, phosphomonoesters. The spectra were referenced to the resonance position of PCr at 0 p.p.m.

This peak was identified as GPC on the basis of the following criteria: (i) by its position (3.1 p.p.m.); (ii) by the addition of GPC to the Ringer's solution, which co-resonated with the peak from the muscle; and (iii) by comparison with other work on tissue extracts (Burt *et al.* 1976). It can also be seen that GPS, which is not a trimethylamine, does not change in concentration with salt adaptation. There was no difference between the levels of ATP (6–7 mmol l^{-1}) or other high-energy phosphates between control and salt-adapted animals, even in animals containing very high levels of urea, indicating that energy metabolism was not altered. The intracellular pH (pH_i) was obtained from the resonance position of P_i. There was no significant difference in its value between control and salt-adapted animals (7.43±0.02, N=8, and 7.37±0.04, N=8, respectively, 4 °C). A final feature shown in Fig. 2 is that there is an extra peak at the extreme left of the spectrum from the salt-adapted animals, labelled PME. This peak was seen in 17 out of 20 salt-adapted toads, whereas it was either absent (N=11) or small (N=3) in control ones. Its identity is not known, but it could represent another osmolyte (see Discussion).

The mean [GPC] in control animals was 3.88±0.38 mmol l^{-1} (N=14). In salt-adapted animals, it was significantly elevated to 6.86±0.41 mmol l^{-1} (N=20; P<0.01). The relationship between the trimethylamine GPC and urea concentrations up to 10 mmol l^{-1} is given in Fig. 3A. The linear fit to the data is highly significant (r^2=0.9; P<0.05). Fig. 3B shows the same relationship for urea levels up to 105 mmol l^{-1}. It can be seen that above 10 mmol l^{-1} urea little further increase in GPC concentration occurred.

^{14}N nuclear magnetic resonance

To establish whether other trimethylamines contribute to the response to elevated urea levels, ^{14}N NMR spectroscopy was also used. This nucleus is difficult to use, because most of the

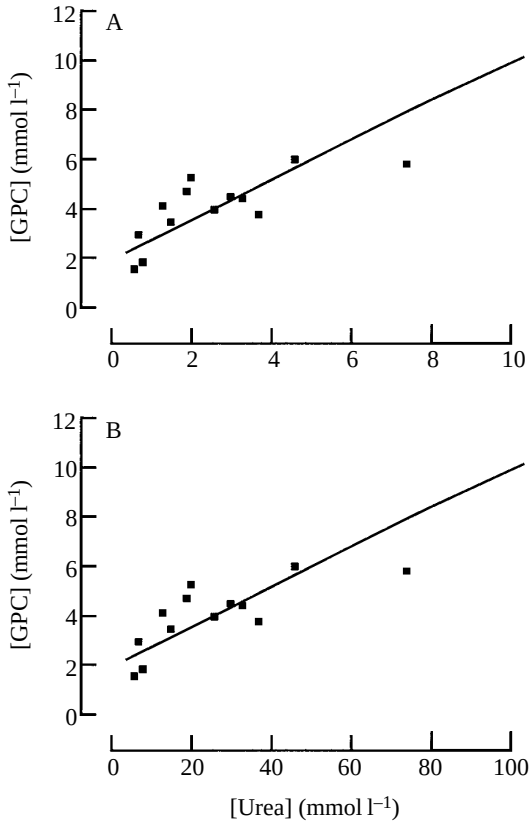

Fig. 3. The relationship between plasma urea and glycerylphosphorylcholine (GPC) levels over the range 0–10 mmol l^{-1} urea (A) and up to 100 mmol l^{-1} urea (B).

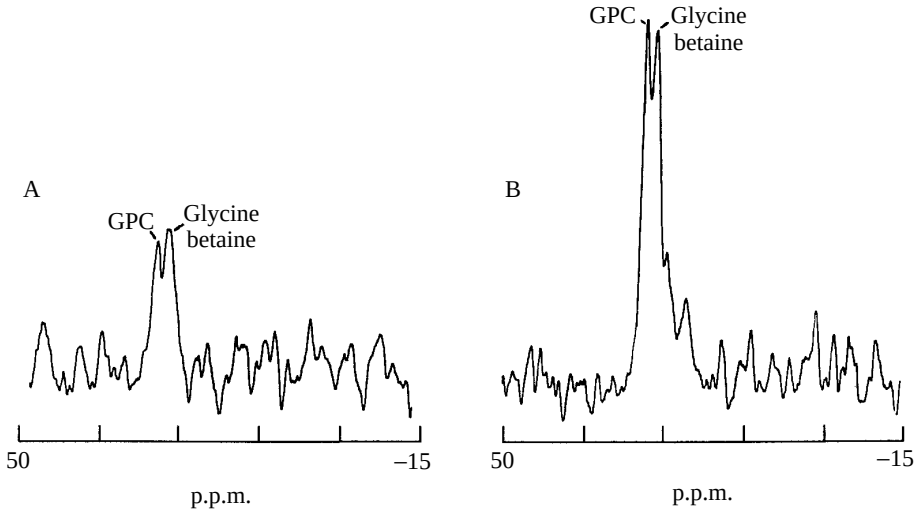

Fig. 4. The ^{14}N NMR spectra of gastrocnemii from control (A) and salt-adapted (B) *Xenopus laevis*. The spectra were referenced to the resonance position of NH_4^+ at 0 p.p.m. GPC, glycerylphosphorylcholine.

signals it gives are enormously broad owing to its quadropolar nature (Mason, 1981). However, metabolites where electron pairs are symmetrical about the nitrogen nucleus can give signals that are sufficiently narrow to provide clear peaks. Both GPC and another trimethylamine, glycine betaine, fall into this category. We therefore examined a further group of *Xenopus* gastrocnemii using ^{14}N NMR. Typical ^{14}N NMR spectra are shown in Fig. 4. The only peaks in this area of the spectrum are from GPC and glycine betaine. Nitrate, which was used as a standard, resonates a long way from this region, at −355 p.p.m. There are clearly higher GPC and glycine betaine levels in the salt-adapted animals. It can also be seen that these two trimethylamines occur in roughly equal ratios in control animals and in salt-adapted ones. For 11 muscles, GPC and glycine betaine levels were quantified (Fig. 5). It can be seen that at both the medium and high urea levels there is still a discrepancy between concentrations of trimethylamines and plasma urea.

Discussion

This work has shown that there is a relationship between GPC, glycine betaine and urea levels *in vivo* in *Xenopus laevis*. Trimethylamine concentrations rose initially as urea levels increased, but then reached a plateau. It is possible that other undetected trimethylamines were also being produced, or that higher urea concentrations are not correspondingly directly counterbalanced by trimethylamine production (see below). ^{31}P and ^{14}N NMR spectroscopy were used to measure GPC and glycine betaine levels in the gastrocnemii. With this muscle it is possible to obtain ^{31}P NMR spectra relatively quickly (in a few minutes), but with ^{14}N NMR spectra took 30 min to acquire. This longer period may therefore limit the usefulness of ^{14}N NMR for routine studies of trimethylamines (see below). However, it was found that GPC and glycine betaine levels increased approximately symmetrically (Fig. 4); thus, it may be valid to assume glycine betaine concentrations from ^{31}P NMR measurements of GPC levels. The ^{31}P NMR spectra also enable metabolic status and pHi to be simultaneously obtained, thus adding to the usefulness of ^{31}P spectra. Urea determinations were made from plasma samples, rather than from gastrocnemii, because of technical difficulties in measuring urea in these large muscles. It is, however, assumed that, owing to its high lipid solubility, plasma urea levels will reflect muscle levels (see Pang *et al.* 1977). The trimethylamines are present intracellularly (Somero, 1986; Balaban and Knepper, 1983; Beck *et al.* 1992).

The relationship between GPC and urea concentrations was linear and highly statistically significant up to urea levels of around 10 mmol l^{-1} (Fig. 3). Above 10 mmol l^{-1}, as shown in Fig. 3B, there is a clear break in the data and little further increase in [GPC] occurs as [urea] is further increased. Glycine betaine levels showed a similar pattern. Thus, in *Xenopus laevis* muscle, GPC and glycine betaine were not found in large

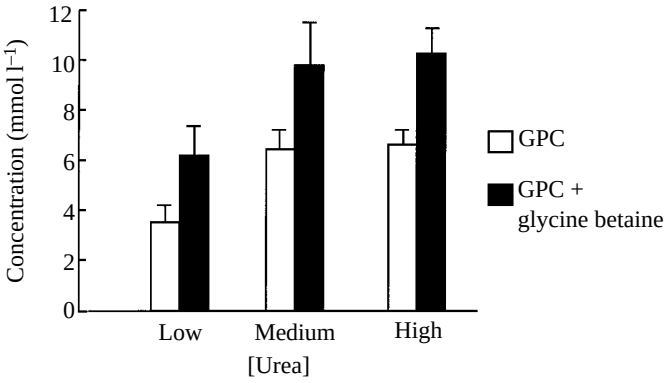

Fig. 5. A histogram of trimethylamine concentration against plasma urea levels. The GPC and betaine concentrations (filled bars) were obtained from ^{14}N NMR spectra and the [GPC] (open bars) from ^{31}P NMR spectra. The vertical lines indicate the S.E.M. of the trimethylamine values. The data were grouped into three ranges of urea concentration: below 10 mmol l^{-1} is indicated as 'low' (N=4), between 10 and 100 mmol l^{-1} is 'medium' (N=4) and above 100 mmol l^{-1} is 'high' (N=3).

enough amounts to counterbalance urea at concentrations much over 10 mmol l^{-1}. In addition, the ratio of 2:1 urea:trimethylamines often found previously (e.g. Somero, 1986) was not apparent in this study. One explanation for this is that other trimethylamines were synthesized, but not detected by the techniques employed in this study. In particular, trimethylamine-*N*-oxide would be such a osmolyte, and it has been shown to be present in elasmobranch tissues and to counteract the effects of urea on proteins (Somero, 1986). No ^{14}N signal from trimethylamine-*N*-oxide was detected, suggesting that either it was present in concentrations below 1 mmol l^{-1} and therefore not detected or, more likely, it produced a signal too broad to be identified. Another possible osmolyte is the peak in the phosphomonoester region of the ^{31}P NMR spectra. As noted in Fig. 2, many spectra from salt-adapted animals contained this peak, but it was absent in most of the spectra from control toads. Its identity is unknown, but it is tempting to speculate that this represents another osmolyte being synthesised to counter high urea levels, although other explanations, such as seasonal variations in metabolites, as have been reported for [ATP] (Briner *et al.* 1959), cannot be excluded.

Changes in trimethylamine levels in response to increased osmolality in muscle tissue have been previously demonstrated and correlated with urea levels. A threefold increase in trimethylamine levels in rat kidney following dehydration has been reported previously, although no measurement of [urea] was made (Gullans *et al.* 1988). Large amounts of GPC have been noted in spectra from rabbit kidney, and the amount increased from cortex to medulla in parallel with rising urea concentrations (Yancey and Burg, 1989; Somero, 1986). Peterson *et al.* (1992) and Nakanishi *et al.* (1993) recently showed *in vivo* in rats that there was a consistent linear correlation between renal or urine urea levels and kidney GPC content. This relationship between trimethylamine levels in mammalian tissues, especially the kidney and brain, during periods of altered osmolarity is now well established (Garcia-Perez and Burg, 1991; Dragolovich and Pierce, 1994).

In a recent study of plasma and urine from human patients with chronic renal failure (plasma urea 21–90 mmol l^{-1}), both trimethylamine-*N*-oxide and dimethylamine were detected, whereas they are absent in plasma and urine from healthy subjects (Bell *et al.* 1991). In another H^+ NMR study of plasma from chronic renal failure patients, high glycine betaine levels were found before dialysis and fell following dialysis (Grasdalen *et al.* 1987). Sizeland *et al.* (1993) found a very similar relationship between [urea] and GPC and glycine betaine levels in the human kidney to that found in the present study. Trimethylamine levels were much higher in the medulla than in the cortex. Interestingly, urea levels have been reported to rise with age in man and it has also been reported that [GPC], measured in the forearm by NMR, rises with age (Chalovich *et al.* 1979). Finally, abnormal trimethylamine excretion has been reported to be responsible for fish odour syndrome (Ayesh *et al.* 1993), producing body malodour in sufferers. It has been suggested that diagnosis is difficult because of the technical difficulties of measuring trimethylamines (Walker, 1993). We have demonstrated the usefulness of both ^{31}P and ^{14}N NMR spectroscopy for detecting high levels of some of these compounds. These methods may be of use, therefore, in helping diagnosis of fish odour syndrome.

As mentioned earlier, trimethylamines have been shown to counteract the effects of urea on a variety of protein functions (Yancey *et al.* 1982; Somero, 1986; Yancey and Somero, 1980). The mechanism underlying this effect is not yet fully understood. It is, at least in part, due to our poor understanding of the structure of water and the solvent shell surrounding proteins. Urea is considered to be a denaturant because it can penetrate this shell; it binds to peptide groups and thereby disrupts the shell. Trimethylamines are thought to stabilize proteins by preventing this disruption, by being preferentially excluded from the immediate domain of the protein (Timasheff, 1992). It should be noted that trimethylamines in the absence of urea would not be beneficial to the organism as the protein structures would become too rigid. In elasmobranchs, a ratio of 2:1 for urea:trimethylamine occurs; this ratio was found to be most effective in maintaining protein function *in vitro* (Somero, 1986).

In conclusion, we have shown directly that trimethylamine levels increase in muscle tissue as plasma [urea] increases; trimethylamines thereby protect muscle proteins from the denaturing effects of urea. This effect was, however, limited to the lower end of the range of urea concentrations studied, but we suggest that other trimethylamines, undetected by ^{31}P and ^{14}N NMR spectroscopy, may account for this discrepancy.

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