bioavailability of lysine. Although citraconylation is reversible under acidic conditions, and hence would not impair the bioavailability of lysine, the nutritional safety of citraconylated proteins is yet to be studied. In this respect phosphorylated proteins may have an advantage over either succinylated or citraconylated yeast proteins. Since most of the milk proteins are phosphoproteins, it may be expected that the phosphorylated yeast proteins will be nutritionally safe and may possess good functional properties.

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Trimethylamine Oxide Prevents Insolubilization of Red Hake Muscle Proteins during Frozen Storage

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The enzymic breakdown of trimethylamine oxide (TMAO) to dimethylamine and formaldehyde has been suggested to cause changes in the physicochemical properties of frozen, stored, gadoid muscle via the interaction of the proteins with formaldehyde. In the experiments reported here we demonstrate that in the absence of significant concentrations of formaldehyde a decrease in the level of TMAO of and by itself leads to a change in solubility of the muscle proteins of red hake during frozen storage. Betaine, which does not participate in the reaction catalyzed by the enzymic system responsible for the degradation of TMAO, also can inhibit the solubility change of protein during frozen storage.

During frozen storage of fish, muscle proteins undergo denaturation due to a variety of causes (Shenouda, 1980). Among these are the denaturation that is caused by the increase in salt concentrations or change in pH following removal of water by ice formation. Interactions with lipids can denature proteins due to their surfactant effects, while lipid oxidation can change protein conformations by modifying side groups or by inducing cross-linking of proteins by a free radical process (Karel et al., 1975). Gadoid fish contain an enzymic system for the breakdown of trimethylamine oxide (TMAO) to dimethylamine and formaldehyde (Amano and Yamada, 1964; Yamada et al., 1969; Dingle et al., 1977; Crawford et al., 1979). It has been suggested that the formaldehyde produced cross-links the proteins in the muscle tissue, forming a three-dimensional network causing textural toughness (Amano and Yamada, 1965; Castell et al., 1973; Gill et al., 1979). There is, however, no direct evidence that cross-linking of proteins in fish muscle is caused by formaldehyde produced enzymically, nor is there any direct evidence that chemical changes in fish muscle proteins are brought about by reaction of the proteins with formaldehyde. Nevertheless, there have been reports that indicate that formaldehyde concentrations equivalent to those found in frozen stored fish muscle can cause polymerization of some proteins (Ohnishi and Rodger, 1979; Owusu-Ansah and Hultin, 1984). We also have evidence that much of the formaldehyde that is produced from the enzymic decomposition of TMAO reacts with the small molecular compounds in the muscle tissue (Banda and Hultin, 1983). Thus, it is probably premature to assign all of the changes in texture in frozen stored gadoid muscle to cross-linking of proteins by formaldehyde.

Recently, Yancey et al. (1982) discussed the evolution of various osmolyte systems that protect cells from water stress. Methylamines, including trimethylamine oxide, are examples of such osmolytes. Water stress in living organisms could be caused by high or fluctuating salinity, dehydration, or freezing. Species tolerant to frost restrict ice formation to the extracellular spaces of the tissue. This concentrates the salts in the cell that would denature the proteins without the protective osmolytes. Freezing fish muscle post-mortem concentrates the solutes of the cell, and we thought it possible that the naturally occurring protective osmolyte of fish muscle, trimethylamine oxide, might protect the muscle proteins from denaturation caused by the stress of solute concentration during freezing. The results reported in this paper show that trimethyl-

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amine oxide does in fact protect against the loss of protein solubility in red hake during frozen storage.

EXPERIMENTAL SECTION

Materials. Red hake (*Urophycis chuss*) were obtained from fishing boats in Gloucester the same day of the catch. The fish were transported to the laboratory on ice and immediately skinned and filleted.

Trimethylamine *N*-oxide (TMAO) and betaine were obtained in their dihydrate and hydrate forms, respectively, from Eastman Kodak, Rochester, NY. All other chemicals were analytical grade from Fisher Scientific.

Methods. *Preparation of "Washed–Minced" and "Reconstituted" Muscle Samples.* The fillets were minced in a Rival electric meat grinder with orifices of 0.4 cm. Minced tissue was centrifuged for 18 h at 15 000 rpm in a No. 19 rotor in a Beckman L5-65B preparative ultracentrifuge. The particulate fraction was suspended in cold, distilled, deionized water (1:10 w/v) and strained through a 0.3 μm mesh screen. The material not passing through the screen was taken up and suspended in cold, distilled, deionized water (1:10 w/v) and again strained. This was repeated 3 times. The final residue was centrifuged at 15 000 rpm in a No. 19 rotor in the Beckman ultracentrifuge to "dewater" the material. This is the "washed–minced" sample.

The supernatant fraction from the first centrifugation, i.e., press juice, was dialyzed in Spectrapor membrane tubing (with a molecular weight cutoff of 6000–8000) against two changes of deionized, distilled water over an 18-h period at 6 °C at a ratio of 1 volume of press juice to 400 volumes of water to remove low molecular weight substances, especially TMAO and the cofactors of the TMAO breakdown reaction (Landolt and Hultin, 1982). The dialyzed supernate was combined with the washed–minced sample and thoroughly mixed to produce the "reconstituted–minced" red hake muscle. This is chemically equivalent to minced muscle with small molecular weight soluble material removed. The concentration of TMAO was determined in the original muscle tissue as well as the final samples.

To both the washed–minced muscle and the reconstituted muscle, TMAO was added at levels equal to that originally present in the muscle tissue and at a level half of that value. Other samples of both types of muscle were used with no added TMAO.

To another sample of reconstituted muscle was added sodium chloride to a final concentration of 0.2 M (based on water content of the muscle) in addition to the TMAO. This was to determine if the salt would further "stress" the muscle during frozen storage and emphasize any protective effect of TMAO. The concentration of 0.2 M was chosen to approximately duplicate the ionic strength of the sarcoplasm. To a sample of washed–minced muscle, betaine was substituted for TMAO. Betaine does not serve as a substrate for the enzyme that breaks down TMAO (Parkin, 1983) but has been reported to stabilize proteins during water stress (Yancey et al., 1982).

For each set of experiments, a single preparation of washed–minced or reconstituted muscle was used. After preparation, the moisture content of the samples was adjusted with distilled, deionized water. The final moisture content of the washed–minced sample was 80% and that of the reconstituted sample was 81%. After thorough mixing for 15 min in a Kitchen Aid mixer, the samples were packed in Whirl-pak bags and stored at −7 °C. This relatively high temperature was chosen to accelerate loss of protein solubility. Samples were removed from storage and analyzed at the times indicated in the figure.

Chemical Analysis. A trichloroacetic acid (TCA) extract of the muscle samples was prepared by homogenizing 25 g of duplicate thawed samples for 1 min in a Waring blender with 25 mL of deionized, distilled water and 50 mL of 10% TCA solution. The extract was filtered through a Whatman No. 1 filter paper, and aliquots were taken for the analyses.

TMAO in the TCA extract was reduced by the procedure of Yamagata et al. (1969) using conditions described by Parkin and Hultin (1982). The TMA produced was analyzed by the procedure of Dyer (1945) using 45% KOH in place of 50% K_2CO_3 as suggested by Shewan et al. (1971). TMAO content was calculated as the difference between this determination and the determination of TMA content before reduction.

Dimethylamine (DMA) in the TCA extract was determined by the modified (Dyer and Mounsey, 1945) copper dimethyldithiocarbamate colorimetric procedure of Dowden (1930).

Free formaldehyde was determined by the procedure described by Castell and Smith (1973) using the Nash reagent (Nash, 1953).

Extractable protein was determined by treating 3 g of sample with 60 mL of ice cold 0.02 M $NaHCO_3$ solution at pH 7.2 containing 5% NaCl (Dyer et al., 1950). The protein content of the extract was determined by the biuret procedure of Gornall et al. (1949).

Moisture content of the samples was determined by drying in an air oven at 110 °C for 12 h.

Statistical Analysis. Simple linear regression analysis was used to fit the data to first-order reaction kinetics, and the correlation coefficients were tested for significance at the 5% level by using the *t* test (Ostle and Mensing, 1975). The insolubilization rate constants were compared by the *t* test at the 5% level of significance (Ostle and Mensing, 1975).

RESULTS AND DISCUSSION

The decrease in percent extractable protein with time of storage at −7 °C for washed–minced muscle tissue, reconstituted–minced muscle, and reconstituted–minced muscle in the presence of 0.2 M NaCl at the different levels of TMAO is shown in Figure 1a–c, while the results with washed–minced muscle in the presence of betaine are shown in Figure 1d. The TMAO level was adjusted in each group of experiments to the concentration that was present in the original tissue. Thus, in each experiment, the highest concentration of TMAO or betaine varied slightly. Also, since the efficiency of TMAO removal was slightly different in each experimental batch, the TMAO content of the samples with no added TMAO also varied slightly. Only very small quantities of DMA (less than 0.06 mmol/100 g of sample) and free formaldehyde (less than 0.02 mmol/100 g of sample) were produced during storage. In all cases over the 12-week storage period, the samples with no added TMAO or betaine had the lowest values of extractable protein.

The plots of percent extractable protein vs. time in Figure 1 were presented on a linear scale to give a clear indication of the change with time. However, when the data in Figure 1 are plotted on a semilog plot, reasonable linearity is obtained (see correlation coefficients in Table I). From these plots insolubilization rate constants were calculated (Table I). In each set of experimental data, the insolubilization rate constants were significantly different among the samples fully reconstituted with TMAO (or with the substitute betaine), the samples reconstituted at the 50% level, and those samples to which no TMAO or betaine had been added.

Table I. Insolubilization Rate Constants of Muscle Proteins with Different Levels of TMAO and Betaine[a]

sample	TMAO/ betaine level, mmol/ 100 g	insolubilization rate constant, week^{-1}	r
washed-minced muscle + TMAO	0.1	0.034[a]	−0.97
	4.6	0.017[b]	−0.95
	8.9	0.014[d]	−0.93
reconstituted muscle + TMAO	0.3	0.034[a]	−0.99
	4.6	0.022[c]	−0.98
	8.7	0.011[d]	−0.86
reconstituted muscle + 0.2 M NaCl + TMAO	0.1	0.032[a]	−0.92
	3.8	0.019[b,c]	−0.85
	7.8	0.013[d]	−0.87
washed-minced muscle + betaine	0	0.035[a]	−0.98
	3.9	0.020[b,c]	−0.90
	7.9	0.014[d]	−0.90

[a] Rate constants bearing different letters are significantly different ($P \lesssim 0.05$). Correlation coefficients (r) represent the linearity of a first-order plot.

In addition, even though there were some small differences in TMAO or betaine levels in the different experiments, there were no significant differences among the samples containing similar levels of TMAO (or betaine) except in the case of the "washed-minced" and "reconstituted" samples containing half of the full complement of TMAO. Significant differences were not observed between either of these samples and the muscle with NaCl containing the intermediate level of TMAO or the washed-minced muscle with the intermediate level of betaine.

It is difficult to ascertain from this study the relative importance of the loss of TMAO on the extractability of red hake muscle proteins compared to other factors such as production of formaldehyde. Most studies with red hake have not closely followed loss in TMAO content. Even in those situations where this has been done, the TMAO concentration is constantly changing. Thus, experimental conditions would be quite different than they were in these experiments where the TMAO concentration was essentially constant. Nevertheless, an earlier study with red hake in our laboratory (Parkin and Hultin, 1982) showed that losses in percent extractable protein were more rapid in minced red hake muscle in which the small molecular weight fraction had not been removed and which was undergoing TMAO breakdown to dimethylamine and formaldehyde than in the samples of this study. This would indicate that more than the stabilizing effect of TMAO is implicated in whatever molecular changes are important that lead to a change in protein extractability. Likely, the formation of formaldehyde (and possibly DMA) is involved.

Our previous observations on the absolute requirement for various cofactor systems in the breakdown of TMAO to dimethylamine and formaldehyde made these particular experiments possible. The very low levels of dimethylamine and formaldehyde that were produced in these samples on the addition of TMAO confirms the earlier observation that it is indeed the cofactors that are rate limiting for the breakdown of this compound in red hake muscle (Landolt and Hultin, 1982; Banda and Hultin, 1983).

It is not known why the TMAO protects the red hake muscle proteins from denaturation during frozen storage. However, Arakawa and Timasheff (1982) studied the stabilizing effects of polyhydroxy compounds on protein stability and showed that polyhydroxy compounds promote hydration of proteins. This results in a positive free

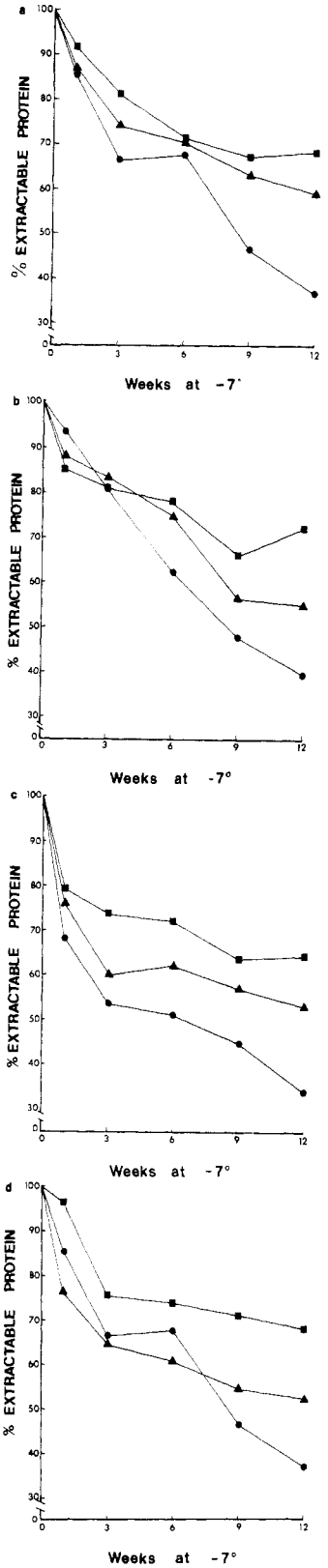

Figure 1. (a) Changes in the protein extractability of washed-minced muscle with storage time at −7 °C [8.9 mmol of TMAO/100 g (■); 4.6 mmol of TMAO/100 g (▲); 0.1 mmol of TMAO/100 g (●)]. (b) Changes in the protein extractability of reconstituted muscle with storage time at −7 °C [8.7 mmol of TMAO/100 g (■); 4.6 mmol of TMAO/100 g (▲); 0.3 mmol of TMAO/100 g (●)]. (c) Changes in the protein extractability of reconstituted muscle plus 0.2 M NaCl with storage time at −7 °C [7.8 mmol of TMAO/100 g (■); 3.8 mmol of TMAO/100 g (▲); 0.1 mmol of TMAO/100 g (●)]. (d) Effect of betaine on the protein extractability of washed-minced muscle with storage time at −7 °C (7.9 mmol of betaine/100 g (■); 3.9 mmol of betaine/100 g (▲); no betaine added (●)].

energy change that is proportional to the surface area of the proteins. Therefore, it was suggested that the denatured form of the protein, which has a larger surface area, is less stable thermodynamically than the nondenatured form, and thus the protein tends not to denature. It is possible that certain amino compounds such as TMAO protect against protein denaturation in a similar way.

Registry No. TMAO, 1184-78-7; betaine, 107-43-7.

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Nutritional Characteristics of Alkali-Treated Zein

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Batches of commercial zein, a lysine-free protein, were treated with either 0.1 N NaOH or $Ca(OH)_2$ (4 h at 85 °C), neutralized, washed, and freeze-dried. Amino acid analysis showed major losses of cystine and lesser losses of serine and threonine. The average proportions of D-amino acids in the NaOH- and $Ca(OH)_2$-treated samples were 20.3 and 15.3% vs. 1.5% in the untreated zein. The order of racemization was Asp and Ser > Phe, Glu, Tyr, and Thr > Met > Ala > Val, Leu, Ile, and Pro. Young rats receiving 4% N from untreated zein and liberal amino acid supplements grew well. With NaOH-treated zein they failed to grow and showed severe diarrhea; effects with $Ca(OH)_2$-treated zein were less severe. These adverse effects were not fully explained by differences in digestibility, nor were they reproduced by adding D-serine or D-alloisoleucine to diets containing untreated zein. Replacing supplementary L-threonine with D-allothreonine gave depressed growth but did not product diarrhea. Our results show that alkali treatment can cause nutritional damage to a protein without the formation of lysinoalanine.

Adverse nutritional effects from the alkaline treatment of proteins have most commonly been linked to the formation of lysinoalanine (LAL), which can be toxic under certain conditions (De Groot and Slump, 1969; Karayiannis et al., 1979). The special interest of work with zein is that is contains no lysine so that there is no possibility of LAL formation by the Michael addition between the ϵ-amino group of lysine units and dehydroalanine, which may be formed by decomposition of cystine and serine. Our plan was to study the chemical changes in zein treated with either NaOH or $Ca(OH)_2$ and to feed it to young rats. Because of the multiple amino acid deficiencies of zein, feeding experiments require that it be supplemented with a range of amino acids. Our first hypothesis was that if the treatments proved nutritionally deleterious, this could be explained by the formation of D-serine, which has been reported to cause severe kidney damage in rats (Wachstein, 1947a; Kaltenbach et al., 1979). When we did find changes not apparently explained by the formation of D-serine, we

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