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Effects of Sodium Nitrite and Formaldehyde on the Lysine Content of Fish Muscle. Model Experiments

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ABSTRACT

Fish muscle pieces were treated under anaerobic conditions and at different pH values with sodium nitrite and formaldehyde alone and in combination. Lysine and tyrosine were the only amino acids affected under the conditions chosen. Lysine was reduced by some 20% by nitrite alone and by nitrite and formaldehyde in combination at 24 h reaction time at pH 5·0, which was the lowest pH value tested. At higher pH values the effect was negligible. Formaldehyde had no effect on lysine. Tyrosine was not affected by nitrite, but formaldehyde alone and in combination with nitrite had a dose-related effect resulting in almost complete loss at the highest levels tested. This effect of formaldehyde was probably an artefact due to a reaction between tyrosine and formaldehyde during hydrolysis of the fish muscle pieces.

INTRODUCTION

Today fish meal is produced almost exclusively from unpreserved raw material soon after it is caught. From about 1955 to the early 'eighties', though, it was necessary to preserve catches of shoaling fish at the reduction plants before production of fish meal. This was due to the fact that production capacity did not match the catch and transport capacities.

Sodium nitrite ($NaNO_2$) was introduced as a preservative in fish meal production after extensive feeding experiments with cattle, sheep, pigs and

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chickens. A joint report of these experiments was published in Norwegian with a general summary in English by Breirem and Flatla (1955). They concluded that the use of sodium nitrite was safe provided the Norwegian regulations for use were followed. These required the sodium nitrite content in the meal not to exceed 200 mg per kg. Fish meal production was extensively controlled on that point. However, in 1964 it was reported that herring meal produced from fish preserved with sodium nitrite might contain a hepatotoxic factor (Ender *et al.*, 1964) which was identified as dimethylnitrosamine (Sakshaug *et al.*, 1965; Ender *et al.*, 1967). How formaldehyde (HCHO) was introduced as a preservative in fish meal production is more obscure, but it was found by experience that a mixture of sodium nitrite and formaldehyde was better than each ingredient alone. The most commonly used preservative, V65, contained 200 g HCHO and 125 g $NaNO_2$ per litre. A typical dose of V65 was about 200 ml per 100 kg fish varying somewhat depending upon the expected time of storage. Experiments with chicks, however, showed that lysine was somewhat less available in meals of preserved than in meals of unpreserved raw material (Anon., 1979). As both formaldehyde and nitrite may react with free amino groups it was anticipated that preservation might reduce the amount of chemically determined lysine (Olley & Watson, 1961).

The experiments reported here were part of a project to study the effects of $NaNO_2$ and HCHO alone and in combination on chemically determined lysine.

MATERIALS AND METHODS

Reagents

Acetic acid (30%, v/v), phosphate buffers (1/15M) of pH 5·0, 5·5, 6·0, 6·5 and 7·0, citrate-phosphate buffers (0·1M) of pH 5·0, 6·0 and 7·0, sodium nitrite ($NaNO_2$) (1M), formaldehyde (HCHO) (about 3·3M), $NaNO_2$/HCHO (1M/3·3M), sodium chloride (NaCl) (1M).

Fillets of saithe (*Pollachius virens*) were bought at the local fish market.

Procedure

Fish muscle pieces, about 200 mg, and 2·5 ml acetic acid or buffer were placed in Thunberg tubes (Fig. 1) and 0·25 ml preservative solution in the stopper. It was found necessary to have anaerobic conditions as erratic results were obtained under aerobic conditions. The tubes were evacuated several times

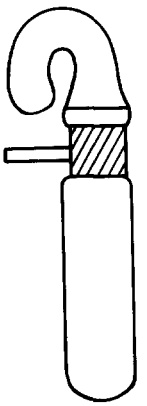

Fig. 1. Thunberg tube, height 15 cm.

and N_2 gas was let in after each evacuation. The contents were mixed and left to react at room temperature for specified times. The muscle pieces were washed three times with acetic acid or buffer before hydrolysis.

In most cases controls with NaCl instead of the preservative solutions were run.

Amino acid analysis

After washing, the muscle pieces were hydrolysed in 6M hydrochloric acid (HCl) at 110°C for 24 h under the ambient atmosphere. The amino acid analyses were performed on an automatic amino acid analyser (Kontron Liquimat III). Preliminary experiments showed that lysine and tyrosine were the only amino acids affected by the treatments. The results are therefore given relative to phenylalanine, which appears between tyrosine and lysine on the chromatograms. The ratios between the heights were used. Normally these ratios are about 1·0 for tyr/phe and 4·5 to 5·5 for lys/phe in untreated samples.

Design of experiments

Experiment 1

Saithe muscle pieces were treated with $NaNO_2$ either in 30% acetic acid or in phosphate buffer, pH 5·0, for 3 h. The results are given in Table 1.

Experiment 2

Saithe muscle pieces were treated with $NaNO_2$ alone or with $NaNO_2$ + HCHO in phosphate buffers of pH 5·0, 5·5, 6·0, 6·5 and 7·0 for 1 h. The results are given in Table 2. Controls with NaCl were not included.

TABLE 1

Peak Height Ratios, lys/phe and tyr/phe, in 6M HCl Hydrolysates of Saithe Muscle after Reaction for 3 h with $NaNO_2$ in Acetic Acid (30%) or Phosphate Buffer, pH 5·0

	Lysine		*Tyrosine*	
	NaCl	*$NaNO_2$*	*NaCl*	*$NaNO_2$*
Acetic acid	4·66	4·52	1·19	1·00
Phosphate buffer pH 5·0	5·31	3·79	1·17	1·13

TABLE 2

Peak Height Ratios, lys/phe and tyr/phe, in 6M HCl Hydrolysates of Saithe Muscle after Reaction for 1 h with $NaNO_2$ or with $NaNO_2$ + HCHO in Phosphate Buffers

	Lysine		*Tyrosine*	
	$NaNO_2$	*$NaNO_2$ + HCHO*	*$NaNO_2$*	*$NaNO_2$ + HCHO*
pH 5·0	4·87	4·81	1·06	0·21
pH 5·5	4·87	4·99	1·11	0·42
pH 6·0	4·43	4·86	1·10	0·15
pH 6·5	—	—	1·07	0·11
pH 7·0	4·85	4·88	1·03	0·04

TABLE 3

Peak Height Ratios, lys/phe and tyr/phe, in 6M HCl Hydrolysates of Saithe Muscle after Reaction for 24 h with $NaNO_2$ or with $NaNO_2$ + HCHO in Citrate-Phosphate Buffers. (The mean of the two runs and the difference between run 1 and run 2 are given.)

	Lysine			*Tyrosine*		
	$NaNO_2$	*$NaNO_2$ + HCHO*	*NaCl*	*$NaNO_2$*	*$NaNO_2$ + HCHO*	*NaCl*
pH 5·0	4·52 − 0·31	3·13 + 0·14	4·63 + 0·04	1·17 − 0·04	0·11 + 0·03	1·18 + 0·05
pH 6·0	4·72 + 0·08	4·34 + 0·16	4·63 + 0·54	1·17 + 0·08	0·10 + 0·01	1·18 + 0·19
pH 7·0	4·72 + 0·08	4·45 + 0·23	4·83 − 0·10	1·13 + 0·05	0·06 − 0·01	1·22 − 0·01

Experiment 3

Saithe muscle pieces were treated with $NaNO_2$ or with $NaNO_2$ + HCHO for 24 h in citrate buffers of pH 5·0, 6·0 and 7·0. The experiment was run twice. The results given in Table 3 are the mean values and the differences between the runs. NaCl was used for the control.

Experiment 4

Saithe muscle pieces were treated on separate days with $NaNO_2$, HCHO and $NaNO_2$ + HCHO in citrate-phosphate buffer, pH 5·0, for 24 h. The preservative solutions were added undiluted (1/1) and diluted (1/2, 1/4, 1/8 and 1/16). One control run with NaCl at full strength was included on each day. The results are given in Table 4.

Experiment 5

Saithe muscle pieces were treated with $NaNO_2$, HCHO and $NaNO_2$ + HCHO in citrate-phosphate buffers of pH 5·0, 6·0 and 7·0 for 24 h. After mixing, one set of Thunberg tubes were left to react for 24 h. They were

TABLE 4

Peak Height Ratios, lys/phe and tyr/phe, in 6M HCl Hydrolysates of Saithe Muscle after Reaction in Citrate-Phosphate Buffer pH 5·0 for 24 h with $NaNO_2$, HCHO and $NaNO_2$ + HCHO of Varying Strength. (The concentration of reactant versus ε-amino-N is given in parentheses). (Experiment 4)

Reactant strength	*Lysine*					
	$NaNO_2$	*NaCl*	*HCHO*	*NaCl*	*$NaNO_2$ + HCHO*	*NaCl*
1 (10)	4·32	4·50	4·63	4·19	3·06	4·19
$\frac{1}{2}$ (5)	4·50		4·57		4·04	
$\frac{1}{4}$ (25)	4·44		4·65		4·44	
$\frac{1}{8}$ (1·25)	4·50		4·47		4·50	
$\frac{1}{16}$ (0·625)	4·46		4·60		4·50	

Reactant strength	*Tyrosine*					
	$NaNO_2$	*NaCl*	*HCHO*	*NaCl*	*$NaNO_2$ + HCHO*	*NaCl*
1 (10)	1·07	1·10	0·05	1·09	0·09	1·09
$\frac{1}{2}$ (5)	1·09		0·10		0·16	
$\frac{1}{4}$ (25)	1·06		0·46		0·32	
$\frac{1}{8}$ (1·25)	1·11		0·78		0·71	
$\frac{1}{16}$ (0·625)	1·10		0·92		0·91	

TABLE 5

Peak Height Ratios, lys/phe and tyr/phe, given Relative to Phenylalanine, in 6M HCl Hydrolysates of Saithe Muscle after Reaction in Citrate-Phosphate Buffers with $NaNO_2$, HCHO and $NaNO_2$ + HCHO. (Muscle + reagents were boiled either before or after the vessel was left to react for 24 h.) (Experiment 5)

	Lysine							
	$NaNO_2$		*HCHO*		*$NaNO_2$ + HCHO*		*NaCl*	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
pH 5·0	3·75	4·44	4·48	4·44	2·99	3·99	—	—
pH 6·0	4·49	4·74	4·51	4·60	4·38	4·45	4·49	4·90
pH 7·0	4·66	4·71	4·36	4·71	4·33	4·70		

	Tyrosine							
	$NaNO_2$		*HCHO*		*$NaNO_2$ + HCHO*		*NaCl*	
	Before	*After*	*Before*	*After*	*Before*	*After*	*Before*	*After*
pH 5·0	0·85	1·00	0·01	0·04	0	0·07	—	—
pH 6·0	0·99	1·10	0	0·01	0	0·05	1·01	1·18
pH 7·0	1·03	0·76	0·03	0·01	0	0	—	—

then heated in a boiling water bath for about 5 min and cooled. Another set of tubes was heated before they were left to react at room temperature for 24 h. The results are given in Table 5.

RESULTS AND DISCUSSION

Lysine is critical in fish meal production because of possible reactions with its ε-amino group. The present experiments were aimed mainly at studying the effects of the preservative solution on chemically determined lysine. The model experiments were performed with the ingredients of the preservative solution, sodium nitrite ($NaNO_2$) and formaldehyde (HCHO), alone and in combination.

Nitrite is reactive and may react with several components of proteins. This has been studied mainly with meat protein (e.g. Cassens *et al.*, 1979). Formaldehyde was shown by Reis & Tunks (1973) to react with some amino acids during hydrolysis of casein with 6M HCl resulting in some loss of lysine and a complete loss of tyrosine. Olley and Watson (1961) found that sodium

nitrite and formaldehyde, alone and in combination, reduced chemically determined lysine when they were used as preservatives for herring.

Effects of nitrite and formaldehyde on lysine

In the model experiments nitrite showed no appreciable effect on lysine in a short time experiment (Table 2). When the standing time before analysis was 24 h the lys/phe ratio was lower at pH 5·0 than at the higher pH-values tested and than the controls (Tables 1, 3 and 4). When the mixture was heated the effect was greater when heating was before the standing time than after (Table 5).

In the fish muscle pieces only the ε-amino groups may have been free to react as the α-amino groups would be tied up in peptide bonds. Some preliminary experiments with di-peptides of L-lysine and either glycine or L-alanine and with α- and ε-acetyl-lysines had shown that the ε-amino group was much less reactive with nitrite than the α-amino group. This is in agreement with the early results of Van Slyke and Birchard (1913–14), with the reports of Van Vunakis *et al.* (1960) and of Wagner *et al.* (1969). Dunn and Lewis (1921) found that treatment with excess nitrite in acetic acid solution completely deaminated the protein so that no lysine was recovered. This is difficult to reconcile with the present finding. In Experiment 1 there was little reaction in acetic acid. Their findings may probably be explained by low pH and use of heat.

When $NaNO_2$ and HCHO were used together, the effects on lysine were similar to those found with $NaNO_2$ alone; the effect at pH 5·0 was probably more evident.

HCHO alone showed no effect on lysine under the conditions used. This is in agreement with the results of Carpenter and Opstvedt (1976) who stored capelin with formalin for 23 days.

Effects of nitrite and formaldehyde on tyrosine

Nitrite alone showed no effect on tyrosine when heating was not involved (Tables 1–4). Tyr/phe ratios were lower when the tubes were heated before the standing time at pH 5·0 and after the standing time at pH 7·0. These observations were not repeated.

The mixture of $NaNO_2$ and HCHO showed a dramatic effect on tyrosine at all pH-values tested. This effect was obvious after 1 h standing time (Tables 2–5). The effect seemed to be greater at the higher pH-values. When HCHO was tested alone the same results were obtained as with the mixture. In the dilution experiment the tyr/phe ratio was negatively correlated to the logarithm of the HCHO concentration ($r = -0{\cdot}970$ for the mixture,

$r = -0{\cdot}978$ for HCHO alone). The results of Reis & Tunks (1973) make it highly probable that this effect of formaldehyde is an artefact due to formaldehyde being bound to the protein followed by reaction when the samples were hydrolysed with 6M HCl.

In the theories presented to explain the reduced availability of lysine from fish meal produced from preserved raw material, especially the reaction of nitrite with the ε-amino group of lysine was thought to be of importance. Calculations showed, however, that the amounts of nitrite used in practice would correspond to a reduction of about 3·5% of the lysine in fish protein if all the nitrite reacted with the ε-amino groups of lysine. This would be within the error of the amino acid analysis.

On the other hand, reduced availability of lysine is not necessarily reflected in a reduction of chemically determined total lysine (Carpenter, 1973, review).

In most of the present experiments the concentrations of nitrite and formaldehyde in the aqueous phase were about 90 mM and 300 mM, respectively. This is about 20 times higher than the concentrations used in practical preservation of the catches of fish used in fish meal production. The nitrite concentration in the model experiments was about 10 times in excess of the ε-amino-groups in the muscle pieces of fish. In the dilution experiment (Experiment 4, Table 4) the conditions approached practical conditions at the greatest dilution.

In the present experiments, which lasted up to 24 h, it may be assumed that only reactions between the preservatives and protein-bound amino acids would be detected, as soluble parts of the fish muscle pieces were removed during the washing procedure. When fish catches are preserved, bacterial action is arrested but proteolysis continues. There will then obviously be possibilities for free amino acids to react with the preservatives.

The experiments show that, under the conditions used, there was little effect of nitrite and formaldehyde on lysine, and that the reaction between formaldehyde and tyrosine is probably an artefact.

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