Effect of Various Cryostabilizers on the Production and Reactivity of Formaldehyde in Frozen-Stored Minced Blue Whiting Muscle

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The production of formaldehyde in frozen-stored minced blue whiting muscle was described by a rectangular hyperbolic model, and the effectiveness of each cryostabilizer is discussed in terms of its parameters. The maltodextrins assayed noticeably inhibited formaldehyde production, this effect being greater at −20 °C than at −10 °C. Sucrose was only effective at −20 °C. It seems that these compounds act by restricting molecular diffusion. The effect of each cryostabilizer on formaldehyde binding was closely regulated by its effect on production. This is discussed in terms of the binding equation parameters. The binding of formaldehyde during frozen storage was dependent on protein rearrangements leading to reactive groups becoming available. The constraints of cryostabilizers on molecular diffusion reduced the exposure of these groups. Consequently, the interpretation of formaldehyde reactivity was biased, leading to conclusions different from those that would be obtained from a study done under standard conditions.

Keywords: *Minced fish; formaldehyde production; cryostabilizers; maltodextrins; sucrose; frozen storage; formaldehyde binding*

INTRODUCTION

Trimethylamine oxide demethylase is an enzyme found in the muscle and viscera of gadoids, which gives rise to the formation of formaldehyde during frozen storage (Harada, 1975; Parkin and Hultin, 1982a; Hultin, 1992). The high reactivity and diffusivity of formaldehyde has led to it being implicated in the textural deterioration of muscular tissue (Castell et al., 1973; Gill et al., 1979; Haard, 1992). A high level of dimethylamine is also responsible for the appearance of unpleasant odors and flavors.

The production and reactivity of formaldehyde are enhanced in minced muscle by the rupture of cellular integrity in the muscle structure, due to the decompartmentalization of the different cell components (Hiltz et al., 1976; Parkin and Hultin, 1982b). The mincing of fish muscle tissue does, however, have its advantages, among which the most prominent are the ability to make use of species with low commercial value as well as scraps from filleting and other parts of the fish that would otherwise go to waste, all of which lead to savings in costs (Pastoriza et al., 1994; Borderías and Pérez Mateos, 1996).

Various alternatives for inhibiting the production of formaldehyde have been proposed, such as refrigerating the fish muscle for a period of 4−5 days or putting it through a freeze−thaw cycle before frozen storage (Reece, 1985); storage under oxidizing conditions (Lundstrom et al., 1982; Reece, 1983); the use of heat treatments (Lall et al., 1975; Tokunaga, 1980); the removal of the substrate and cofactors by washing (Landolt and Hultin, 1981); or the addition of enzymatic inhibitors (Parkin and Hultin, 1982b; Careche and Tejada, 1990).

It therefore follows that preventing or inhibiting the activity of this enzyme system leads to an improvement in quality and a greater stability of the frozen product. To be more precise, these ends can be achieved by inhibiting the reactivity of formaldehyde or by reducing the amount of formaldehyde that can interact with the various muscle components.

Sucrose and sorbitol are commercially used as cryoprotectants during the storage of minced fish muscle and play a large role in the conservation of its functional properties, but they give a flavor to the end product that is too sweet (Park et al., 1988; Sych et al., 1990). Additionally, these two additives, as well as polydextrose, are able to diminish formaldehyde production in frozen-stored minced muscle (MacDonald et al., 1990; Herrera and Mackie, 1994; Simpson et al., 1994; Chang and Regenstein, 1997), thus slowing down textural deterioration.

By adding polymers with high molecular weight, such as maltodextrins, to food systems, their glass transition temperature (T_g') can be increased and they can therefore be stored at higher temperatures with greater stability and longer storage life (Levine and Slade, 1988; Slade and Levine, 1991; Roos and Karel, 1991; Kerr et al., 1993). The effect maltodextrins have on the stability of a system depends on several intrinsic factors: their molecular weight, i.e., dextrose equivalent (DE), their structure, their origin, etc., as well as on other extrinsic factors such as storage temperature, freezing rate, etc. The addition of carbohydrates with low molecular weight, such as sucrose, can also allow T_g' to be increased, albeit to a lesser extent (Levine and Slade, 1988), so one would expect the addition of maltodextrins to have a greater cryostabilizing effect. Thus, the addition of maltodextrins to fish muscle could prove to be a means of inhibiting the production and reactivity of formaldehyde. Furthermore, this process would not be incompatible with any of the various above-mentioned protocols.

It was therefore decided to perform a study of the effect of a series of maltodextrins with a wide DE range, as well as that of sucrose, on the production and reactivity of formaldehyde in minced blue whiting muscle, frozen-stored at two different temperatures.

10.1021/jf9809986 CCC: $18.00
Published on Web 05/20/1999

MATERIALS AND METHODS

Blue whiting (*Micromesistius potassou*), caught in offshore Galician waters, was brought layered between crushed ice in polyfoam boxes to the laboratory within the first 24 h postcapture, where it was immediately gutted and beheaded and fillets were taken off and skinned. Subsequently, muscle was minced in a meat mincer (Cutter DITO SAMA K-35) and then mixed manually with a number of different maltodextrins (MD DE 4-28) (Cerestar Ibérica, S.A.), as well as with sucrose, to reach an additive concentration of 8% (w/w). A batch with no additive was used as a control.

Samples (ca. 30 g) about 0.5−0.8 cm thick were then placed in individual plastic bags, which were vacuum-sealed and frozen in a freezer cabinet set at −20 °C. One day later, one-half of the samples were transferred to a freezer cabinet set at −10 °C whereas the remaining half was kept at −20 °C. Samples stored at −20 and −10 °C were periodically taken out of the freezer cabinets, and their respective acid extracts were analyzed for both dimethylamine (DMA) and formaldehyde (FA).

Formaldehyde production was monitored by determining the content of DMA-N according to Dyer and Mounsey (1945), as FA and DMA are produced in equimolar amounts, whereas the content of bound formaldehyde was calculated as the difference between total formaldehyde content and free formaldehyde content (Castell and Smith, 1973). To make the interpretation of results easier, these contents are expressed as μmol/100 g of wet weight. The notations used for the cryostabilized samples correspond to the cryostabilizer added, e.g., MD DE 9 for the sample with maltodextrin DE 9.

The inhibition (I) of formaldehyde production or binding was determined as follows

$$I = \left(1 - \frac{FA_{cryost}}{FA_{ctrol}}\right)100$$

where FA_{cryost} and FA_{ctrol} are total (or bound) formaldehyde contents in the cryostabilized and control samples, respectively, at each sampling period.

The reactivity of formaldehyde was calculated for each sample as the ratio between bound and total formaldehyde contents at each sampling period.

The parameters of both production and binding equations were determined according to the bootstrap method (Efrom, 1981), with 1000 iterations, after linearizing the experimental data according to transformations analogous to those of Lineweaver−Burk and Eadie for the Michaelis−Menten equation. Nonetheless, the fits shown on the graphs were obtained from a minimum squares method carried out for the experimental values. In both cases, Microsoft Excel was used for calculations.

RESULTS AND DISCUSSION

1. Inhibition of Formaldehyde Production. The production of formaldehyde in frozen-stored minced blue whiting muscle was markedly affected by the storage temperature. Thus, the control batches showed contents of over 600 μmol of FA/100 g of wet weight after 12 weeks of storage at −20 °C and over 700 μmol of FA/100 g of wet weight after only 2 weeks at −10 °C, at which temperature the value reached after 12 weeks was 1800 μmol of FA/100 g of wet weight. The importance of this variable has been noted by numerous authors (Tokunaga, 1980; Licciardello et al., 1982; Sotelo et al., 1994).

The experimental values of formaldehyde production (P), obtained from the different samples throughout the storage period, were defined according to a rectangular-type hyperbolic model as follows

$$FA = \frac{P_m t}{k + t} \quad (1)$$

Table 1. Parameters of the Equation for Formaldehyde Production (eq 1) in Minced Blue Whiting Muscle Stored at −20 °C[a]

sample	P_m	k
control	1052.0a	7.34a
MD DE 4	414.6b	4.77b
MD DE 9	374.6c	3.80c
MD DE 12	401.97d	4.70b
MD DE 18	305.2e	5.55d
MD DE 28	356.2f	4.32e
sucrose	369.3c	2.20f

[a] a−f: values in the same column with different letters are significantly different ($\alpha = 0.05$).

Table 2. Parameters of the Equation for Formaldehyde Production (eq 1) in Minced Blue Whiting Muscle Stored at −10 °C[a]

sample	P_m	k
control	2198.7a	3.33a
MD DE 4	1888.4b	5.46b
MD DE 9	1487.2c	3.88c
MD DE 12	1496.4c	4.28d
MD DE 18	1384.7d	4.14e
MD DE 28	1606.5e	3.92c
sucrose	1947.0b	3.79c

[a] a−e: values in the same column with different letters are significantly different ($\alpha = 0.05$).

where, by analogy with the Michaelis−Menten equation (Slade and Levine, 1993), P_m is the asymptotic value that would correspond to the maximum expected production of formaldehyde, t is the storage time, and k represents the time (in weeks) that it would take to reach one-half of the expected maximum production P_m.

A similar trend has been found in many other studies, not only in fish minces (Dingle et al., 1977; Tokunaga, 1980; Reece, 1983; Jahncke et al., 1992; Simpson et al., 1994), but also in fillets and in whole fish muscle (Castell et al., 1971; Licciardello et al., 1982; Parkin and Hultin, 1982b). In this sense, it can be easily inferred that an enzyme that follows Michaelis−Menten kinetics, such as the TMAOase (Parkin and Hultin, 1986; Gill et al., 1992), also describes a hyperbolic profile as a function of the time of assay, i.e., storage period, for a fixed substrate concentration. However, under conditions of very low rates of production, such as a low temperature or the existence of a physical barrier between some of the pools of the reactants, such as in fillets, this pattern can be preceded by a lag phase (Tokunaga, 1980; Licciardello et al., 1982). Nonetheless, no lag phase was found under the experimental conditions of the present study and the initial rates of production were, within the experimental range, maximum, decreasing with the storage time as a result of a gradual depletion either of TMAO or of any of the cofactors involved in the process due to formaldehyde production.

The values of the parameters P_m and k, obtained for each sample at both storage temperatures, are shown in Tables 1 and 2. The experimental values obtained, together with the expected hyperbolic functions obtained by a minimum squares fitting to those values, are shown in graph form in Figures 1 and 3.

So that the experimental values used in the fitting would only show the enzymatic production of formaldehyde during the frozen-storage period, the content of dimethylamine present in the fish muscle prior to freezing, approximately 123 μmol of DMA-N/100 g of wet

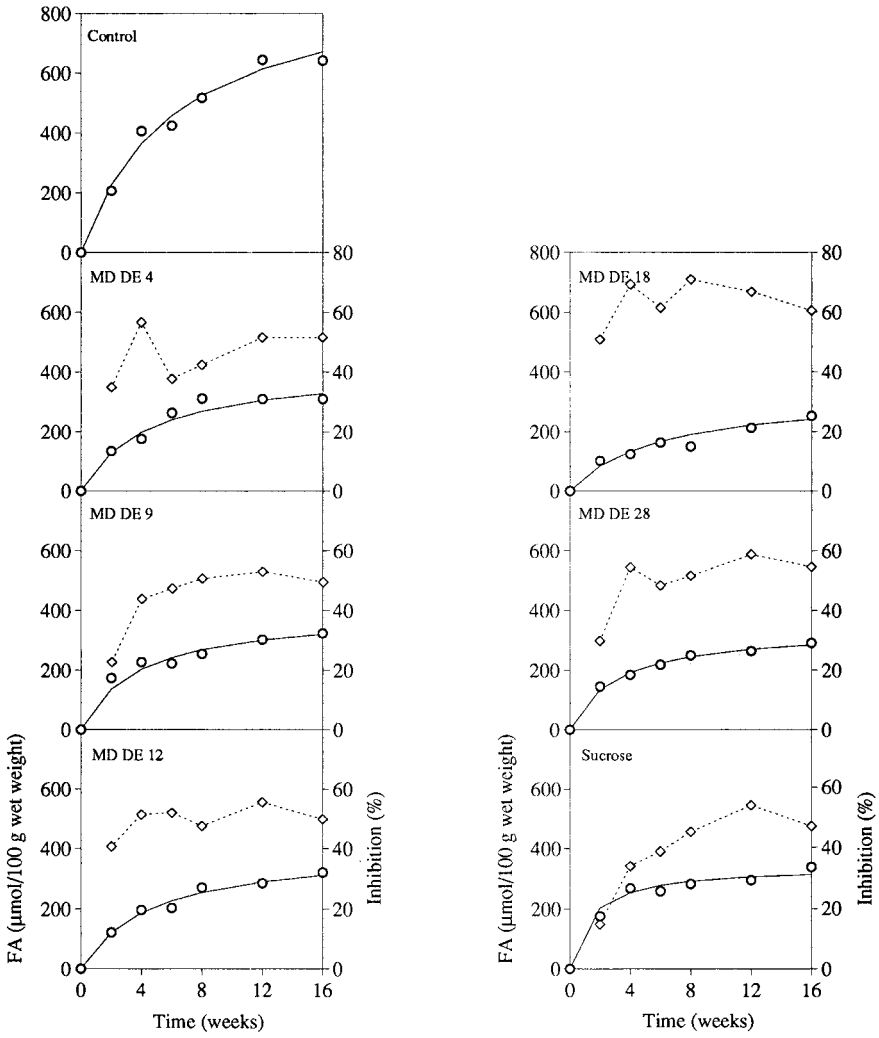

Figure 1. Production (○) and inhibition of production (◇) of formaldehyde in minced blue whiting muscle stored at −20 °C in the presence of various cryostabilizers. The solid line represents the expected values of production obtained from the fit of the experimental values to eq 1. The remaining notations follow the usual criterion.

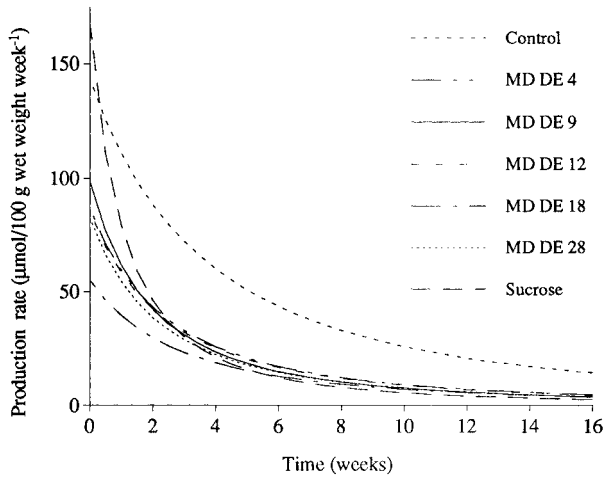

Figure 2. Evolution of the expected values for the rate of formaldehyde production, according to eq 2, in minced blue whiting muscle stored at −20 °C in the presence of various cryostabilizers. The notations follow the usual criterion.

weight, was subtracted from the values obtained throughout the storage period. Bearing in mind that this consideration may not be considered strictly appropriate from a biochemical standpoint, given that the equimolarity of the production of formaldehyde and dimethylamine would only occur as a result of the decomposition of the TMAO, it was taken to be valid.

Similar experiments have shown that the production parameters were dependent on the initial state of the fish muscle (results not shown). The biological state of the fish and its freshness are factors that bear on the concentration of the reactants involved in TMAO demethylation and on the redox potential of the muscle and, therefore, on the production of formaldehyde during the frozen-storage period (Lall et al., 1975; Kelleher et al., 1981; Parkin and Hultin, 1982b, 1986). The latter authors obtained a wide variation in the inhibition/activation factors for a number of compounds tested in frozen red hake minced muscle, which was attributed to fish sample variation rather than to variation in the chemical analyses (Parkin and Hultin, 1982b). Thus, the values obtained for the parameters must not be considered in absolute terms, as they can change from batch to batch, but as a means to compare the effectiveness of the different cryostabilizers assayed, among them and in relation to an untreated control. Nonetheless, the phenomenological behavior is expected to be found to be the same for similar experiments.

The correlation between the production values obtained experimentally and the expected values obtained from the production equation (eq 1) verified the good-

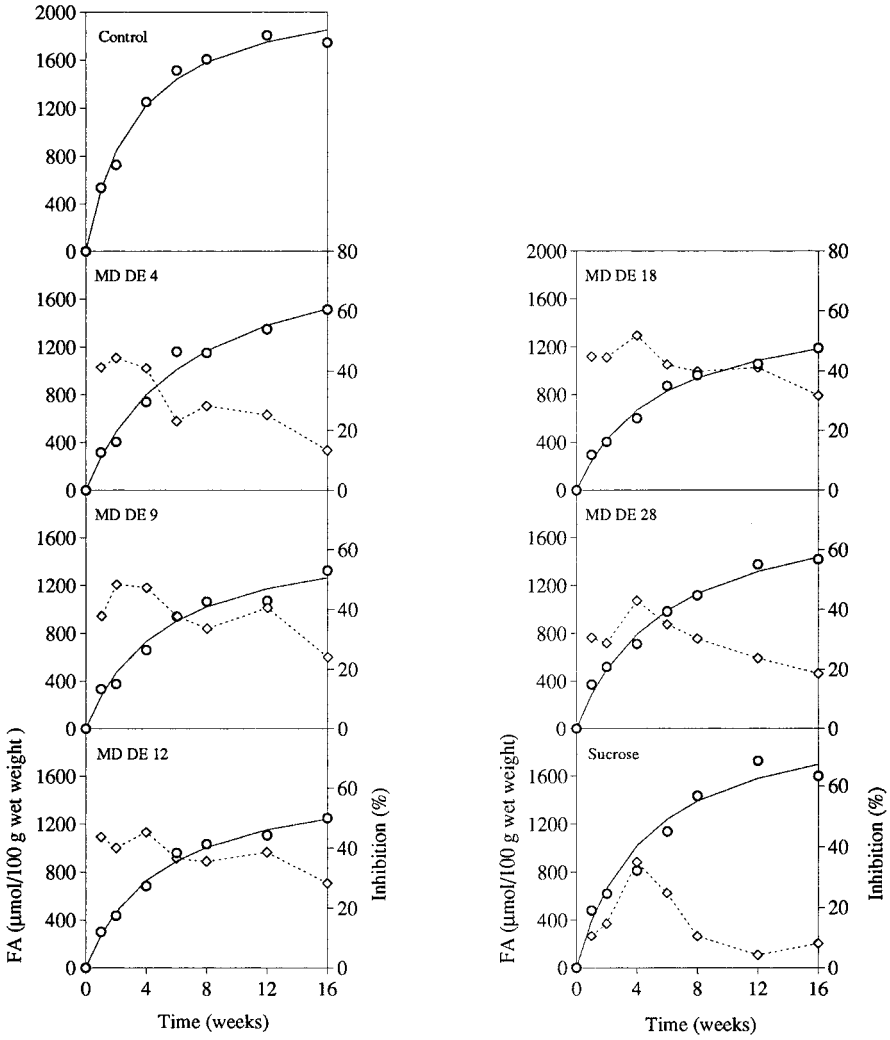

Figure 3. Production (○) and inhibition of production (◇) of formaldehyde in minced blue whiting muscle stored at −10 °C in the presence of various cryostabilizers. The solid line represents the expected values of production obtained from the fit of the experimental values to eq 1. The notations used follow the usual criterion.

ness of the fit that was performed ($r = 0.993$, $f(x) = 0.991 + 6.13$).

1.1. Effects during Storage at −20 °C. The production of formaldehyde increased considerably in the control batch during the first 2 weeks of storage at −20 °C (Figure 1), diminishing gradually due to the limitation imposed by the storage temperature on the interactions between the reactants (Fennema, 1975), associated with the gradual consumption of one or more of the cofactors needed by the enzymatic system or of the substrate itself (Banda and Hultin, 1983). The molecular interactions are proportional to the concentration of reactants in the unfrozen water fraction. Thus, Reece (1985) found that the production of formaldehyde in minced cod muscle could be significantly reduced, if the storage conditions favored the oxidation of NADH.

Furthermore, the possible inhibiting effect caused by the products of the reaction should, in principle, also be taken into account. In this respect, dimethylamine slightly inhibited the production of formaldehyde in minced red hake muscle (Parkin and Hultin, 1982b). At the same time, Sikorski and Kostuch (1982) observed that the rate of production decreased in in vitro systems when formaldehyde was added. Sotelo et al. (1994) later observed that the addition of formaldehyde inhibited the activity of the TMAOase in minced hake muscle proportional to the amount added.

In the present study, the inhibition of formaldehyde production brought about by the cryostabilizers was considerable and increased gradually over the first 12 weeks of storage, as a consequence of a percentage decrease in the rate of production that was less marked in the control batch than in the cryostabilized samples (Figure 2). Moreover, the latter reached the stationary stage several weeks before the former, as is clear from the values for k shown in Table 1. Each of the cryostabilizers showed different increments, with factors ranging between 30% and 70% (Figure 1). Thus, in the samples containing DE 18 maltodextrin, inhibition went from 50% after 2 weeks to 70% after 8 weeks, while those containing DE 28 maltodextrin increased from 30% after 2 weeks to 60% after 12 weeks of frozen storage. The inhibiting effect of sucrose was somewhat less, going from 15% after 2 weeks to 54% after 12 weeks. The other maltodextrins showed factors between 35% and 55%.

Parkin and Hultin (1982b) studied the effect of a number of compounds on the production of formaldehyde, establishing an operational classification, according to which an additive was taken to be a strong inhibitor when it reduced the formaldehyde content by over 30% and a weak one if the reduction was between 10% and 30%. If this classification is applied to the present study, then the cryostabilizers added would be

considered strong inhibitors of formaldehyde production in minced blue whiting muscle stored at −20 °C.

When 12% sucrose was added to minced hoki muscle, frozen-stored at −20 °C, formaldehyde production was reduced by between 30% and 60% (MacDonald et al., 1990; Simpson et al., 1994). Those values agree with those of the present study, although the proportion of sucrose added in such studies was considerably greater. In contrast, the presence of hydrocolloids had only a slight effect on the inhibition of formaldehyde production in minced whiting muscle stored at −18 °C, with factors of less than 15% (da Ponte et al., 1986).

On the other hand, the addition of oxidized lipids inhibited formaldehyde production in frozen-stored hake muscle (Careche and Tejada, 1990), with factors ranging between 50% and 70% during the storage period. Although this brought about an improvement in protein functionality, it also caused the progressive disappearance of the electrophoretic band of the heavy myosin chain, thus suggesting the use of a combination of minced muscle of gadoid and fatty fish as a protocol for the inhibition of formaldehyde production. However, a recent study has shown that the combination of hake and sardine muscle neither slowed protein alterations nor reduced formaldehyde formation (Huidobro et al., 1998).

The effect of the cryostabilizers significantly reduced P_m, with values of less than one-half that of the control batch, as well as k, at −20 °C ($p < 0.05$) (Table 1). This latter effect resulted in an increase of the inhibition factors throughout the storage period. The samples with DE 18 maltodextrin showed a significantly lower value of P_m and a significantly higher value of k than the other cryostabilized samples ($p < 0.05$), as a result of a lower rate of formaldehyde production associated with its greater effectiveness. On the other hand, the DE 4 maltodextrin-containing samples showed the highest P_m value among the cryostabilized samples ($p < 0.05$).

The above-mentioned results would appear to show that the effect, associated with the restriction of molecular interactions, on the inhibition of the reaction was greater than the effect that of a hypothetical product inhibition, especially in the case of the cryostabilized samples in which the reaction finished sooner and with lower formaldehyde contents than that in the control batch. In addition, the changes in protein conformation/configuration also ought to be more restricted in the presence of cryostabilizers. For these reasons, it is hard to justify a large role for product inhibition in these samples. The possible contribution of this effect to the decrease in production in the control batch during the storage period would only serve to augment the inhibiting effect of the cryostabilizers.

It therefore follows that the decrease in P_m shows that the limitation of the interactions between the reactants, as a consequence of the storage temperature, was enhanced by the presence of cryostabilizers, which, in addition, also explains the decrease in k since the stationary stage was reached sooner.

The time derivative of eq 1 defined the evolution of the rate of production (V) during the storage period, according to a negative hyperbola of the form

$$V = \frac{P_m k}{(k + t)^2} \qquad (2)$$

The control batch stored at −20 °C showed an initial production rate higher than 150 μmoles/100 g of wet weight per week, which gradually decreased until approximately 10 μmoles/100 g of wet weight per week after 16 weeks of storage (Figure 2).

The freezing of a cryostabilized system gives rise to a marked increase in the viscosity of the unfrozen water fraction, compared to an untreated control, and therefore the rate of diffusion is lowered (Levine and Slade, 1988). In this respect, the production rate was lower throughout the storage period in the cryostabilized samples. Nevertheless, the higher production in the control batch caused a progressive depletion of reactants that led to a gradual approximation of the reaction rate to those of the cryostabilized samples in the last 8 weeks of storage.

As shown in Figure 2, among the different cryostabilizers assayed, DE 18 maltodextrin showed the highest effectiveness and, according to the P_m values, it seems that the addition of DE 28 maltodextrin was slightly more effective than the other treatments, among which only very slight differences could be appreciated. Nevertheless, the samples containing sucrose showed an initial rate close to that of the control batch, which subsequently decreased more rapidly than that of the latter. In a similar way, Simpson et al. (1994) obtained similar DMA contents for unstabilized mince and sucrose-added stabilized mince during the first month of storage at −20 °C but not subsequently. For all batches, however, the production rates decreased progressively during the storage period, so no differences could be observed among the production rates of the various cryostabilized samples after 8 weeks at −20 °C.

1.2. Effects during Storage at −10 °C. The formaldehyde content of the control batch increased dramatically during the first weeks of storage at −10 °C, reaching values of over 1200 μmol/100 g of wet weight after 4 weeks (Figure 3).

The large difference observed between the values for P_m obtained for the control batches stored at −20 and −10 °C (Tables 1 and 2) is due to the fact that the physical restriction, associated with the storage temperature, on the diffusion of the reactants increases as the temperature diminishes. Accordingly, it was noted that if samples that had been initially frozen at −20 °C were later stored at a higher temperature, an increase in the production rate occurred that corresponded to the new storage temperature (data not shown). This would appear to be the opposite of the effect that would occur if the enzyme had been denatured by the action of formaldehyde. In this respect, Fennema (1975) pointed out that enzymatic reactions that take place in partially frozen systems do not go to completion, with production being a function of the storage temperature, and that, therefore, a later increase in temperature causes the enzyme to resume reaction, until a point is reached that corresponds to the new temperature. Nevertheless, these results indicate the marginal importance, if any, of product inhibition in all samples stored at −20 °C but not in those stored at −10 °C.

Formaldehyde production in minced blue whiting muscle stored at −10 °C was also markedly inhibited by the various cryostabilizers added, although to a lesser extent than at −20 °C (Figure 3). Thus, the inhibition factors showed lower increases than those obtained at −20 °C or remained approximately constant during the first 4 weeks of storage and then decreased markedly, due to the diminishing production rate for the control

batch, which even reached values lower than those of some of the cryostabilized samples.

DE 9, DE 12, and DE 18 maltodextrins were strong inhibitors of formaldehyde production at −10 °C, according to the classification established by Parkin and Hultin (1982b), since they showed inhibition levels greater than 30% over almost the whole storage period. On the other hand, DE 28 and DE 4 maltodextrins were strong inhibitors for the first 8 and 4 weeks of storage, respectively, thereafter becoming weak ones, while sucrose was a weak inhibitor for the first 4 weeks and then had no effect (i.e., less than 10%) for the remaining period of storage. It is interesting to note that those authors based the inclusion of a compound in a specific group according to the average value taken over a 2 or 3 week period of storage at −12 °C, and, therefore, if we go by this criterion, all maltodextrins would be considered strong inhibitors of formaldehyde production at −10 °C while only sucrose would be considered a weak one.

The addition of cryostabilizers led to significant decreases in P_m and to k values significantly higher than that for an untreated control ($p < 0.05$) (Table 2), which accounts for the drop observed in the inhibition factors after the fourth week of storage (Figure 3).

Contrary to what was observed at −20 °C, there seems to be no reason for rejecting product inhibition in the cryostabilized samples stored at −10 °C. Thus, formaldehyde production reached the stationary phase in these samples later than in the control batch. Nevertheless, if this is so, the effect would be greater in the unstabilized control batch containing a greater content of formaldehyde and dimethylamine, and therefore, the reaction inhibition factors, due to the effect of the cryostabilizers on molecular diffusion, would be greater than those obtained.

The differences in the effectiveness of inhibition among the various cryostabilizers were greater at −10 °C than at −20 °C. In this respect, the samples with DE 18 maltodextrin also showed the lowest value for P_m at −10 °C and those containing DE 9 and DE 12 maltodextrins showed P_m values significantly lower than those of the remaining treatments ($p < 0.05$). On the other hand, sucrose and maltodextrin DE 4 were the least effective whereas DE 28 maltodextrin had an intermediate value for P_m.

Nevertheless, although DE 4 maltodextrin had a small effect on the asymptote of production, it had a value of k significantly higher than the other cryostabilized samples, thus showing that its effectiveness was based on a slower production rate due to the restriction of molecular diffusion.

The rate of formaldehyde production dropped dramatically in the control batch during the first 2 weeks of storage at −10 °C, as a result of a high initial production that led to rapid consumption of cofactors and substrate (Figure 4). This decrease continued to show itself during the subsequent storage period, although at a much lower rate, until values of less than 20 μmol of FA/100 g of wet weight per week were reached after 16 weeks of storage.

During the first weeks of storage at −10 °C, the cryostabilized samples showed a lower rate of formaldehyde production than did the control batch, with a more gradual decrease; thus, contrary to what was observed during storage at −20 °C, they gave values

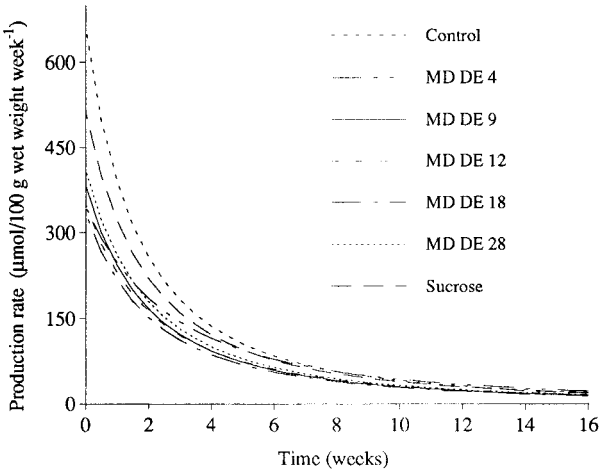

Figure 4. Evolution of the expected values for the rate of formaldehyde production according to eq 2, in minced blue whiting muscle stored at −10 °C in the presence of various cryostabilizers. The notations follow the usual criterion.

for k that were higher than that of the control batch. Consequently, as shown in Figure 4, the curve corresponding to the production rate of this latter batch progressively approached those of the cryostabilized samples over the storage period, crossing that of the sample containing DE 4 maltodextrins after 8 weeks of storage, whereas it practically equalized that of the sample with sucrose after 16 weeks.

A comparative analysis of the effect of the cryostabilizers on the production parameters at both storage temperatures used in the present study shows a higher percentage decrease for P_m, in relation to an untreated control, at −20 °C, i.e., a greater inhibition of production, whereas the values of k hardly changed at both storage temperatures, thus being greater than that for the control batch during storage at −10 °C but lower at −20 °C.

The importance of cryostabilization, as an alternative for inhibiting the production of formaldehyde in frozen minced fish muscle, is based on the fact that the use of cryoprotectants is a common and necessary practice in preventing the functional and biochemical alterations that take place in frozen-stored minced fish muscle (Suzuki, 1981; MacDonald and Lanier, 1991; Herrera, 1993) and on the fact that their use is neither incompatible with nor detrimental to the effectiveness of any other proposed alternative. Furthermore, their use in combination may favor the inactivation of the TMAOase, probably via a decrease of P_m, compared to that of an untreated control batch, since the restriction of molecular diffusion would be enhanced as a consequence of a lessening of the concentration of the reactants that intervene in the reaction together with the inhibiting effect, itself, of the other protocol.

2. Inhibition of Formaldehyde Binding. The binding (B) of formaldehyde to the cellular constituents of minced blue whiting muscle during frozen storage was closely linked to its production, i.e., to the formaldehyde content present in the sample. The correlation coefficients between the content of formaldehyde that was produced and that of bound formaldehyde for each of the samples at both storage temperatures were therefore very high, which was also verified when the data were grouped by temperature or globally (Table 3).

Table 3. Coefficients of Correlation between the Content of Formaldehyde Produced and the Content of Bound Formaldehyde

sample	−20 °C	−10 °C
all data	0.947	0.957
control	0.956	0.961
MD DE 4	0.973	0.973
MD DE 9	0.983	0.983
MD DE 12	0.981	0.981
MD DE 18	0.971	0.971
MD DE 28	0.970	0.970
sucrose	0.964	0.964

Table 4. Parameters of the Equation for Formaldehyde Binding (eq 3) in Minced Blue Whiting Muscle Stored at −20 °C[a]

sample	B_m	k'
control	749.3a	6.65a
MD DE 4	319.7b	4.79b
MD DE 9	335.4b	4.17c
MD DE 12	323.5b	4.65d
MD DE 18	241.0c	5.18e
MD DE 28	288.6d	3.42f
sucrose	324.2b	2.43g

[a] a−g: values in the same column with different letters are significantly different ($\alpha = 0.05$).

Table 5. Parameters of the Equation for Formaldehyde Binding (eq 3) in Minced Blue Whiting Muscle Stored at −10 °C[a]

sample	B_m	k'
control	1816.0a	3.370a
MD DE 4	1322.3b	4.750b
MD DE 9	1034.5c	3.500c
MD DE 12	1095.1c	4.110d
MD DE 18	963.3d	3.760e
MD DE 28	1073.1c	3.330a
sucrose	1316.3b	3,600c

[a] a−f: values in the same column with different letters are significantly different ($\alpha = 0.05$).

Thus, the experimental values for bound formaldehyde obtained from the various samples during the storage period were also fitted, in principle and only with comparative purposes, to a hyperbolic equation analogous to that for production

$$B = \frac{B_m t}{k' + t} \quad (3)$$

in which the parameter B_m represents the maximum amount of formaldehyde that would be bound to the components of the minced muscle during its frozen-stored period and k' the time (in weeks) that it would take for one-half of B_m to bind. These two parameters were determined for each sample at each storage temperature, following the same procedure used for the production equation (Tables 4 and 5). Just as in the case of the experimental production values, the initial content of bound formaldehyde in unfrozen minced muscle, i.e., 80 μmol of FA/100 g of wet weight, was subtracted from the contents obtained during the storage period, so that the experimental values used in the fitting would represent the formaldehyde that had bound during the storage period.

The correlation between the experimental values and those obtained from the binding equation (eq 3) also verified the goodness of the fit carried out ($r = 0.986$, $f(x) = 0.9876x + 2.59$).

2.1. Effects during Storage at −20 °C. The content of bound formaldehyde increased noticeably in the control batch during the first weeks of storage at −20 °C, reaching 300 μmol/100 g of wet weight after only 4 weeks (Figure 5). However, the rate of binding gradually lessened thereafter, and after 16 weeks, the content was 495 μmol of FA/100 g of wet weight.

The effect of the cryostabilizers on the content of bound formaldehyde, in minced blue whiting muscle stored at −20 °C, was parallel to their effect on the production of formaldehyde. In other words, their inhibiting effect on the production of formaldehyde acted as a moderator of the subsequent binding, which appeared as significant decreases in B_m and k' ($p < 0.05$) (Table 4). The inhibition factors consequently showed an upward trend during the storage period (Figure 5).

The samples containing DE 18 maltodextrin gave the lowest values of B_m and the highest values of k' for all the cryostabilized samples ($p < 0.05$), corresponding to a greater effectiveness in reducing the content of bound formaldehyde, with factors of over 60% after the fourth week of storage. No differences were noticed among the P_m values of the remaining treatments except for DE 28 maltodextrin that showed a lower value ($p < 0.05$).

2.2. Effects during Storage at −10 °C. The formaldehyde molecules produced in the control batch bound rapidly with the various muscle components during the first 6 weeks of storage at −10°C, reaching a content of bound formaldehyde of 1400 μmol/100 g of wet weight, but no significant increase occurred subsequently (Figure 6), due to the saturation of available binding groups, although production did continue, at a lesser rate, as seen in Figure 4. Consequently, the content of free formaldehyde, which had maintained an almost constant level during the first 8 weeks of storage, increased noticeably thereafter (although not shown, these values can be obtained by subtracting the content of bound formaldehyde from the total), despite a reduction in the production of formaldehyde during this period.

The content of bound formaldehyde also increased gradually in the cryostabilized samples stored at −10 °C, in a way that was closely linked to the inhibiting effect on production for each cryostabilizer, which was reflected in a value of B_m significantly lower than that for the control batch ($p < 0.05$). Consequently, inhibition increased during the first 4 weeks, reaching factors of over 50%, then diminished progressively thereafter.

The minced muscle containing DE 18 maltodextrin also showed the highest effectiveness against formaldehyde binding during storage at −10 °C, showing a value of B_m significantly lower than those for the other cryostabilized samples ($p < 0.05$) (Table 5). However, the differences among the values of k' for the different treatments were too small to overcome their effects on B_m, so hardly any differences were noticed among the maltodextrins DE 9, DE 12, and DE 28, which, nonetheless, were more effective than maltodextrin DE 4 and sucrose at this temperature.

Contrary to what was observed in the control batch, the content of free formaldehyde increased in the cryostabilized samples during the first 6 weeks of storage but later remained almost constant, even decreasing in the presence of DE 18 and DE 4 maltodextrins as the production rate decreased. This would appear to be a consequence, first, of the effect of the cryostabilizers themselves, in the form of a restriction of molecular interactions, and therefore of the cryoprotection of molecular stability and of a lesser exposure

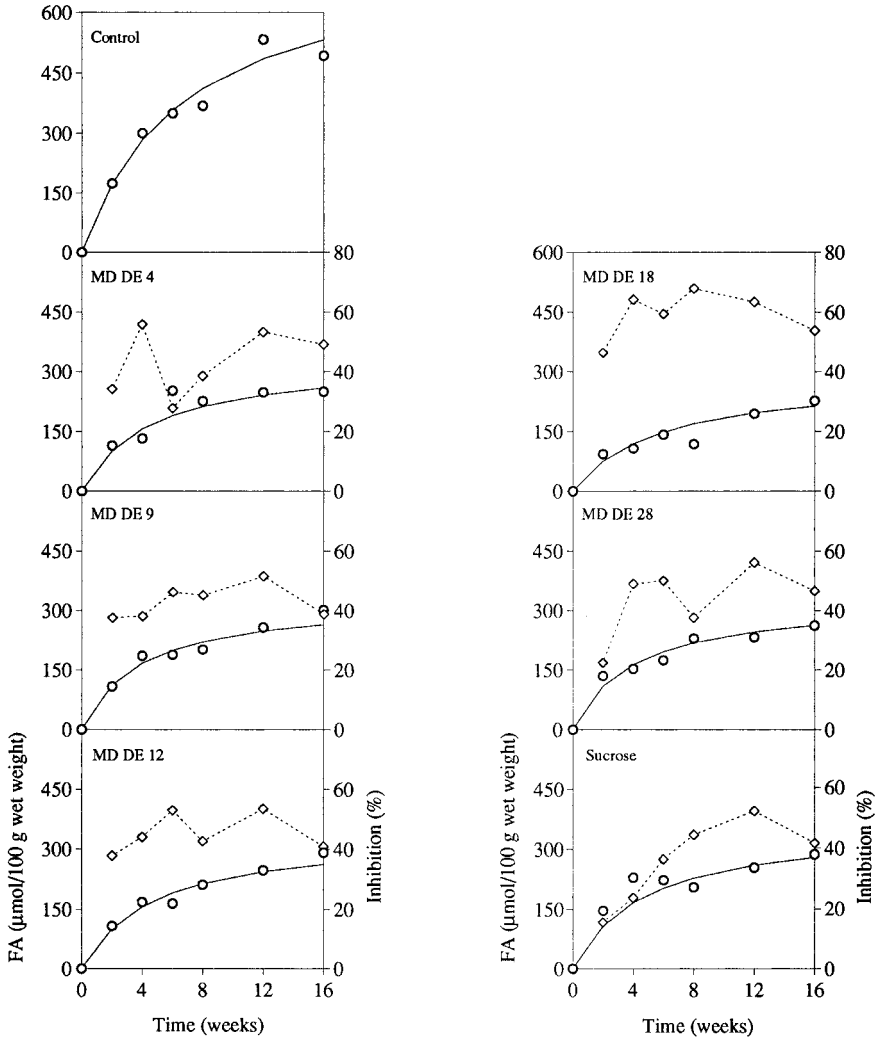

Figure 5. Binding (○) and inhibition of binding (◇) of formaldehyde in minced blue whiting muscle stored at −20 °C in the presence of various cryostabilizers. The solid line represents the expected values of binding obtained from the fit of the experimental values to eq 3. The notations follow the usual criterion.

of potential interacting groups to formaldehyde, and second, of a diminishing rate of binding throughout the storage period due to the occupation of the most easily accessible binding centers. Concerning this effect, Rehbein (1988) noted that the binding of formaldehyde with the components of fish muscle depended not only on its production, but also on the rate and extent of the reaction between them. Thus, binding of the formaldehyde produced also seems to depend to a great extent on the availability and accessibility of the reactive groups.

In this respect, although eq 3 was of great help in comparing the effectiveness of the different treatments, it was found that for the samples with sucrose and DE 28 maltodextrin as well as for the control batch, a detailed analysis of the binding of formaldehyde revealed two different steps, the former of which, extending during the first 2−4 weeks of storage, seemed to describe a hyperbolic profile while the latter showed a sigmoidal pattern. Thus, the evolution of the bound formaldehyde content during storage at −10 °C was fitted to a composite model of the form

$$P = \frac{A_m t}{k_0 + t} + \frac{B_m}{1 + be^{-ct}} \qquad (4)$$

where A_m and B_m are the respective asymptotes of the hyperbolic and logistic terms of the equation, k_0 has an analogous meaning to the aforementioned but referred to the hyperbolic part of the pattern, and b and c are two parameters specific of the logistic equation.

As seen in Figure 7, the fitting of this model to the experimental values excellently described the profiles obtained for these three batches. The values obtained for A_m and B_m showed, on one hand, that A_m was about the same for the three batches whereas, on the other hand, B_m did vary notably among the treatments, being the highest for the control batch (Table 6). Furthermore, the total of both asymptotes approached the values of P_m for both cryostabilized samples, thus supporting the validity of the use of the eq 3 to compare the effectiveness of the different treatments.

From a mechanistic standpoint, it is thought that the first term of eq 4 would correspond to the binding of formaldehyde to the readily accessible reactive groups, mostly exposed on the protein surface, which would be progressively occupied until saturation, describing a typical hyperbolic profile. However, the interactions of formaldehyde with proteins hastens its denaturation, favoring unfolding and, thus, further reactive groups are exposed and become accessible for binding. Thus, the second term would correspond to a sigmoidal autocatalytic process that would start once the former is in

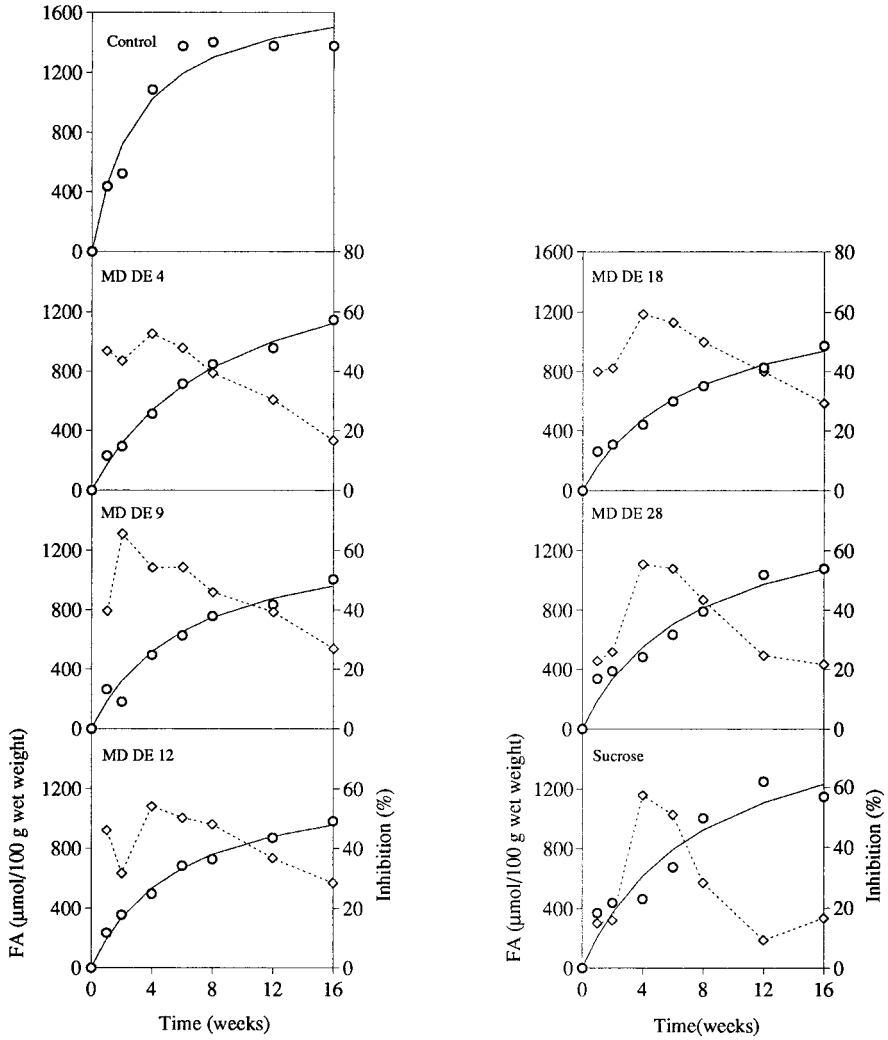

Figure 6. Binding (○) and inhibition of binding (◇) of formaldehyde in minced blue whiting muscle stored at −10 °C in the presence of various cryostabilizers. The solid line represents the expected values of binding obtained from the fit of the experimental values to eq 3. The notations follow the usual criterion.

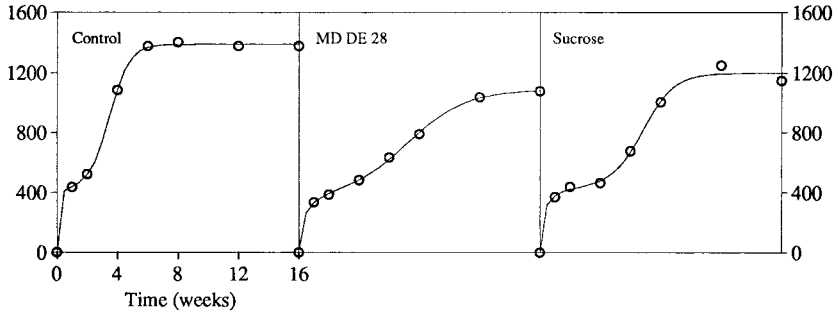

Figure 7. Binding (○) of formaldehyde in minced blue whiting muscle stored at −10 °C in the presence of various cryostabilizers. The solid line represents the expected values of binding obtained from the fit of the experimental values to eq 4. The notations follow the usual criterion.

progress, so that different degrees of overlapping could be observed. In this way, in samples in which the initial rate of formaldehyde production is high, such as in those with sucrose or DE 28 maldextrin (see Figure 4), the readily accessible reactive groups would be rapidly occupied and both processes would be separated in time. This, however, would be less evident for an untreated control, in which protein denaturation is much faster than in cryostabilized samples.

On the other hand, in cryostabilized samples with a low formaldehyde rate of production, both processes would show some overlapping, obstructing a clear separation between both of them, as other factors related to the physicochemical perturbations of the frozen system would also act, promoting protein denaturation. In this sense, studies carried out in our laboratory have shown that the loss of protein functionality was much more dependent upon the amount of formaldehyde produced in minced fish stored at −10 °C than in minces at −20 °C, in which the rate of production was lower (Herrera et al., unpublished results).

Thus, although the results obtained for the remaining samples suggest that this type of composite pattern seems also to be described (especially in DE 18 and DE

Table 6. Parameters of the Equation for Formaldehyde Binding (eq 4) in Blue Whiting Minces Stored at −10 °C[a]

sample	control	MD DE 28	sucrose
A_m	439.7	422.3	462.67
K_0	0.048	0.359	0.224
B_m	950.9	674.8	744.34
b	206.1	49.67	902.70
c	1.53	0.533	0.992

[a] a−f: different letters within a same column refer to significant differents ($\alpha = 0.05$).

12 containing samples), unfortunately the sampling periods used avoided the fittings and gave rise to meaningful values of the parameters, from a mechanistic viewpoint. For this to be confirmed in all samples, a higher number of data should be obtained during the first weeks of storage, defining the transition step with a higher precision.

2.3. Comments on the Reactivity of the Formaldehyde Produced. In those samples stored at −20 °C, the reactivity of the formaldehyde seemed to show the highest values initially, later showing only some oscillations, in keeping with the low rates of production, and it increased from the twelfth week of storage, reaching factors close to those initially obtained, due to the lowering of the production rate (Table 7).

However, in the control batch stored at −10 °C, the reactivity factor increased during the first 6 weeks of storage, reaching close to 90%, then decreased subsequently (Table 7). The high initial production would lead to a saturation of the available groups in the original system; alterations in the proteins would need to take place to allow new groups to be exposed and thus facilitate these interactions. That is, the rate of production would be initially higher than the rate of binding, which in this case would be controlled by configurational and conformational rearrangements of the different protein structures present in the system. Once further reactive groups became available, formaldehyde molecules would bind with them rapidly so that their availability would decrease, thus becoming a limiting factor, and the reactivity factors would diminish.

In this respect, formaldehyde would react to a higher extent if the protein structure were partially denatured due to the exposure of a greater number of reactive groups (Connell, 1975). On the other hand, Regenstein et al. (1982) noted that the proteins of fish muscle underwent a series of conformational changes during the early stages of frozen storage, leading to molecular aggregation. Later, Herrera (1993), working with natural actomyosin of rainbow trout, observed an increase in the surface hydrophobicity and in the number of reactive sulfhydryl groups during the first weeks of frozen storage.

On the other hand, the cryostabilized samples stored at −10 °C gave maximum initial reactivities when most reactive groups were available, which then decreased until the sixth week of storage, since the cryoprotective effect of the various maltodextrins and the sucrose on the protein structure prevented rapid exposure of new binding groups. These values later increased as a result of the decrease in production (Table 7).

During frozen storage of fish muscle, the interactions between formaldehyde and the constituents of the muscle therefore depend on the diffusion of the formaldehyde molecules toward the binding groups, as well as on the availability and accessibility of the latter.

The diffusion of the formaldehyde molecules toward the reactive groups takes place in the unfrozen water fraction that is cryoconcentrated in the fish muscle and is directly proportional to the amount of formaldehyde present in the sample. Formaldehyde is a small molecule, possessing a great ability to diffuse and a high reactivity toward several functional groups (Poulter and Lawrie, 1979; Tome et al., 1985). This allows it to react in fish muscle with sarcoplasmic, myofibrillar, and stroma proteins, as well as with various compounds of low molecular weight, in a nonspecific manner (Castell et al., 1973; Banda and Hultin, 1983; Montero and Borderías, 1990). As a result, these molecules should not need to diffuse any great distance to interact with the reactive groups present in fish muscle, in a fashion similar to that observed in free radicals (Dean et al., 1991), provided that their availability is not a limiting factor.

The number of reactive groups decreases during the storage period as a result of their interaction with formaldehyde molecules, and thus their availability is inversely proportional to the amount of formaldehyde present in the medium. These interactions would finally lead to saturation of these binding groups.

However, the proteins present in fish muscle undergo a series of conformational and configurational rearrangements during frozen storage, allowing for the exposure, on the surface of the protein, of reactive groups that were initially buried inside the core of the protein structure (Buttkus, 1970). Taking into account the denaturing effect of formaldehyde, the exposure of binding groups may depend to a certain extent on the amount of formaldehyde present in the sample, since it

Table 7. Reactivity of the Formaldehyde Produced (%) in Minced Blue Whiting Muscle during Storage at −20 and −10 °C[a]

time	control	MD DE 4	MD DE 9	MD DE 12	MD DE 18	MD DE 28	sucrose
				Stored at −20 °C			
2 weeks	83.96	86.19	83.05	88.04	91.60	92.84	83.18
4 weeks	73.93	75.34	81.56	85.12	86.27	82.70	85.36
6 weeks	82.56	85.10	84.49	80.91	87.05	79.84	85.80
8 weeks	71.26	75.10	79.13	77.92	78.81	91.84	72.25
12 weeks	82.73	79.71	85.09	86.52	91.25	87.92	85.88
16 weeks	87.59	80.68	93.05	90.64	89.91	90.04	84.65
				Stored at −10 °C			
1 week	81.19	73.46	78.77	77.72	88.27	90.29	77.25
2 weeks	71.66	72.75	63.78	81.60	76.03	74.49	70.58
4 weeks	86.77	69.51	75.32	72.79	73.73	67.83	57.08
6 weeks	89.07	61.59	66.61	71.34	68.40	64.48	59.48
8 weeks	87.35	73.66	71.15	70.49	72.71	70.80	70.00
12 weeks	76.30	70.98	77.88	78.65	77.92	75.40	72.33
16 weeks	78.92	75.87	75.86	78.65	81.67	75.98	71.71

would act as a destabilizer of the protein structure and favor the exposure of new groups with which it could interact (Ang and Hultin, 1989).

Thus, the hyperbolic model defined by the binding of formaldehyde in the cryostabilized samples, with a smaller content than in the control batch, would seem to suggest that, to a large extent, the interactions between these molecules and the cell components are due to protein unfolding and the subsequent exposure of new binding groups. Concerning this effect, Herrera (1993) observed that increases in the number of reactive sulfhydryl groups and in the surface hydrophobicity of natural actomyosin of rainbow trout during storage at −20 °C were noticeably reduced in the presence of various cryoprotective agents.

Cryostabilizers should therefore play an important part in the inhibition of the reactivity of formaldehyde by reducing the exposure and thus the availability of reactive groups, as well as by restricting the diffusion of the formaldehyde molecules toward these groups. However, the interactions of formaldehyde with the various components of minced blue whiting muscle showed a close dependence on the content of formaldehyde present in the sample, which in turn was regulated by the differing inhibiting effect of each cryostabilizer. This effect greatly influenced the state of the system when it came to performing a comparative evaluation of the effect of these cryostabilizer compounds on the reactivity of formaldehyde, giving rise to a biased interpretation of the results and, in consequence, to a series of conclusions that differed from those that would have been obtained if this study had been carried out under standard conditions, allowing the effect of cryostabilizers on reactivity to be seen independently.

Thus, the reactivity factors for the cryostabilized samples were considerably higher during storage at −20 °C than at −10 °C, ranging from between 80% and 90% in the first case and between 65% and 80% in the second (Table 7), contrary to the results that would be expected from a kinetic study of reaction rates under standard initial conditions. This apparent anomaly is due to the higher production rate and therefore the greater content of formaldehyde in the samples stored at −10 °C, which reduces the number of available reactive groups.

Similarly, the cryostabilized samples stored at −20 °C showed high levels of reactivity equal to or even higher than those of the control batches at both temperatures due to a lower production rate and thus a lower content of formaldehyde, favoring its interaction with the various reactive groups, which never became a limiting factor.

In addition, the gradual decrease of the reactivity factors observed in cryostabilizer-containing samples during the first 6 or 8 weeks of storage was, on the whole, more marked at −10 °C than at −20 °C, in keeping with a higher level of occupation of the reactive groups. These factors reached relatively low levels, e.g., the samples containing sucrose showed factors of below 60% after 4 weeks of storage. This gave rise to an increase in the content of free formaldehyde that lacked sufficient groups to bind with, despite its greater diffusibility at the higher temperature, meaning that it had to diffuse greater distances in order to interact or, alternatively, wait until new groups were exposed. The reactivity did not continue to decrease and instead rose gradually over the subsequent weeks of storage at both temperatures, corresponding to the marked decrease in the production rate.

ACKNOWLEDGMENT

We thank Cerestar Ibérica S.A. for making the various maltodextrins available to us, Mr. Carlos Suárez and Lorena Barros Tajes for their collaboration in sampling and the application of analytical techniques, and Dr. M. Murado and Dr. P. González for their advice concerning the statistical treatment of data.

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Received for review September 4, 1998. Revised manuscript received March 22, 1999. Accepted March 31, 1999. This work was carried out with the financial support of the Interministerial Commission for Science and Technology (CICYT) within Research Project ALI97-0589.

JF9809986