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Cryoprotection of frozen-stored actomyosin of farmed rainbow trout (*Oncorhynchus mykiss*) by some sugars and polyols

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Abstract

A study on the effectiveness of several cryoprotectants (polydextrose, lactitol, glucose syrup and a mixture of sucrose and sorbitol [1:1]) in preventing freeze-induced perturbations of fish proteins was carried out in in vitro systems of natural actomyosin of rainbow trout (*Oncorhyncus mykiss*) muscle. Adding these cryoprotectants prevented drastic decreases of ATPase activity, as well as a rapid exposure of hydrophobic and sulphydryl groups on the protein surface. Cryoprotectants therefore slowed down the kinetics of aggregation via intermolecular secondary forces and disulphide bonds, thus greatly reducing losses in solubility.

Keywords: Natural actomycin; Fish muscle; Cryoprotectants; Freeze-induced perturbations

1. Introduction

Freezing is a widely used and highly effective means for long-term preservation of muscle systems, since deteriorative (bio)chemical changes are slowed down and inactivation of microbiological activity occurs. However, frozen storage is often carried out at relatively high temperatures and for excessively long periods, so proteins become denatured and, as a result, there is a loss in quality of the products (Mackie, 1993; Shenouda, 1980; Sikorski, Olley, & Kostuch, 1976).

Myofibrillar proteins of fish muscle—particularly myosin or actomyosin—are highly susceptible to physico-chemical and structural modifications, due to freezing and frozen storage, causing intramolecular conformational rearrangements and intermolecular aggregation. Hydrophobic interactions, hydrogen bonds and also disulphide bridges are primarily involved in these processes (Alvarez et al., 1999; Connell, 1965; Owusu-Ansah & Hultin, 1986).

A number of compounds have been tested against such freeze-induced perturbations. Carbohydrates, polyols, some aminoacids and related compounds have shown the highest cryoprotective effectiveness (Matsumoto, 1980; Noguchi, 1974; Park, 1994). Nevertheless, many of them cannot be used for various reasons such as high cost, not permitted by food regulations, or adverse sensory properties.

Sucrose and sorbitol (4:4% w/w) are commonly used to process fish mince-derived products. However, they impart a taste that is too sweet, and it has led to a search for other cryoprotectants (Dondero, Gandolfo, & Cifuentes, 1994; Dondero, Sepulveda, & Curotto, 1996; Park, Lanier, & Green, 1988; Sych, Lacroix, Adambounou, & Castaigne, 1990, 1991). To this end, further studies, related to the role of cryoprotectants in the prevention of denaturation and aggregation of myofibrillar proteins, are evidently needed.

This work has therefore been directed at studying how some cryoprotectants prevent freeze-induced perturbations in in vitro systems of natural actomyosin (NAM) of fish muscle.

2. Materials and methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish farm and kept alive in tanks until required for experimental studies. Then,

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fish were killed, gutted and kept in ice for about 24 h. Once in the rigor state, the fish were skinned and filleted by hand, and fillets were used for actomyosin extraction.

2.2. Chemicals

The hemimagnesium salt of 8-anilino 1-naphthalene sulphonic acid (ANS) and adenosine 5-triphosphate (disodium salt) (ATP) were obtained from SIGMA, 5,5-dithiobis 2-nitrobenzoic acid (grade A) (DTNB) was provided by Calbiochem, and urea and sodium dodecyl sulphate (SDS) by Bio-Rad. All other chemicals were of reagent grade.

Cryoprotectants were kind gifts from the following sources: sorbitol (Neosorb 20/60) and glucose syrup (Flolys* B6382) by Roquette Ltd., Polydextrose-K by Pfizer Ltd., and lactitol (Lacty) by CCA biochem b.v.

2.3. Preparation of samples

Natural actomyosin (NAM) was extracted according to the method of Kawashima, Arai, and Saito (1973) with some modifications.

Minced fish (50 g) was thoroughly mixed with 300 ml of low ionic strength sodium phosphate buffer ($\mu = 0.05$, pH 7.5) and then centrifuged (5000×*g*, 3 °C, 10 min). After discarding the supernatant, this step was repeated twice. Subsequently, the washed mince was homogenized with 150 ml of salt-extraction solution (0.8 M KCl in 50 mM phosphate buffer, pH 7.5) by using an Omnimixer (Sorvall), 100 ml of salt-extraction solution were further added, and the whole was allowed to stand for 45 min. The crude extract was then centrifuged (5000×*g*, 3 °C, 15 min) and the supernatant was collected and diluted into 10 volumes of cold-distilled water. After precipitation of the myofibrillar protein, most of the water was suctioned off, the remainder being removed by centrifugation (5000×*g*, 3 °C, 10 min). The protein extract was then dissolved in 1.5 M KCl-25 mM imidazole, pH 6.9 solution down to 0.6 M KCl, filtered through a gauze, and eventually dialysed against 0.6 M KCl-25 mM imidazole solution, pH 6.9 for about 18–20 h.

Subsequently, each of the cryoprotectants [polydextrose, lactitol, glucose syrup, and a combination of sucrose and sorbitol (1:1)] was added to a final concentration of 8% (w/w), and the protein concentration was adjusted to 14 mg/g of extract. A batch with no additive was used as a control.

Subsequently, NAM extracts (ca. 14 ml), were poured into polyethylene test tubes, and then frozen in a freezer cabinet set at −20 °C. Samples were taken out periodically, thawed under cold running tap water, homogenized by using a 25 ml Potter-Elvejhem glass homogenizer, and subjected to a number of analyses.

2.4. Solubility

NAM extracts were centrifuged at 15 000×*g*, 3 °C, for 1 h and the supernatant solution was collected. The protein fraction contained in this solution was defined as salt-soluble protein. The protein concentration was determined by the ultraviolet method of Kalb and Bernlohr (1977), and protein solubility was determined as the percentage ratio between salt-soluble protein and total protein contents.

2.5. Differential solubility analyses

An aliquot (1 ml) of NAM extracts was mixed with 10 ml of a 2% (w/v) SDS—0.5% (w/v) borax solution, pH 8.9, with or without 1% (w/v) 2-mercaptoethanol (ME) (Rehbein & Karl, 1985). Both mixtures were gently shaken at 60 °C for 2 h, and then centrifuged at 15 000×*g* for 30 min. The protein fractions contained in each of the supernatant solutions were defined as ME-soluble fraction and SDS-soluble fraction, respectively.

The protein concentration was determined in each fraction according to a modified Biuret method (de Wreede & Stegemann, 1981), which lacks interference due to SDS and ME. Nonetheless, polydextrose, lactitol as well as the mixture of sucrose and sorbitol had to be added to standard protein solutions to reduce their interference. This method thus became acceptable for comparative purposes.

2.6. ATPase activities

ATPase activity assays were carried out at low ionic strength (0.06 M KCl) and pH 7.0 (25 mM Tris maleate). The substrate for reaction was 1 mM ATP. The remaining conditions were 5 mM calcium chloride and 0.5 mg protein/ml for Ca^{2+} ATPase activity, and 1 mM magnesium chloride and 0.2 mg protein/ml for Mg^{2+} ATPase activity.

Samples were incubated for 3 min at 25 °C, and the reaction was stopped by adding 0.5 ml of a 15% perchloric acid solution to 2 ml of the reaction mixture. The amount of inorganic phosphorus (P_i) liberated was determined (Fiske & Subarow, 1925), and the results were expressed as μmol of P_i per mg of protein per min at 25 °C.

2.7. Sulphydryl (SH) groups

The total content of SH groups was determined by adding 0.25 mg protein to a reaction solution consisting of 0.6 M KCl, 8 M urea, 6 mM ethylenediamine tetraacetic acid (EDTA) and 50 mM sodium phosphate buffer, pH 8.0, in the ratio 1:19 (v/v) (Buttkus, 1971). After bubbling through a stream of nitrogen, samples (3 ml)

were incubated at 25 °C for 10 min, and then 20 μl of 10 mM DTNB were added. The reaction mixture was left to stand for 5 min, and then the absorbance was read out at 412 nm.

The content of reactive SH groups was determined similarly, but in the absence of urea, and samples were left to stand for 30 min after adding DTNB.

The content of SH groups was calculated in both cases by using a molar absorptivity coefficient of 13 600 (Ellman, 1959), and expressed as SH moles per $5{\cdot}10^5$ g of protein.

2.8. Surface hydrophobicity (S_0)

Surface hydrophobicity (S_0) was measured by using ANS as fluorescent probe (Kato & Nakai, 1980). The hemimagnesium salt of ANS was initially eluted through a Dowex-50 column (potassium form) to obtain the potassium salt of ANS. Subsequent atomic absorption spectroscopy analysis revealed an extremely low level of remaining magnesium ions (< 10 ppm).

An aliquot (20 μl) of 8 mM ANS solution was added to 2 ml of a number of NAM extracts, ranging from 0.4 to 0.05 mg protein/ml. The mixture was left to stand for 5 min in a ice-water bath, and then fluorescence was measured at excitation and emission wavelengths of 383 and 472 nm, respectively. Fluorescence was also measured in those same extracts in the absence of ANS. The difference between the two fluorescence values was defined as net fluorescence intensity. Fluorescence readings were always referred to a value of 30 in a 100 full scale set for a standard solution consisting of 10 μl of 8 mM ANS solution in 2 ml of methanol.

The initial slope of plotting net fluorescence intensity versus protein concentration was defined as surface hydrophobicity.

3. Results and discussion

3.1. Salt-solubility

Salt-solubility decreased markedly in the control during the first 24 h of frozen storage (Fig. 1). This decrease is most likely due to changes occurring in the protein microenvironment associated with the process of freezing and thawing (Franks, 1985; Love, 1966; Shenouda, 1980). Salt-solubility decreased still further, but to a lower extent subsequently, so that more than half of NAM was no longer soluble after 4 weeks of storage.

The addition of cryoprotectants maintained solubility levels significantly higher than those of the control throughout the entire period of storage. Polydextrose and lactitol showed a higher effectiveness than glucose syrup and the mixture of sucrose and sorbitol. It seems clear that these cryoprotectants prevented the drastic changes of proteins associated with freezing and thawing, so aggregation occurred more slowly.

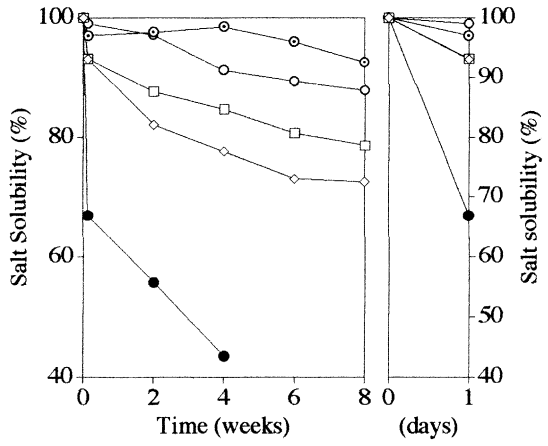

Fig. 1. Effects of freezing and frozen storage on salt solubility of rainbow trout actomyosin in the absence (control: ●) or presence of cryoprotectants (lactitol: ○; polydextrose: ⊙; glucose syrup: ◇; sucrose and sorbitol: □). Right figure shows the effects after 24 h of frozen storage.

3.2. ATPase activity

Ca^{2+} ATPase activity is a good index of the structural state of myosin. Accordingly, the sharp decrease of Ca^{2+} ATPase activity in the control after overnight frozen storage indicates that freezing and thawing notably perturbed myosin (Fig. 2). Watanabe, Kitabatake, and Doi (1988) had found that the Ca^{2+} ATPase activity of rabbit myosin stored at −5 °C remained intact under supercooling conditions (i.e. non-frozen), but decreased notably if frozen and thawed. Ca^{2+} ATPase activity decreased still further during the subsequent period of storage, but at a much lower rate. Studies on bovine actomyosin had shown that myosin head was much affected during the first weeks

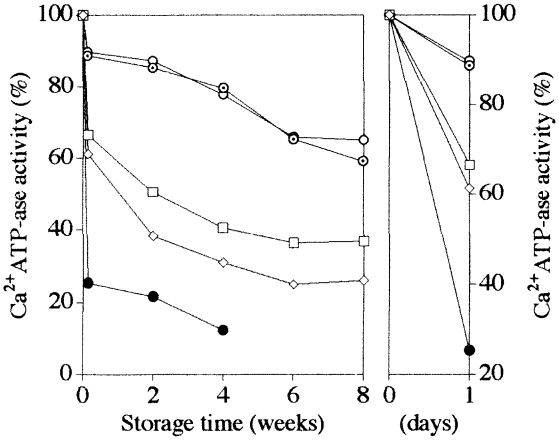

Fig. 2. Effects of freezing and frozen storage on Ca^{2+} ATPase activity of rainbow trout actomyosin in the presence and absence of cryoprotectants. Right figure shows the effects after overnight frozen storage. Symbols follow the notations of Fig. 1.

of storage, slight alterations occurring subsequently (Wagner & Añon, 1986).

Polydextrose and lactitol were highly effective in preventing Ca^{2+} ATPase activity decreasing sharply during the first 24 h of storage. They appeared to have preserved myosin from microenvironmental perturbations associated with freezing. Such perturbations would have affected myosin progressively and to a much smaller extent than in the control during the entire period of storage. Glucose syrup and the mixture of sucrose and sorbitol also reduced the initial drop of Ca^{2+} ATPase activity, but much less effectively than polydextrose and lactitol.

As shown by Mg^{2+} ATPase activity measurements (Fig. 3), the interactions between myosin and actin were notably disturbed in the control after freezing. A further loss of activity was found during the subsequent storage, but at a slower rate. The addition of cryoprotectants reduced the drop of Mg^{2+} ATPase activity, but no differences were found amongst them.

ATPase activity assays were much more sensitive than solubility measurements to changes affecting NAM extracts. The head of myosin had been reported to be highly susceptible to denaturation, but a higher degree of alteration would be needed for aggregation and loss of solubility to occur (Wagner & Añon, 1986). On the other hand, solubility was found to be a more adequate index for discriminating between different treatments in the present case, presumably because the kinetics of denaturation were excessively rapid.

3.3. Surface hydrophobicity (S_0)

Surface hydrophobicity increased greatly (nearly twofold) in the control after 24 h of storage (Table 1). On the other hand, it had hardly changed when NAM extracts were chilled-stored (results not shown). It is

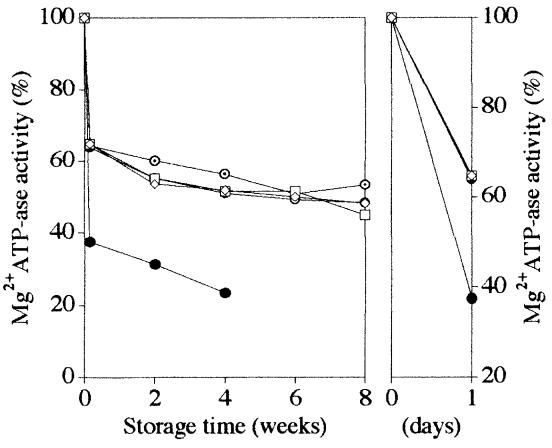

Fig. 3. Effects of freezing and frozen storage on Mg^{2+} ATPase activity of rainbow trout actomyosin in the presence and absence of cryoprotectants. Right figure shows the effects after overnight frozen storage. Symbols follow the notations of Fig. 1.

Table 1
Variation of surface hydrophobicity (S_0) in NAM solutions during frozen storage in the absence (control) or presence of cryoprotectants

Time	Control	Polydextrose	Lactitol	Glucose syrup	Sucrose: sorbitol
Unfrozen	24.8	30.0	32.3	27.9	29.0
1 day	43.1	39.6	37.4	39.0	36.2
2 weeks		38.4	40.0	44.1	40.9
4 weeks		41.9	44.1	53.0	45.6
6 weeks		39.4	37.7	48.0	46.9
8 weeks		41.8	42.6	54.9	50.0

therefore clear that freezing strongly perturbed the stability of actomyosin. Freezing modifies the structural organization of water, and therefore the network of hydrogen bonds so that many buried hydrophobic residues in the native protein become exposed (Ang & Hultin, 1989; Franks, 1985). Unfortunately, these perturbations gave rise to large size aggregates responsible for light scattering, making it impossible for further measurements to be taken.

The addition of cryoprotectants diminished the initial increase of S_0 considerably, but it increased further during the subsequent period of storage. The effectiveness of cryoprotectants seemed to have followed a similar order to that of previous measurements. That is, polydextrose and lactitol were found to be more effective than glucose syrup and the mixture of sucrose and sorbitol.

Therefore, these cryoprotectants reduced the exposure of hydrophobic residues on the surface of actomyosin and, consequently, the tendency to cluster through intermolecular hydrophobic interactions and to form aggregates. The strengthening of intramolecular hydrophobic interactions and the maintenance of the structural organization of water were postulated to be the main effects of the cryoprotection of proteins, which would thus remain in the native state (Arakawa, Carpenter, Kita, & Crowe, 1990; Carpenter & Crowe, 1988).

Proteins undergo continuous conformational changes during frozen storage and, as a result, the location of non-polar residues varies. However, ANS only binds to non-polar residues exposed at the time of measuring, but not those that are buried, no matter whether they are in the native or in a different location. This shortcoming accounts for some fluctuations found for S_0.

It would also account for the higher S_0 values obtained for the samples with glucose syrup or sucrose and sorbitol than for the control. Non-polar regions tend to hide away from water. Therefore, hydrophobic interactions would have taken place rapidly in the latter once non-polar residues became exposed, and ANS could not bind them, but much more slowly in the presence of these cryoprotectants (see solubility results), so

more hydrophobic groups would have been accessible for ANS binding.

3.4. Sulphydryl (SH) groups

The total number of SH groups diminished significantly in the control after 24 h of frozen storage, and still further during the following 2 weeks of storage (Table 2). The formation of large size aggregates prevented measurements subsequently being taken.

The addition of polydextrose, lactitol, glucose syrup and the mixture of sucrose and sorbitol reduced such decreases notably. However, no differences were found between cryoprotectants. Similar decreases were thus found for the control after 2 weeks of storage and for the samples with cryoprotectants after 6–8 weeks of storage. Considering that large differences were found in solubility between these samples, this result seems to stress the importance of secondary forces being responsible for denaturation and aggregation of actomyosin.

The number of reactive sulphydryl groups only showed slight changes in all the samples after 24 h of storage (Table 3). However, it decreased noticeably in the samples with lactitol or polydextrose during the subsequent 2 weeks of storage, but not beyond this time, whereas a progressive increase was found in the sample with glucose syrup during the first 6 weeks of storage.

Conformational changes, taking place in proteins during frozen storage, lead to a continuous exposure and burial of SH groups. SH groups are prone to oxidation but are also involved in interchange reactions as well as in metallic complexes (Buttkus, 1971). Accordingly, in the samples with polydextrose or lactitol, oxidation and steric hindrance of SH groups would be faster than unfolding, leading to exposure of natively-buried SH groups, so that the number of reactive SH groups decreased. On the other hand, the exposure of natively-buried SH groups would have prevailed in the sample with glucose syrup, so it increased. In similar terms, unfolding and aggregation took place rapidly in the control, i.e. during the first 24 h of frozen storage, and it would have masked changes in the number of reactive SH groups.

Table 2
Variation in the total number of sulphydryl groups in NAM solutions during frozen storage in the absence (control) or presence of cryoprotectants

Time	Control	Polydextrose	Lactitol	Glucose syrup	Sucrose: sorbitol
Unfrozen	35.7	31.2	32.0	31.6	31.7
1 day	31.9	29.4	29.9	29.3	30.5
2 weeks	28.3	27.3	27.8	27.5	29.2
4 weeks		26.6	27.4	26.7	27.4
6 weeks		26.1	26.9	26.4	26.2
8 weeks		25.2	26.0	24.6	25.3

Table 3
Variation in the number of reactive sulphydryl groups in NAM solutions during frozen storage in the absence (control) or presence of cryoprotectants

Time	Control	Polydextrose	Lactitol	Glucose syrup	Sucrose: sorbitol
Unfrozen	27.4	24.4	25.2	24.5	24.7
1 day	27.6	24.1	24.4	25.1	24.3
2 weeks	26.8	22.9	23.0	25.5	24.9
4 weeks		22.5	23.7	26.1	24.9
6 weeks		22.6	22.9	26.7	24.7
8 weeks		22.5	23.6	24.6	22.8

3.5. Differential solubility

The effectiveness of cryoprotectants in preventing denaturation and aggregation of actomyosin via secondary forces and disulphide bonds led a subsequent study aimed at finding out how they reduced the role of these interactions in the loss of solubility.

Although actomyosin was extracted by using NaCl instead of KCl as extractant, since potassium ions precipitate SDS, the pattern of salt solubility hardly changed (Fig. 4). That is, solubility dropped markedly after overnight frozen storage—particularly in the case of the control—and much more slowly during the subsequent period of storage. However, higher values and smaller size aggregates—according to visual observation— were found. Polydextrose, lactitol, glucose syrup and the combination of sucrose and sorbitol afforded an excellent cryoprotection of actomyosin, the two former showing the highest effectiveness again.

The protein content of the SDS-soluble fraction decreased only slightly in all the samples after 24 h of frozen storage, but more noticeably thereafter. Polydextrose and lactitol were most effective in preventing such decreases. Differences between SDS-solubility and salt-solubility revealed the importance of intermolecular secondary forces during the first weeks of frozen storage.

Solubility hardly decreased in any sample during the first 2 weeks of storage in the presence of ME and SDS, but some protein became insoluble in the control and in the glucose syrup-containing samples thereafter. Polydextrose, lactitol, as well as the combination of sucrose and sorbitol, seemed to have completely prevented the formation of non-disulphide covalent bonds. Differences in protein contents between SDS-soluble and ME-soluble fractions indicated that intermolecular disulphide bonds had already formed after overnight frozen storage. These bonds developed further during the subsequent period of storage, but to a much less extent than secondary forces (Buttkus, 1971; Lim & Haard, 1984; Owusu-Ansah & Hultin, 1987).

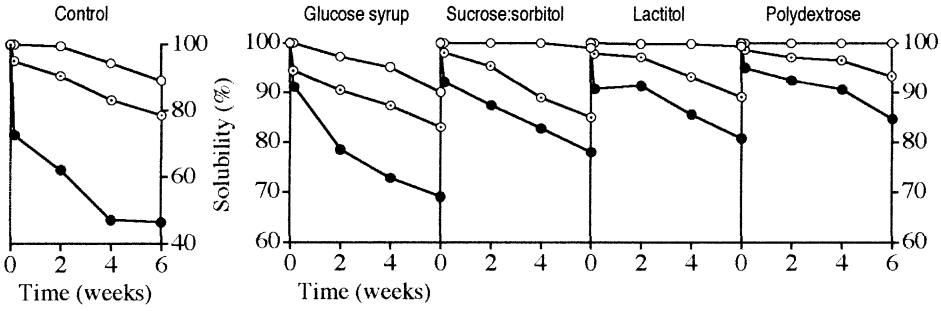

Fig. 4. Effects of freezing and frozen storage on the solubility in different media (salt-medium: ●; SDS-medium: ⊙; SDS + ME-medium: ○) of rainbow trout actomyosin in the presence and absence of cryoprotectants.

These results clearly showed a highly developed aggregation phenomenon in frozen-stored NAM solutions, with even the possible presence of non-disulphide covalent bonds or a greater strength of secondary forces and a less accessible location of disulphide bonds in some of the samples. Polydextrose, lactitol and the mixture of sucrose and sorbitol slowed down the formation of intermolecular secondary interactions and disulphide bonds with respect to the control, and apparently prevented the formation of non-disulphide covalent bonds throughout the period of storage.

4. Conclusions

Polydextrose, lactitol, glucose syrup and the mixture of sucrose and sorbitol were highly effective in preventing changes taking place in frozen-stored NAM extracts of rainbow trout. Thus, the addition of these cryoprotectants preserved the structural stability of myosin to some extent and slowed down the exposure of buried hydrophobic residues on the protein surface. As a consequence, the kinetics of protein aggregation were slowed down.

Polydextrose appeared to be the most effective cryoprotectant. Whether it acts by kinetically inmobilizing the system (Herrera & Roos, 2001) or by thermodynamically preventing protein unfolding (Carpenter et al., 1988) is a matter for further study. To this end, however, it should be noted that 60% polydextrose solutions had a much higher viscosity (results not shown) than 60% solutions of lactitol, glucose syrup, or sucrose and sorbitol at chilled temperatures.

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