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# Studies on protein denaturation of frozen fish--comparison between super rapid freezing by liquid nitrogen (-196°C) and air (-20°C) freezing

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# Studies on Protein Denaturation of Frozen Fish*
## Comparison between Super Rapid Freezing by Liquid Nitrogen (-196°C) and Air (-20°C) Freezing

Taneko Suzuki, Koichi Kanna and Takeo Tanaka**

Translated by Makoto Inaba, Ph.D.

## SUMMARY (copied from the original text)

1 The effects of freezing

With the view of the denaturation of fish muscle protein, effect of super rapid freezing by immersing fish meat in liquid nitrogen was examined with a reference sample which had been frozen at -20°C. Sea bass, carp and Alaska pollack were frozen at temperatures of -196°C (liquid nitrogen) or -20°C (see Fig. 1) and the "myosins" (a mixture of actomyosin and myosin) extracted from the muscle were examined for their physico-chemical properties.

As seen in Table 3, no significant difference was observed in ultracentifugal characteristics, viscometric behavior, ATP-sensitivity and the amounts of salt extractable proteins between unfrozen muscle and muscle frozen in liquid nitrogen.

On the other hand, there were considerable differences in the characteristics of protein from unfrozen fish and from those frozen at -20°C. Increase of the amounts of the salt extractable proteins and dependence of the viscosity of myosins solution on the protein concentration and decrease of sedimentation constant ($S_{20}$) value of actomyosin were found in muscles frozen at air of -20°C (Fig. 2, 4(b), Table 1).

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2 The effects of cold storage

No distinct change occured in the characteristics of "myosins" in the fish muscle frozen in liquid nitrogen, even after being stored for 1 week at -20°C, however, appreciable changes took place in samples after storage period for longer than 3 weeks at this temperature (Fig. 7,8). When storage temperatures of -30°C or -30°C were employed, little changes in the ultracentrifugal properties were detected even after 11 weeks (Fig. 10).

On the other hand, proteins from muscle frozen and held at -20°C showed drastic changes in ultracentrifugal patterns and $S_{20}$ value of actomyosin after only 1 week's storage (Fig. 7,8).

Fine ice crystals were formed inside the muscle fiber when the tissue was frozen in liquid nitrogen and stored at -20°C for 3 weeks. There were pronounced differences in the microscopic structures of tissues frozen and sotred at -20°C for the same period (Plate 1).

These findings suggest that super rapid freezing by means of liquid nitrogen can control protein denaturation, if sufficiently low storage temperatures (e.g. below -30°C) are used subsequently.

## INTRODUCTION

Freezing of food at a low temperature of -196°C by salking in liquid nitrogen has been investigated on asparagus, cakes and pork[1]. Love[2], Heen[3], Weld[4] and Taylor[5] reported on denaturation of proteins by such low temperature freezing and storage. Some of these reports describe non-occurence of denaturation of meat proteins by freezing at the extremely low temperature and storage at the same temperature, while others reports unfavorable results when the storage was done at the extremely low temperature, and there has not been any agreeable conclusion.

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For the freezing with liquid nitrogen, two methods are usually applied; one is placing the food directly into liquid nitrogen and the other is spraying liquid nitrogn to the food. In this report, the former, placing the food into liquid nitrogen and freezing at -196°C, was applied. The food thus frozen was melted immediately after freezing, and the effect of freezing (melting) to the physical properties of myosin protein of fish muscle protein was studied (Experimental I), and also the optimal storage temperature of the liquid nitrogen frozen protein was investigated by comparing the degrees of denaturation of the muscle protein during the storage period (Experimental II). The authors also conducted a set of parallel experiments by freezing the protein at -20°C with air and the two sets of data were compared.

## Experimental I. The Effect of Freezing (Melting)

### EXPERIMENTAL METHOD

1. Material

As a live sample, Lateolabrax japonicus ("Seigo", sea bass) and Cyprinus Carpio ("Koi", carp) were used. Samples were prepared of these two fishes immediately after sacrificed and before rigor mortis, during rigor mortis, and after rigor mortis, and thus they represent three classes of freshness. Besides these, a sample was prepared using Theragra chalcogramma ("Suketodara", Alaska pollack) which is said to show remarkably large degree of protein denaturation. The Alaska pollack has been fished off the coast of Abashiri area in Hokkaido, and stored frozen in ice for 1 week, and therefore its freshness is the third class or after rigor mortis.

2. Method of Freezing

The sample was prepared in a piece of about 20 g of muscle protein with 1 cm of thickness, 4 cm of width and 5 cm of length. A thermocouple was inserted into the sample and the sample was pulled out of liquid nitrogen when the temperature of the sample became -196°, and the other sample was pulled out of freezer adjusted to -20°C when the temperature of the muscle protein was -19°C. The freezing curves of the samples of different fishes were nearly identical, and therefore only the results of sea bass are shown in the Fig. 1. As shown in the figure, it took only 40 seconds (Approximately 5 seconds to pass the so-called maximum ice-crystal zone) for the temperature of the center of the sample to reach at -196°C, but it took about 100 minutes (or 38 minutes to pass the ice-crystal zone) to reach at -19°C when the freezing was done by air at -20°C.

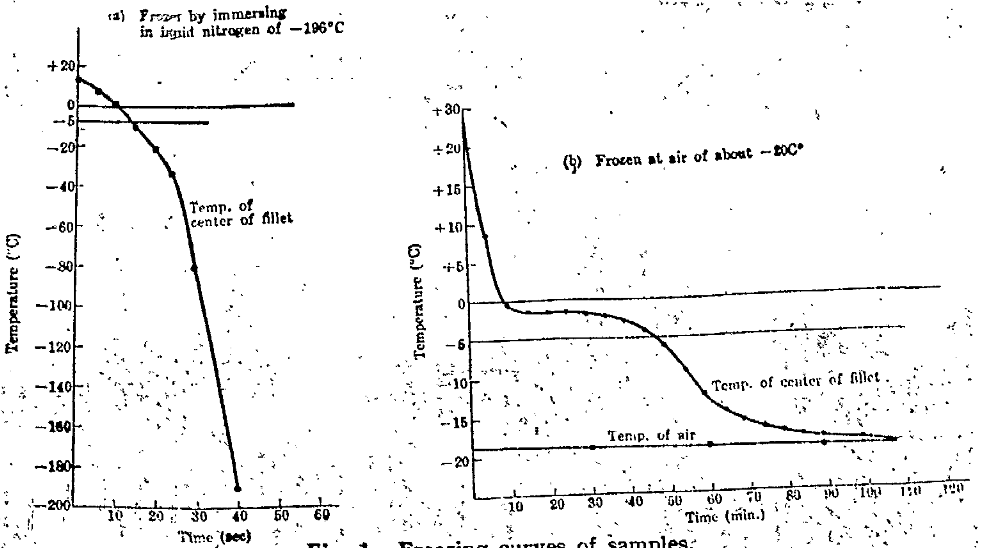

**Fig. 1.** Freezing curves of samples.
Size of fillets used for samples: ***high*** about 1 cm, ***width*** about 4 cm, ***length*** about 5 cm, ***weight*** about 20 g.

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3. Method of Melting

To a frozen sample, about 20 volumes of an extractor to extract salt extractable protein, 0.6M KCl solution, was added, and the sample was melted in a refrigerator (at 2 - 5°C) in about 1 hour. If the frozen sample by liquid nitrogen method was stirred with 0.6M KCl solution in a blender to extract the proteins, the mixture foams up considerably. Therefore, the frozen protein samples, frozen with liquid nitrogen as well as with −20°C air, were melted first in a refrigerator and then the following extraction procedures were applied.

4. Determination of the Degree of Denaturation of the Protein

(1) Salt Extractable Protein.......The protein sample after melting together with the 0.6M KCl solution that was used for melting the muscle protein was extracted by mixing with a blender for 1.5 minutes and centrifuged for 20 minutes at 7000 r.p.m. The clear supernatant thus obtained was subjected to a biurett method6) of determination of protein concentration. This protein is salt extractable protein.

(2) Viscosity.......Using the salt extractable protein solution described in (1), relative viscosity at various protein concentration at 20°C was determined with an Ostwald viscometer that has more than 500 of average velocity gradient. The restored viscosity and specific viscosity were calculated according to the following equations.

$$\eta_r = \frac{t_s}{t_o}$$

$$\eta_{sp} = \eta_r - 1$$

$$[\eta_{sp}/c]_{c\to o} = [\eta]_{c\to o}$$

where $\eta_r$......relative viscosity

$t_s$......running down time of protein solution

$t_o$......running down time of solvent

$\eta_{sp}/c$......restored (or reduced) viscosity

(3) ATP-Sensitivity.......Sodium salt of ATP was added to a salt extractable protein solution containing about 1 mg of protein nitrogen per 1 cc to the extent that the final concentration of the added ATP is $10^{-3}$ M. The relative viscosity before and after addition of ATP was determined.

(4) Sedimentation Diagram and Sedimentation Constant.......A protein solution with a known concentration (about 1 mg of protein nitrogen per 1 cc of solution) was centrifuged at 20°C, 51090 r.p.m., using a Hitachi analytical ultracentrifuge apparatus (Model UCA-I). Feature of the sedimentation was measured for about 60 minutes at 5 minute interval. The sedimentation constant was shown by Svedberg unit [S], and in this report $S_{20}$ was used as a sedimentation constant of a solution at 20°C.

## EXPERIMENTAL RESULTS

From the many data obtained on each fish species or on the same species (sea bass or carp) and also on each sample of various different degree of freshness, only one of those that show the same data is given as a representative result. However, if there is any marked difference depending on the degree of freshness, the result is shown in the following tables and figures.

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1. The Amount of Salt Extractable Protein.......Table 1 shows the experimental result on the amounts of salt extractable protein of -196°C freezing* and -20°C freezing*. Namely the amount of the protein does not change before and after freezing at -196°C,

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* Correctly freezing and melting. Hereafter they are abbreviated as freezing.

Table 1. Amounts of salt extractable protein of muscle before and after freezing.

| Sample fish | Salt extractable protein nitrogen* | | Sample fish | Salt extractable protein nitrogen* | |
|---|---|---|---|---|---|
| | Before freezing | After freezing at −196°C | | Before freezing | After freezing at −20°C |
| Sea bass (1) | | | Sea bass (2) | | |
| *pre-rigor* | 28.9 | 27.9 | *pre-rigor* | 22.0 | 25.3 |
| *full-rigor* | 23.9 | 23.5 | *full-rigor* | 23.2 | 29.0 |
| Sea bass (5)<br>*full-rigor* | 18.1 | 20.1 | Sea bass (3)<br>*full-rigor* | 27.6 | 29.2 |
| Sea bass (4)<br>*post-rigor* | 23.2 | 24.4 | Sea bass (6)<br>*full-rigor* | 27.6 | 28.4 |
| Carp (1)<br>*full-rigor* | 27.6 | 27.6 | Sea bass (4)<br>*post-rigor* | 23.2 | 24.4 |
| Carp (2)<br>*post-rigor* | 26.0 | 26.4 | Carp (4)<br>*post-rigor* | 26.0 | 32.4 |
| Alaska pollack (1)<br>*post-rigor* | 21.4 | 23.8 | Alaska pollack (1)<br>*post-rigor* | 18.8 | 25.6 |

( ) Sample number

* Protein N mg per 1 g muscle

although there a few exceptions. However, there is a marked tendency that the protein amount increases after freezing in case the freezing was carried out at -20°C. At the same time it is seen in the table that the amount of the increase in case of the -20°C freezing is larger than the amount of the exceptionally observed increase by the -196° freezing.

2. Viscosity.......As seen in the Fig. 2 the viscosity of salt extractable protein obtained from the protein sample frozen at -196°C is not different from that determined on the same sample before freezing. Namely the specific viscosity $[\eta]$ is nearly the same before and after the freezing, and also there was no change in the relationship between the restored (reduced) viscosity ($\eta_{sp}/c$) and the concentration of the protein. On the contrary, although there was no difference in its specific viscosity by a protein sample frozen at -20°C, the concentration dependency of the restored (reduced) viscosity increased by the frozen sample than that of unfrozen sample.

3. ATP-Sensitivity.......As seen in the Fig. 3, degree of decrease of viscosity ?? addition of ATP and of its restoration, was not different from that of unfrozen sample. There was not any difference on this degree depending on the freezing method. The slight difference of the initial relative viscosity at the starting point depending on the freezing methods (?) due to the difference of the concentration of the protein in the samples used for the determination.

Fig. 2

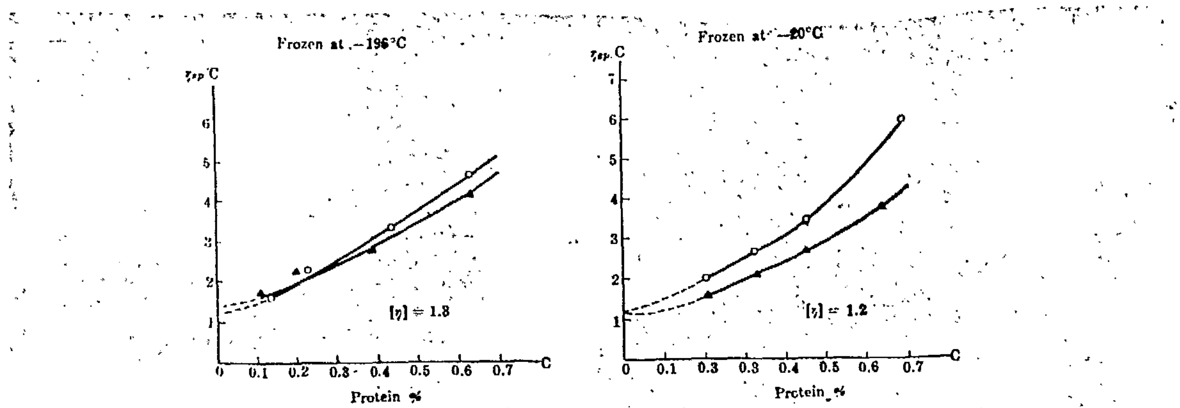

**Fig. 2.** Viscosity of salt extractable protein from sea bass muscle (*full-rigor*) before ard after freezing.
▲—▲ Before freezing  ○—○ After freezing at -196°C or -20°C

Fig. 3

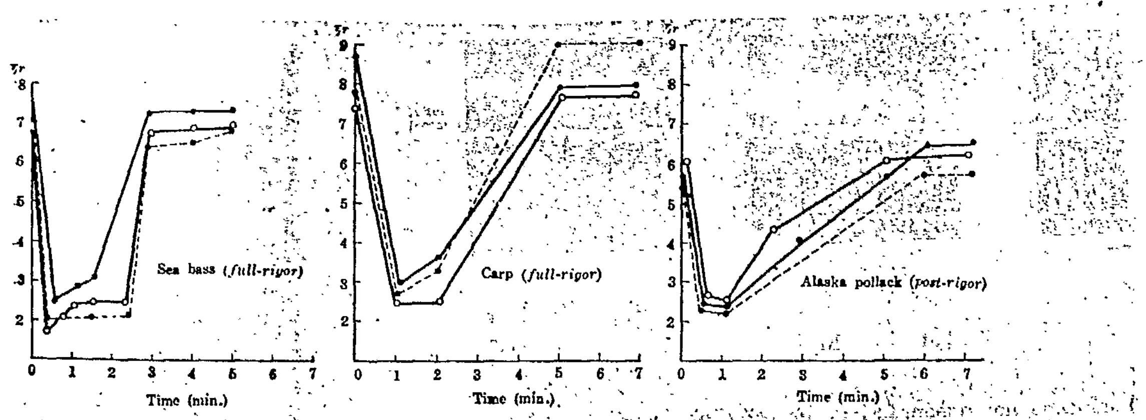

**Fig. 3.** ATP-sensitivity of salt extractable protein from fish muscle before and after freezing.
○—○ Before freezing
●—● After freezing at -196°C
●--● 〃 at -20°C

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4. Sedimentation Diagram and Sedimentation Constant ($S_{20}$).......The results are shown in the Fig. 4. The (a) of the figure shows the sedimentation diagram and the $S_{20}$ of each component of the muscle protein of sea bass, carp and Alaska pollack frozen at -196°C. These sedimentation diagrams do not show any marked difference before and after freezing. Each sedimentation diagram has 5 ~ 6 peaks (3 for carp), and $S_{20}$ value calculated on the peak 1, 2 and 4, 5* (peak 1 and 3 for carp) suggests that the peak 1, 2 (peak 1 for carp) is

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* Although the distinction between the peak 4 and 5 is not clear by the diagram shown in the text, it becomes clear after rotation for more than 30 minutes.

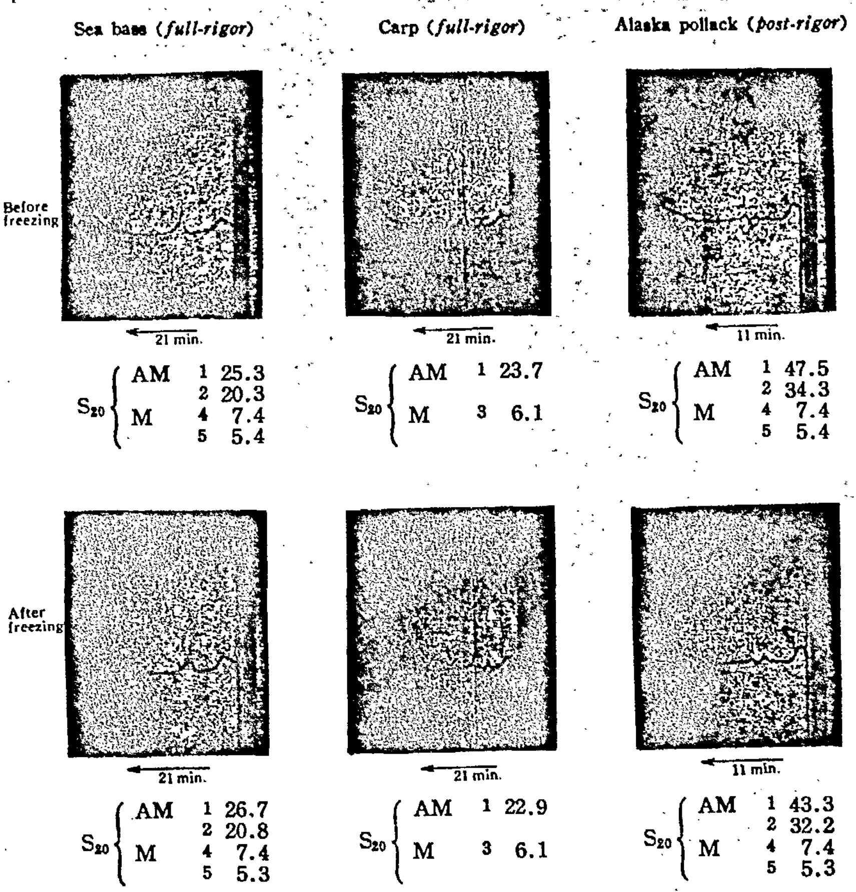

**Fig. 4 (a).** Sedimentation diagram and sedimentation constant ($S_{20}$) of salt extractable protein from fish muscle before and after freezing at −196°C.

AM…actomyosin M…myosin

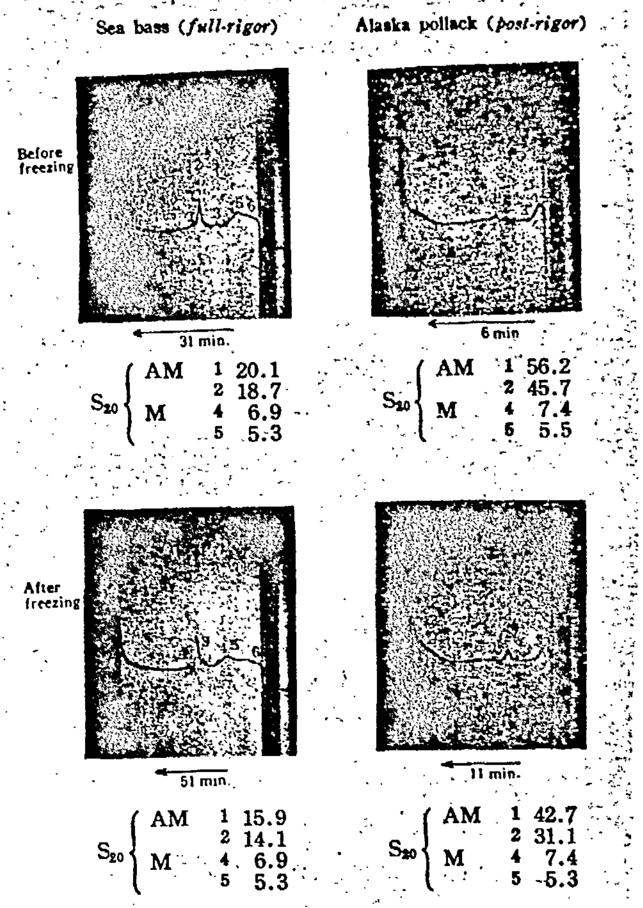

**Fig. 4 (b).** Sedimentation diagram and sedimentation constant ($S_{20}$) of salt extractable protein from fish muscle befare and after freezing at −20°C.

AM…actomyosin M…myosin

consisted of actomyosin and the peak 4, 5 (peak 3 for carp) is consisted of myosin, as seen in the Fig. 4. The (b) shows sedimentation diagram and $S_{20}$ value of sea bass and Alaska pollack before and after freezing at -20°C. These diagrams are different before and after freezing. As shown in the (a), the (b) has six peaks by sea bass and five peaks by Alaska Pollack. Calculation of $S_{20}$ on each peak indicated that the peak 1, 2, which was considered to be mainly consisted of actomyosin, had a tendency to decrease after freezing at -20°C. However the $S_{20}$ of myosin is stable and does not change by freezing. The peak 3 (2 for carp) of the sedimentation diagrams of the both group (a) and (b) of the Fig. 4 had about 10 of $S_{20}$ value and it contained a component that does not change at all by freezing.

## Experimental II. The Denaturation of the Protein During Storage under Freezing

### § Storage under Freezing at -20$^{\circ}$C

### 1. Denaturation of Protein; Experimental Method and Results

The material for experiment, method of freezing and determination method of the degree of denaturation of protein are the same as described in the Experimental I.

The storage method was consisted of the rapping the frozen sample with aluminum foil, placing in a tin can, and storing at -20$^{\circ}$C in a freezer. The storage period is 35 weeks at the maximum and the sample was taken out at a certain interval of storage period.

(1) Salt Extractable Protein.......As shown in the Table 2, storage of sea bass muscle protein at -20$^{\circ}$C for 11 weeks showed a tendency that the salt extractable protein became less soluble (or decrease of the amount of the salt extractable protein*) in both cases of the freezing at -196$^{\circ}$C and at -20$^{\circ}$C, and the degree of this transformation was nearly identical by both freezing methods. The results of determination by Alaska pollack muscle protein are shown in the Fig. 5, and the decrease of the amount of the salt extractable protein was observed in this case. But the amount of its decrease was less for the protein sample frozen at -196$^{\circ}$C.

**Table 2.** Amounts ot salt extractable protein from sea bass muscle frozen at −196°C or −20°C and stored at −20°C for 11 weeks.

Tabl

| Condition of sample fish | Temperature of freezing | Salt extractable protein nitrogen* | |
|---|---|---|---|
| | | After freezing | After storing at −20°C for 11 weeks |
| *Pre-rigor* | −196°C | 26.4 | 20.1 |
| | − 20°C | 26.4 | 20.0 |
| *Full-rigor* | −196°C | 21.0 | 17.4 |
| | − 20°C | 22.0 | 17.1 |
| *Post-rigor* | −196°C | 24.0 | 19.6 |
| | − 20°C | 26.0 | 18.9 |

* Protein N mg per 1 g muscle

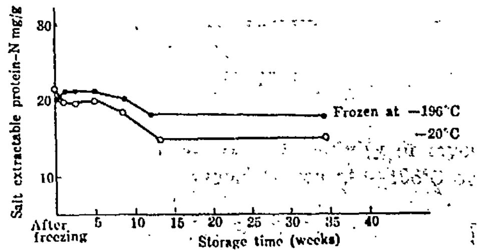

**Fig. 5.** Salt extractable protein from Alaska pollack muscle (*post-rigor*) frozen at −196°C or −20°C and stored at −20°C.

(2) ATP-Sensitivity.......The results of the determination of frozen Alaska pollack after storage for 1, 3, 5 and 8 week periods

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* Added by the translator.

are shown in the Fig. 6. Addition of ATP decreased the viscosity, (I and II of the Fig.), on any samples of different storage periods, and the amount of the decrease is not changed by changing the period of storage. The once lowered viscosity was restored and elevated to the original viscosity in either sample of the different storage periods (III and IV of the Fig.). However, the period between the stage II and III is affected by the period of the storage, and the longer period of storage necessitated the longer phase of this change. This indicates that the activity of the ATPase is deteriorated. The same results were seen by sea bass muscle protein samples.

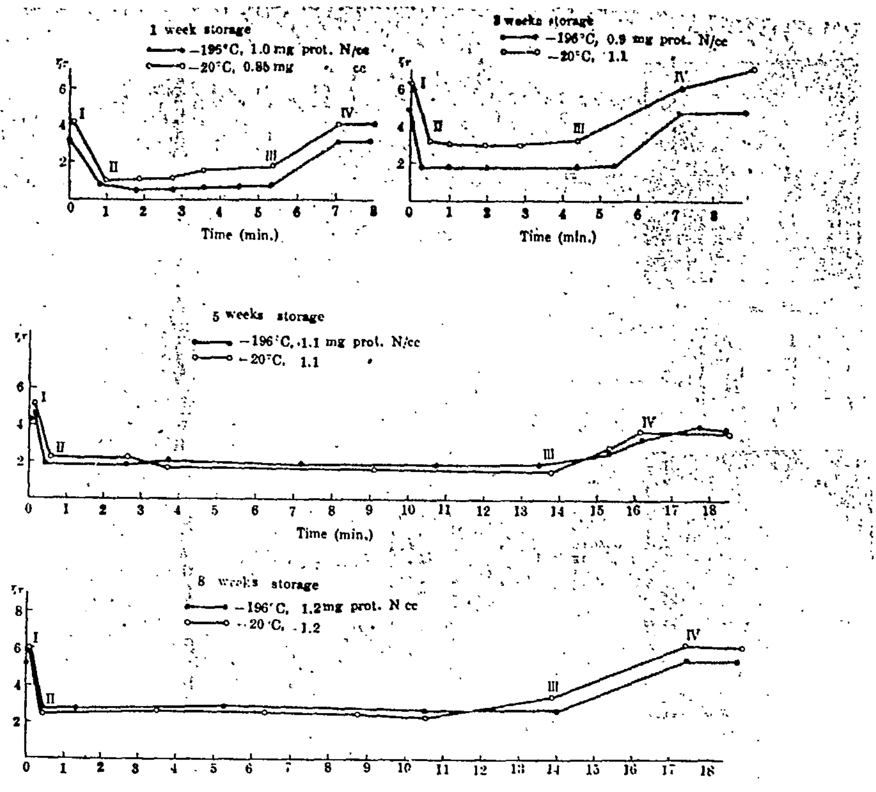

Fig. 6. ATP-sensitivity of myosins fraction of Alaska pollack muscle (*post-rigor*) frozen at −196°C or −20°C and stored at −20°C.

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(3) Sedimentation Diagrams and the $S_{20}$ Value.......The results are shown of sea bass in the Fig. 7 and of Alaska pollack in the Fig. 8. The Figs. 7 and 8 show that there is no marked difference of the sedimentation diagram and the $S_{20}$ value of each component after the sample was frozen and the values determined immediately after the freezing or after the same frozen sample was stored at -20°C for 1 week. However, after storage for more than three weeks, the

sedimentation diagrams change considerably and also the $S_{20}$ value of the actomyosin increases comparing to those determined immediately after freezing (Fig. 7 and 8). On the other hand, if the protein sample is frozen at -20°C, only after 1 week of storage under freezing, the sedimentation diagram changes and the $S_{20}$ of actomyosin also changes considerably comparing with the values taken immediately after freezing. However the $S_{20}$ value of myosin did not show any change throughout the storage period regardless the freezing methods, -196°C or -20°C.

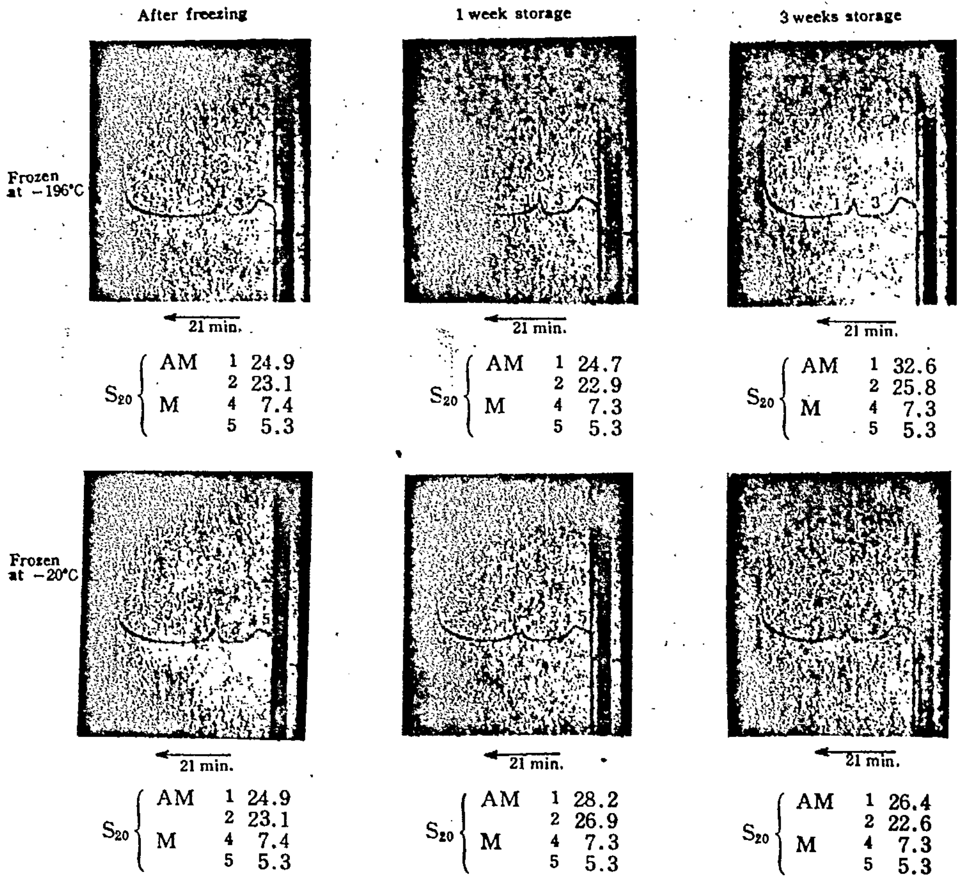

Fig. 7

**Fig. 7.** Changes of sedimentation diagram and sedimentation constant ($S_{20}$) of salt extractable protein from sea bass muscle (*full-rigor*) frozen at −196°C or −20°C and stored at −20°C.

## 2. The Change of Muscle Tissue; Experimental Method and Results

Among the sample fish species, the muscle protein of Alaska pollack which showed marked difference of tissue arrangements by examination with unaided eyes depending on the freezing methods was examined visually and also microscopically using samples stored under freezing for three weeks. The samples for examinations were taken from the frozen sample in order to investigate the presence of ice crystals.

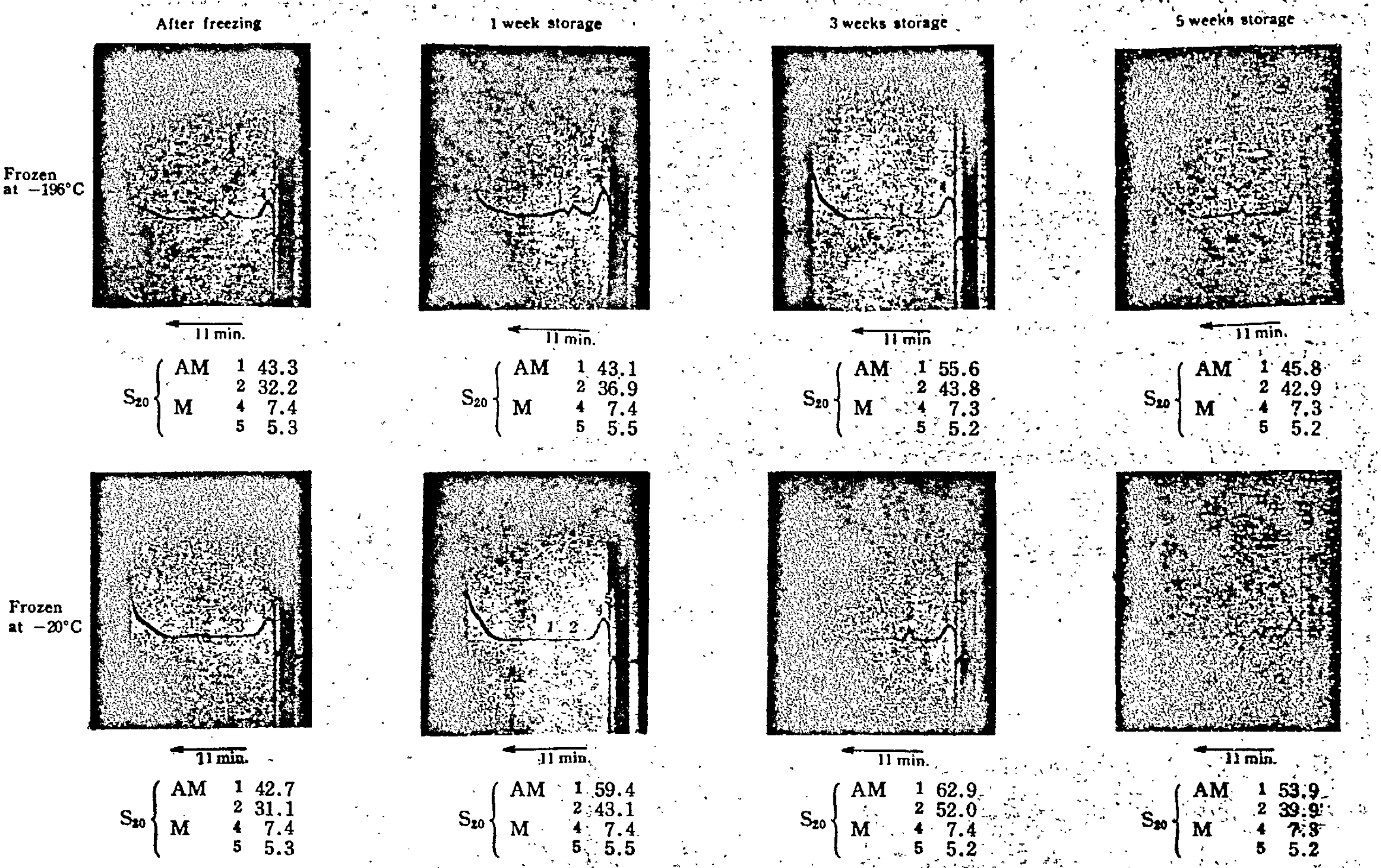

**Fig. 8.** Changes of sedimentation diagram and sedimentation constant ($S_{20}$) of salt extractable protein from Alaska pollack muscle (*post-rigor*) frozen at −196°C or −20°C and stored at −20°C.

Fig. 8

(1) Examination with Unaided Eyes.......Following the method reported previously by one of the authors, Tanaka[7)], on the freeze storage of cod fish, the feature of the presence of ice on a cross section (perpendicular to the direction of the muscle fibre) of the frozen protein sample was mainly examined. The sample frozen at -196°C did not show any visible ice crystals that are separated from the tissue. The sample showed nearly white color perhaps because the ice crystals are dispersed homogeneously throughout the muscle tissue. On the other hand, the sample frozen at -20°C showed, as observed previously by Tanaka[7)], separation of ice crystals and growing of the ice crystals (as a result; presence of small particles of ice), and the sample already started to form a sponge[8)]. The color of the sample was rather dark.

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(2) Observation under Microscope.......The slides for microscopical observations were prepared following the previously reported easy method7). Namely, 4 or 5 tissue pieces with the size of about 0.5 × 0.5 × 1 cm were taken from the back part of each frozen fish sample, and while they were frozen, they were treated with Bouin's solution and washed with running water and gelatinated. The gelatinated samples were sliced to slices of 10 - $15\mu$ of thickness with a Sartorius type frozen-microtome, and dyed with eosin and sealed with Apáthy rubber syrup. The cutting surface by the microtome was prepared only at cross section. The same sample pieces were prepared from the sample before freezing. The sample slides thus prepared, 40 - 50 pieces each, were examined under a microscope at 30 - 400 magnification.

The results are shown in the Plate 1. The sample frozen at -196°C ((B) of the Plate) show nearly identical arrangement of the muscle fibre, interval of the fibre, and size of the individual fibre as those of the fresh sample (A), but the (B) had a large number of extremely small holes or small cracks which were thought to be remains of the ice crystals. The holes and cracks were homogeneously dispersed throughout the muscle fibres and the size of the small holes is about 0.5 - $5\mu$ in its diameter and the size of the cracks is about 5 - $15\mu$ long by determination with a microtome. These holes and cracks were smaller than the "rosette" shape ice crystals observed by Love9) in muscle fibre of pre-rigor Cod frozen within 6.5 minutes. The shapes of the author's samples and those of the cod fish sample by Love are also different. Presence of such ice crystals indicates that the muscle fibre membrane was intact and that there occured a typical intracellular freezing. On the contrary, the frozen sample at -20°C (C) showed remains of large ice crystals outside of muscle fibres, and thus there was so-called sponge formation at the frozen state. These ice crystals were, as will be described later in the part of Discussion, formed by sublimation of the ice within the muscle fibre to the outside of the fibre without breaking the muscle fibre membrane (cf. the photograph of the (C) at ×300 magnification), during the storage period. As a result, each muscle fibre became generally thinner comparing to those of fresh protein, and the shape of the individual fibre lost its round feature and became uneven. These tissue images are nearly identical with those of the corresponding samples treated under the same conditions by Tanaka7).

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## § Storage at the Temperature other than -20$^{\circ}$C

The aforementioned experiments showed that if -196$^{\circ}$C frozen sample is stored at -20$^{\circ}$C for less than 1 week period, the nature of the protein is nearly the same as that of the sample before freezing, and that if the frozen sample, frozen at either -196$^{\circ}$C or -20$^{\circ}$C, is stored at -20$^{\circ}$C for more than 1 week period, its protein is subjected to denaturation to some degree and its degree of the denaturation is nearly the same by both freezing methods. Therefore the authors carried out the following experiment to investigate if there is any advantage in lowering the storage temperature than -20$^{\circ}$C to retain the various merits of extremely low temperature freezing method at -196$^{\circ}$.

## EXPERIMENTAL METHODS AND THE RESULTS

As a sample, carp muscle protein in full-rigor was used, and the freezing methods and others are the same as the experimental I. The sample wrapped in aluminum foil was placed in a tin can and stored in an ice-chamber (-8 - -10$^{\circ}$C) or in a freezer at -30$^{\circ}$C or -80$^{\circ}$C. The samples were taken out of storage after 1, 3, 6, and 11 weeks, and determination of salt extractable protein and sedimentation were conducted. The results are as follows.

(1) Salt Extractable Protein.......As seen in the Fig. 9, regardless the storage temperature, the amount of the salt extractable protein increased during the initial period of the storage, and during the subsequent storage period, the amount of the protein did not decrease much at the lower temperature of storage (-80$^{\circ}$C and -30$^{\circ}$C) but the decrease of the salt extractable protein was remarkable at the higher storage temperature (-8 - -10$^{\circ}$C).

(2) Sedimentation Diagram and the $S_{20}$ Value.......As seen in the Fig. 10, both the sedimentation diagram and the $S_{20}$ value of each component of the samples stored at -30$^{\circ}$C and -80$^{\circ}$C for 11 weeks were nearly identical with those of the sample immediately after the freezing. Comparison of the $S_{20}$ values of actomyosin of the samples stored for 11 weeks at -80$^{\circ}$C and at -30$^{\circ}$C showed that the one stored at -30$^{\circ}$C had a slightly larger $S_{20}$ value. However, the samples stored at -8 - -10$^{\circ}$C showed a remarkably different sedimentation diagram

Fig. 9

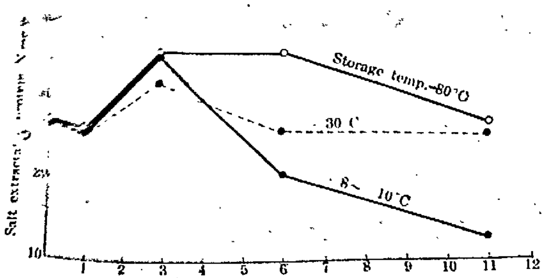


**Fig. 9.** Amounts of salt extractable protein of carp muscle (*full-rigor*) frozen at −196°C and stored at various temperatures.

after storage of three weeks and the $S_{20}$ value of the actomyosin became larger when the sample is stored longer. The $S_{20}$ value of myosin did not change after storage at any temperature.

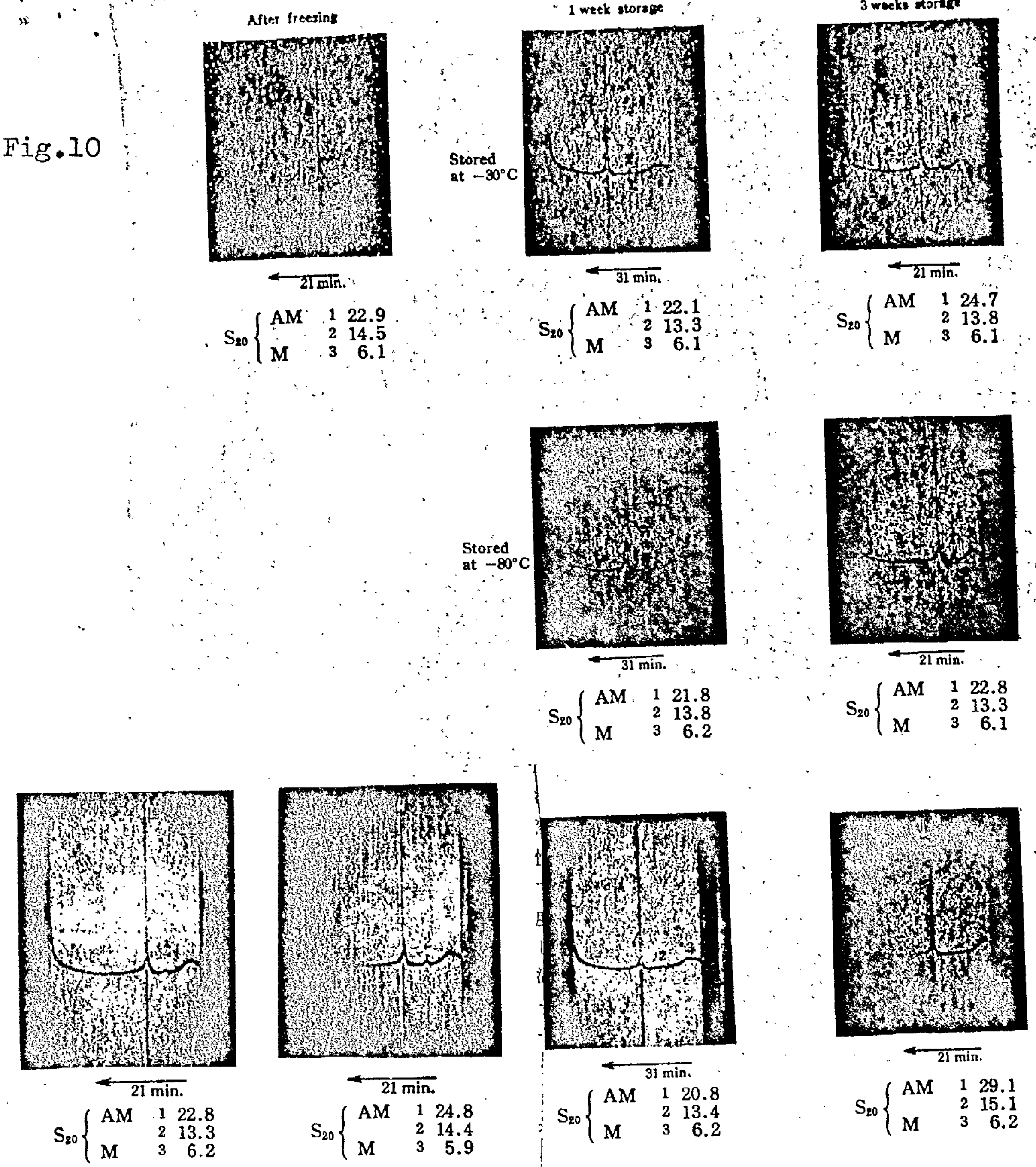
## DISCUSSION

### I Effect of Freezing (Melting)

The protein samples frozen at -196°C by salking in liquid nitrogen and at -20°C in a freezer were melted immediately and their myosin proteins were extracted and the physico-chemical properties of the proteins were compared with those of the sample before freezing. The results of this set of experiments I are summarized in the Table 3. As seen in the table, none of the physico-chemical characteristics of the myosin proteins of the sample frozen rapidly with liquid nitrogen was different from that of unfrozen sample. Therefore such rapid freezing protein does not cause denaturation of protein by freezing (melting). On the contrary, freezing at -20°C caused the lowered $S_{20}$ value of actomyosin after freezing and the concentration dependency of the restored viscosity of the salt extractable protein became larger after freezing, although there was no change in specific viscosity of the salt extractable protein, ATP-sensitivity and the $S_{20}$ value (sedimentation constant) of myosin before and after freezing (melting).

Hanafusa(10) determined the denaturation of actomyosin gel of rabbit after freezing and melting, by measuring the viscosity, ATPase activity, and sedimentation constant, and found that the denaturation was smaller if the freezing velocity was larger at the constant, final freezing temperature but the different freezing velocity, and

Fig.10



After freezing

21 min.

$S_{20}$ AM 1 22.9
2 14.5
M 3 6.1

Stored at −30°C

1 week storage

31 min.

$S_{20}$ AM 1 22.1
2 13.3
M 3 6.1

3 weeks storage

21 min.

$S_{20}$ AM 1 24.7
2 13.8
M 3 6.1

Stored at −80°C

31 min.

$S_{20}$ AM 1 21.8
2 13.8
M 3 6.2

21 min.

$S_{20}$ AM 1 22.8
2 13.3
M 3 6.1

21 min.

$S_{20}$ AM 1 22.8
2 13.3
M 3 6.2

21 min.

$S_{20}$ AM 1 24.8
2 14.4
M 3 5.9

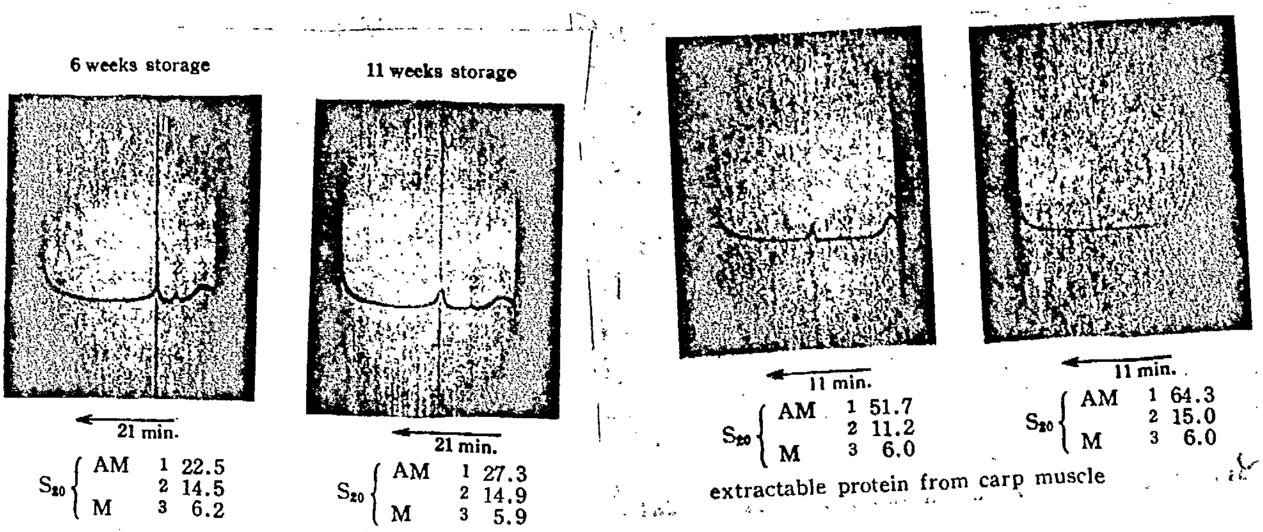
31 min.

$S_{20}$ AM 1 20.8
2 13.4
M 3 6.2

21 min.

$S_{20}$ AM 1 29.1
2 15.1
M 3 6.2

**Fig. 10.** Changes of sedimentation diagram and sedimentation constant ($S_{20}$) of salt (*full-rigor*) frozen at −196°C and stored at various temperatures.



6 weeks storage

21 min.

$S_{20}$ AM 1 22.5
2 14.5
M 3 6.2

11 weeks storage

21 min.

$S_{20}$ AM 1 27.3
2 14.9
M 3 5.9

11 min.

$S_{20}$ AM 1 51.7
2 11.2
M 3 6.0

11 min.

$S_{20}$ AM 1 64.3
2 15.0
M 3 6.0

extractable protein from carp muscle

that the denaturation was larger if the final freezing temperature was lower at the same freezing velocity but different final freezing temperature. However, the present authors' experiments showed that freezing at the extremely low temperature -196°C gave the smaller denaturation than that at -20°C. On the other hand, as shown by the curve of Fig. 1 freezing velocity of the liquid nitrogen freezing is only 5 seconds from 0 to -5°C while that of the -20°C freezing is about 38 minutes. Therefore the authors consider that the effect of the freezing velocity on the denaturation of protein is much larger than the final freezing temperature. Since the present authors experimented on the protein solution prepared by extracting the protein sample with KCl and they did not isolate actomyosin. Therefore the changes of the nature of the actomyosin - for example, the decreased $S_{20}$ value, and the increased concentration dependency of viscosity - by freezing at -20°C are not sufficient clues to develop the discussion on the change of the actomyosin molecules, but it is unmistakenly true that the actomyosin of the muscle were subjected to some change by freezing at -20°C. The reason why the amount of the salt extractable protein by extraction of the -20°C frozen sample with 0.6M KCl increases slightly after freezing is not clear at this stage. These problems remain to be studied in future.

**Table 3.** Comparison of frozen and unfrozen muscle.

|  | Items of determination | Comparison with unfrozen muscle |  |
|---|---|---|---|
|  |  | Frozen at −196°C | Frozan at −20°C |
| Physico-chemical characteristics of "myosins" from frozen muscle | Sedimentation constant ($S_{20}$) of actomyosin and myosin | Actomyosin and myosin: no change | Actomyosin: smaller than unfrozen one.<br>Myosin: no change |
|  | [$\eta$] | no change | no change |
|  | Relation between $\eta_{sp}/c$ and $c$ | do | concentration dependence of $\eta_{sp}/c$ became high |
|  | ATP-sensitivity | do | no change |
| Amounts of 0.6 M KCl extractable proteins |  | do | increased |
| Organoleptical test of frozen muscle |  | white color and fine texture | dark color and icy texture |

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In recent years,[11] the denaturation of protein is considered to be the result of cleavage of the protein hydrophobic bond due to the structural change of water of hydration. Therefore, if the freezing velocity is small, crystallization of solvent water in the process of freezing causes change of the bonding and the orientation of the hydration water by interaction of the ice crystals and water of hydration. If the freezing velocity is enormously large, the ice crystals may rapidly take the same structure as the hydration water without causing the mutual interaction between the ice crystals and hydration water. As a result, the protein denaturation does not occur. Perhaps we need study the change of the structures of water by freezing, using some physical methods such as proton magnetic resonance spectrum or dielectric constant measurement which may clear the nature and the extent of the change of the protein structures.

## II Denaturation of the Protein during Storage under Freezing

### 1. Denaturation of Protein

At the industrial view points, the quality of the protein stored for a certain period after being frozen with liquid nitrogen is very important. Therefore we must study the denaturation of the fish muscle protein during this period. There was practically no denaturation when sea bass and Alaska pollack were stored at -20°C after being frozen at -196°C, comparing to the denaturation of the same fish protein samples when they were stored at -20°C for 1 week, but, when they were stored at -20°C for more than three weeks after being frozen at -196°, there was about the same extent of denaturation as they were frozen at -20°C(cf. Fig. 7 and 8). There was no change in the ATP-sensitivity of the salt extractable protein regardless of the storage period after being frozen at -196°C or at -20°C. However, there was a weakened ATPase activity by prolonged storage, as shown by the elongated phase length between II and III of the Fig. 6. Connell[12)] reported that the ATPase decreased as much as 55% after storage of cod fish sample at -22°C or at -14°C for 140 - 180 weeks although it did not decrease to the recognizable extent after storage at -29° for the same period. He also reported that the decrease of the ATPase activity was not shown as sharply as the decrease of salt extractable mysoin prtein. In the authors' experiment, by any of the freezing methods that they applied, the storage at -20°C for periods between 5 and 8 weeks decreased the ATPase activity.

The decrease of the amount of the salt extractable protein of sea bass and Alaska pllack does not represent the denaturation tendency by freezing and during storage, but the $S_{20}$ value, viscosity and ATP-sensitivity showed the denaturation clearly. It appears that the reason is because these latter determination values, $S_{20}$, viscosity and ATP-sensitivity, are the results of microscopic observation of the protein denaturation, while the former, extraction of the salt extractable protein, is relatively macroscopic observation. Therefore, even though there was no marked difference in the amount of salt extractable protein, the segimentation diagrams, the $S_{20}$ values, viscosity values and the ATP-sensitivity values show some difference. Love and others13)14)15) measured the degree of the denaturation of protein during storage by the decrease of the soluble protein of the samples. According to the authors' experimental results, the decrease of the soluble protein of stored sample after -20°C freezing was not quite as remarkable as recorded in the afore-cited references. Perhaps this difference occured because of the different size of protein sample frozen, and, as shown by the freezing curve of the Fig. 1, the freezing velocity under the authors' experimental conditions is within the category of the so-called rapid freezing, and thus the decrease of soluble protein during storage took place relatively slow.

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In the storage experiment of carp fish, frozen at -196°C, at other than -20°C, the sample stored at -8 - -10°C showed a remarkable decrease of salt extractable protein, change of sedimentation diagram and a large increase of the $S_{20}$ value of actomyosin. On the contrary, there was practically no change in these characteristics when the same frozen sample was stored at -30°C or -80°C even after 11 weeks of storage. Therefore the authors consider that the storage temperature of the -196°C frozen fishes must be lower than -30°C in order to avoid the protein denaturation.

## 2. Relationship Between the Change of Muscle Tissue and Protein Denaturation

According to the microscopical observation (Plate I) of the protein sample of Alaska pollack stored at -20°C for 3 weeks, the sample frozen at -196°C had extremely small ice crystals dispersed homogeneously within the muscle fibres but the one frozen at -20°C had rather large ice crystals even outside of the muscle fibres. The difference of the size of ice crystals and the mode of their distribution resulted in the difference in their outlook, namely white color and fine texture of the former and dark color and icy texture of the latter. Previously Tanaka[7)] reported that there was no ice formation outside of the muscle fibres when fillet of Alaska pollack was frozen by air blast within 25 minutes (the period in which the temperature at the center of the fillet passed 0° to 5°C), but there was gradual formation of ice outside of the muscle fibres when the same sample was stored at -20°C for 50 days. Considering this experimental results, the -20°C frozen sample of the present experiment probably did not have ice formed outside of the muscle fibres, i.e., only intracellular freezing, immediately after freezing, since the freezing velocity was 38 minutes for the center temperature to pass 0° to 5°C (cf. Fig. 1(b)). Later during the storage period of 3 weeks, the ice moved from inside of the muscle fibre to outside of the muscle fibre by sublimation and consequently rather large pieces of ice crystals were formed outside of the muscle fibre, as shown in the Plate I(c). On the other hand, the sample frozen at -196°C but stored at the same temperature, -20°C, did not show any tendency of the moving of the ice to outside of the muscle fibres by sublimation. The present authors are unable to explain this difference because both freezings at -196°C and at -20°C are rapid freezing and there must have been intracellular freezing immediately after the samples werefrozen. It seems to be most reasonable to assume that the freezing velocity of the former, 5 seconds, was extremely rapid comparing to that, 38 minutes, of the latter, and consequently the state of the ice crystals formed was different in both frozen samples. Also, since the final freezing temperature -196°C by liquid nitrogen freezing must have restored as high as -20°C within the initial period of storage, perhaps the effect of the final freezing temperature is not very important regarding this problem.

Next the relationship of the tissue change and the protein denaturation will be discussed. According to Love's theory[2)] after experiments using cod fish, the protein denaturation occurs more easily if large ice crystals are formed continuously outside of the muscle fibres. One of the authors, Tanaka[16)], also suggested that the easier protein denaturation of Alaska pollack during the freeze storage than yellowtail is due to the freezing outside of the cells and the resulting acceleration of the aggregation of tissues. The protein denaturation in these reports are mainly based on the decrease of soluble, myosin proteins, and the results of our experiment on the change of the amount of soluble myosin proteins (Fig. 5) supported this. Namely, the decrease of the soluble, myosin protins was very small and slow when the sample was frozen at -196°C, i.e., typical intracellular freezing, and stored at -20°C, but the -20°C frozen sample showed remarkably larger decrease of the soluble protein than the -196°C frozen sample, perhaps because the ice moved outside of the fibre thus resulting in extracellular freezing during the process of freezing. The reason that the progress of the decrease is not as remarkable as those of Love and Tanaka has been already discussed in the part II.1 of the discussion.

On the other hand, the protein denaturation during the storage period, based on the determination of ATP-sensitivity, ultracentrifuge sedimentation diagram and $S_{20}$ value, is not related with the change of muscle tissue structure during the storage period, and this type of denaturation is completely different in its nature from that determined by decrease of the soluble, myosin protein. That is, this type of denaturation has taken place and progressed rapidly even for the -196° frozen sample that was frozen intracellularly and whose frozen state was maintained during the freezing. This fact indicates that the denaturation which takes place at the ultramicro stage of molecular unit progresses regardless the macroscopic transition of tissue structure which can be observed under a microscope. This is perhaps one of the most important problems to be studied in the future, especially because the factors that control the denaturation can be numerous. For example, water that perhaps exists as a bridge between the two types of protein denaturation and its role in the protein denaturation should be studied by nuclear magnetic resonance or dielectric constant determination on its various state of existance, i.e., freely movable liquid water, non-movable bound water by a polymer and various types of ice crystals. In any case, studies on the effect of the size and distribution of the ice crystals and, if their effect is significant, the mechanism of their role in the denaturation processes are perhaps the most important problem to be studied.

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## SUMMARY

Fish samples (carp, sea bass, Alaska pollack) were prepared in a size of 1 × 4 × 5 cm and salked in liquid nitrogen and rapidly frozen at $-196^{\circ}$ (Fig. 1(a)). As a control, the same samples were frozen in a freezer at $-20^{\circ}C$ (Fig. 1(b)). The frozen samples were melted, and physico-chemical characteristics of myosin proteins were investigated on both samples by determining the amount of salt extractable protein, viscosity, ATP-sensitivity, ultracentrifuge sedimentation diagram, and sedimentation constant, and these characteristics were compared.

After freezing, myosin proteins changed considerably by $-20^{\circ}C$ freezing, but those of $-196^{\circ}C$ frozen sample did not change (Table 3). However, when the $-196^{\circ}C$ frozen sample was stored at $-20^{\circ}C$, although the velocity of the denaturation of the myosin proteins was small comparing to that of $-20^{\circ}C$ frozen sample, the amount of the denaturation became nearly the same if the sample was stored for more than 3 weeks (Table 2, Fig. 5 - 8). But, if the storage temperature was lowered to $-30^{\circ}C$ or $-80^{\circ}C$ from $-20^{\circ}C$, the denaturation of myosin proteins did not occur even after 11 weeks of storage (Fig. 9, 10).

Microscopical observation of muscle protein sample of Alaska pollack, frozen at $-196^{\circ}C$ and at $-20^{\circ}C$, and stored at $-20^{\circ}C$ for 3 weeks, revealed presence of extremely small ice crystals inside of muscle fibre for the $-196^{\circ}C$ frozen sample but large ice crystals outside of muscle fibre for the $-20^{\circ}C$ frozen sample (Plate I).

The authors discussed the significance of the aforementioned experimental results.

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*Translator's Note: These may not be the same as their formal English title, if they have any.

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