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Denaturation of Enzyme Protein by Freeze-Thawing and Freeze-Drying

1. Freeze-thawing and freeze-drying of myosin and some other muscle proteins*

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Abstract

Changes in physico-chemical properties and in enzymatic activities of some muscle proteins by freeze-thawing or freeze-drying were measured for the purpose of clarifying the mechanism of protein denaturation by freeze-thawing and freeze-drying.

The results showed following: Freeze-thawing unfolded the conformation of the rod-like proteins and decreased their enzymatic activities. The two effects increased with the lowering of the final freezing temperature as well as with the slowing of the cooling rate. Freeze-drying unfolded the protein molecules more than freeze-thawing did. Some additives such as sugars or amino acids protected the protein against denaturation by freezing.

The effects of freeze-thawing seemed to depend on the shape of the protein molecule.

It was suggested that protein denaturation by freeze-thawing might be basically due to the crystallization of solvent water which loosened the hydrophobic bonds of the protein molecules and which broke the intramolecular hydrogen bonds in such a manner that the hydration shell of protein was disturbed.

1. Introduction

Freeze-thawing and freeze-drying have been widely used for the prepara-

* Contribution No. 1196 from the institute of Low Temperature Science
tion and preservation of various kinds of biological materials because both procedures are believed to be effective to maintain the biological activities of the materials over a long period of time. It is known, however, that under certain conditions of freeze-thawing or freeze-drying the biological activities suffer damage to a certain extent, or in other words, the materials are denatured.

Freeze-thawing and freeze-drying is fundamentally to change the state and the structure of water or to transfer water molecule.

On the one hand, denaturation in general seems to be caused by changes in the structure and state of solvent water, on which, as has been pointed out by NÉMETHY and SCHERAGA, BRANDT and many other authors, the function and structure of biological macromolecules heavily depend. Therefore, it may be considered that, in comparison with other chemical or physical denaturants, freeze-thawing and freeze-drying are such unique denaturants as change the structure and state of solvent water neither with chemical factors nor with high temperatures.

Recently, some studies have been reported on the denaturation of enzyme protein by freeze-thawing or freeze-drying. For instance, the reduction of enzymatic activities of myosin-ATPase and catalase by freeze-thawing was reported by SHIKAMA, and that of myosin-ATPase by freeze-drying by YASUI. But most of them have mainly dealt with changes in enzymatic activities and little inquiry has been made on change in protein conformation by freeze-thawing or freeze-drying.

Because of the importance role of water molecule to protein conformation, it is very worthy to investigate the relationship between protein conformation and changes of the state and the structure of water which caused by freeze-thawing and freeze-drying.

The investigation along this line will contribute in two respects: One is to ascertain the role of water plays in the structure and function of protein and the other is to establish fundamental evidence regarding the mechanism of freezing or drying injury of living organisms.

From this standpoint, examinations were made as to changes caused by freeze-thawing or freeze-drying in physicochemical properties thereof, such as viscosity, difference spectrum, optical rotatory dispersion and ultracentrifugal properties, which were conjectured to affect on enzymatic activity. The effects of some additives on denaturation were also examined.

In this paper, the results of experiments with myosin B, myosin, H- and L-meromyosin and actin will be reported. It was considered that these muscle proteins were adequate for the investigation of denaturation by fre-
ezing or drying, because they had some specific physico-chemical properties and their functions were closely related with their conformations.

II. Materials and Methods

Materials

Myosin B was prepared from rabbit skeletal muscle according to Szent-Györgyi's method with such modifications that the extract in KCl solution was precipitated by adjusting the KCl concentration to 0.3 M and the contaminated myosin was removed by centrifugation. The sediment was then resolved in 0.6 M KCl solution and again precipitated for purification. The purification procedure was repeated twice. Myosion B preparation was stored in 0.6 M KCl solution.

The isolation of myosin from rabbit skeletal muscle was made by Szent-Györgyi's method as modified by Mommaerts. Contaminated myosin B was removed by ultracentrifugation at 30000 rpm by adjusting of ionic strength to 0.28. The purification procedure was repeated three times. Myosin was then resolved in 0.5 M KCl solution.

H- and L-meromyosin, which were prepared from myosin by trypsin digestion, were purified according to Szent-Györgyi's method and stored in aqueous and 0.5 M KCl solution, respectively.

G-actin was prepared by Straub's method from acetone-dried muscle powder, from which myosin had been removed, and stored as an aqueous solution at neutral pH.

All of the protein solutions were stored at 5°C and used within two weeks after extraction.

Methods

For freeze-thawing, a glass tube of 16 mm in diameter containing 4 ml of protein solution was immersed in an alcohol or iso-pentane bath of a certain temperature cooled by dry ice or liquid nitrogen. Temperature of the solution was recorded by using a thermocouple inserted in the solution.

The rate of cooling was determined from the slope of the approximately linear portion of a cooling curve after it had passed the plateau, which represents a freezing point.

The freezing temperature and the rate of cooling were controlled by various procedures: Use of a double-jacket tube, stepwise addition of dry ice or liquid nitrogen to the bath, stepwise transfer of the tube to a number of baths which had been cooled to various temperatures, or direct immersion of the tube in liquid nitrogen. After being immersed in the final bath for
15 min, which was enough for the solution to reach the bath temperature and to freeze completely, the tube was transferred to the bath kept at 30°C for the rapid thawing.

For freeze-drying, 2 ml of the protein solution in a glass container of 20 mm in diameter and 50 mm in depth were frozen at a given temperature. The container was then transferred into liquid nitrogen for arrangement of the final temperature at which drying began and the solution was dried in 6 hours in a freeze-drying apparatus with an oil diffusion pump. The freeze-dried proteins were stored at 5°C before measurement.

Viscosity was measured at 26°C with an Ostwald-type or an Ubbelohde-type viscosimeter, whose flow time of water was 7 sec. and 120 sec. at the temperature respectively.

Ultracentrifugal analysis was made with a Spinco Model E or a Hitachi Model UCA-1 analytical ultracentrifuge.

Optical rotatory dispersion was measured with a Rudolph Model 200S photometric spectropolarimeter or a Shimadzu polarimeter attached to a Shimadzu Model QR-50 spectrophotometer. The concentration of the specimen was between 0.8 and 1%, and the length of the cell was 100 mm. Moffitt-Yang's equation\(^6\) was used to determine the parameter of helix contents of protein, \(b_0\) and \(a_0\), using 212 m\(\mu\) as the value of \(\lambda_0\).

Difference spectra were measured with a Cary Model 14 automatic recording spectrophotometer or a Hitachi-Perkin Elmer Model 139 spectrophotometer, using matched 10 mm quartz cells.

ATPase activities of myosin B, myosin and H-meromyosin were determined by measuring the amount of inorganic phosphate liberated from 1 mM ATP in the presence of 1 mM Ca\(^{2+}\) at 26°C. The reaction mixture was composed of 1 mM ATP, 1 mM CaCl\(_2\), 0.02 M tris-HCl buffer (pH 6.9) and 0.5 M KCl. The inorganic phosphate was measured by Fiske-Subbarow’s method.

The concentration of protein was determined by Biuret method corrected with micro-Kjeldahl method, using a factor of 6.

III. Results

FREEZE-THAWING.

Myosin B

Myosin B in 0.6 MKCl and 0.02 M tris-HCl buffer solution (pH 6.8) were frozen by direct immersion into the baths of \(-30°C\), \(-79°C\) and \(-196°C\), and thawed rapidly at 30°C. Neither turbidity nor precipitation was found
Table I. Effect of freeze-thawing on myosin B

<table>
<thead>
<tr>
<th>Freezing Temp.</th>
<th>Specific viscosity $\eta_{sp}$</th>
<th>ATPase Relative Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C (Cooling rate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.87</td>
<td>100</td>
</tr>
<tr>
<td>$-30^a$ (very slow)</td>
<td>0.48</td>
<td>42</td>
</tr>
<tr>
<td>$-79^b$ (very rapid)</td>
<td>0.58</td>
<td>58</td>
</tr>
<tr>
<td>$-196^c$</td>
<td>0.63</td>
<td>65</td>
</tr>
</tbody>
</table>

a, Freezing in cold room at $-30^\circ$C  
b, Freezing in dry-ice-alcohol bath  
c, Freezing in liquid nitrogen bath

after thawing. As shown in Table I, the viscosity and ATPase activity were decreased by freeze-thawing. The extent of decrease was the largest in myosin B frozen at $-30^\circ$C, very slow rate of cooling. This suggested some relation between freezing condition and the extent of the denaturation.

The examination was made as to a relation between the protein concentration and the extent of denaturation by freeze-thawing that was defined by the change in viscosity of myosin B in 0.6 M KCl and 0.02 M tris-HCl. As shown in Fig. 1, the extent of the denaturation converged to a finite value, decreasing with an increase in protein concentration. To avoid the effect of protein concentration, specimens of protein concentration higher
than 0.1% were used in all of the subsequent experiment.

The effect of freezing conditions on the extent of denaturation was then investigated by measuring specific viscosity and ATPase activity. The freezing conditions were either of various freezing temperatures at the same cooling rate (about $-20^\circ\text{C/min}$) or of the same freezing temperature ($-50^\circ\text{C}$) at various cooling rates. The results were shown in Figs. 2 and 3.

**Fig. 2.** Effect of freezing temperature on the viscosity $\eta_{sp}$ (○) and ATPase activity (●) of myosin B in 0.6 M KCl, 0.02 M Tris-HCl buffer (pH 6.8). Cooling rate, $-20^\circ\text{C/min}$

**Fig. 3.** Effect of cooling rate on the viscosity $\eta_{sp}$ (○) and ATPase activity (●) of myosin B in 0.6 M KCl, 0.02 M Tris-HCl buffer (pH 6.8). Final freezing temperature, $-50^\circ\text{C}$
As shown in the results, ATPase activity and viscosity of freeze-thawed myosin B solution decreased with lowering of the freezing temperature at a constant rate of cooling. They also decreased with slowing of the cooling rate to a given temperature. Therefore, it was considered that the extent of denaturation by freeze-thawing depended on the two factors of freezing conditions: The extent increased with the lowering of the final freezing temperature and with the slowing of the rate of cooling.

The ultracentrifugal analysis showed no difference in sedimentation pattern and sedimentation constant between the treated specimen and the non-treated specimen, indicating that neither dissociation of a molecule nor association of molecules was caused by freeze-thawing. Therefore, the decrease of viscosity of myosin B by freeze-thawing should be considered to be due to a change in intramolecular conformation.

The changes of viscosity of myosin B with time after the addition of 1 mM ATP in the presence of 1 mM Ca\textsuperscript{2+} were shown in Fig. 4. The ATP sensitivity, that is, viscosity change at the time of the addition, was almost the same for every specimen, while the recovery rate of viscosity was slower.

**Fig. 4.** Time course of viscosity change by adding of ATP on frozen-thawed myosin B
Ordinate: Specific viscosity
Abscissa: Time after adding of 1 mM ATP at 26°C
O, control; \(\oplus\), cooling rate \(-15^\circ\mathrm{C}/\mathrm{min}\); \(\bullet\), \(-1^\circ\mathrm{C}/\mathrm{min}\)
Solvent: 0.6 M KCl, 0.02 M Tris-HCl buffer (pH 6.8), 1 mM Ca\textsuperscript{2+}
for the treated specimens than for non-treated one. The initial viscosity and the rate of recovery more decreased in the specimen frozen slowly. This showed the similar trend as the result in Fig. 3. The former evidence suggested that the actin-myosin binding site of myosin B might suffer little damage by freeze-thawing, while the latter might be explained by the decrease of ATPase activity of the treated myosin B.

Reconstituted actomyosin

To confirm that the binding of actin and myosin might not be affected by freeze-thawing, ATPase activity, initial viscosity and ATP sensitivity were measured of the four kinds of reconstituted actomyosin. Namely, they had been constituted by all four possible combinations of F-actin and myosin in mole ratio 1:1, in which case F-actin may be either freeze-thawed or not, and myosin may be either freeze-thawed or not. As shown in Fig. 5, viscosity of the reconstituted actomyosin, of which each component was

Fig. 5. Time course of viscosity change by adding of ATP on frozen-thawed reconstituted actomyosin
Ordinate: Specific viscosity. Abscissa: Time after adding of 1 mM ATP at 26°C. A., actin; M., myosin B; O, control (A.M.); ⊙, A.M*.; ⊙, A.M.; ●, A.M*. Solvent: 0.6 M KCl, 0.02 M Tris-HCl buffer (pH 6.8), 1 mM Ca²⁺
* Shows frozen-thawed specimen. Final freezing temperature, −170°C
treated, decreased to the same value as the control by an addition of ATP and finally recovered to their original values, although their initial viscosity decreased by freeze-thawing. In these specimens, there was a slight difference in the rate of recovery, in parallel with the ATPase activity. The extent was larger in the specimen of which myosin was treated.

These results suggested that the binding of actin and myosin sustain hardly and damage by freezing, despite their exposed state.

**Actin**

Myosin B is composed of F-actin and myosin. In order to confirm which part of myosin B molecule is more unstable against freezing, one should examine the effect of freeze-thawing on myosin and on actin separately. Here F-actin was examined together with G-actin.

After freeze-thawing, the viscosity of F-actin decreased extremely while that of G-actin increased slightly, as shown in Table II. As shown in Fig. 6, the rate of polymerization of G-actin by the addition of 0.1 M KCl decreased with the lowering of the freezing temperature but the final value of viscosity for the treated G-actin was almost the same as that for the non-

<table>
<thead>
<tr>
<th></th>
<th>Reduced Viscosity (dl/g)</th>
<th>Sedimentation coefficient (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Myosin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.3</td>
<td>5.0^b</td>
</tr>
<tr>
<td>Fr-th.</td>
<td>3.1</td>
<td>4.6^b</td>
</tr>
<tr>
<td><strong>G-actin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.32</td>
<td>4.8^d</td>
</tr>
<tr>
<td>Fr-th.</td>
<td>0.35</td>
<td>5.0^d</td>
</tr>
<tr>
<td><strong>F-actin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.60</td>
<td>4.6^c</td>
</tr>
<tr>
<td>Fr-th.</td>
<td>0.70</td>
<td>4.5^c</td>
</tr>
</tbody>
</table>

Freezing temperature, –80°C
a, 0.5 M KCl and 0.02 M tris-HCl buffer solution (pH 6.9)
b, Protein Concentration, 0.33%
c, Aqueous solution
d, Protein concentration, 0.25%
e, 0.1 M KCl solution
f, Protein concentration, 0.25%
Fig. 6. Effect of freeze-thawing on the polymerization of G-actin

Ordinate: Specific viscosity
Abscissa: Time after adding of KCl
O, control; ⊙, frozen to −50°C; ●, frozen to −170°C. Protein concentration, 2.5 mg/ml. Cooling rate, −50°C/min

treated one. Neither dissociation nor association of the molecule was found by ultracentrifugal analysis both in the treated G- and F-actin.

From the extent of the decrease of viscosity it was suggested that freeze-thawing caused a certain configurational change of F-actin and a slight change of G-actin.

Myosin

Of myosin were measured not only viscosity and ATPase activity but optical rotatory dispersion and difference spectrum, which might give direct evidence of the unfolding of the protein molecule. This was also the fact suggested by the result of the experiments on Myosin B. Myosin B is too opaque for these optical measurements to be carried out.

In rapid freezing, 1% myosin in 0.5 M KCl and 0.02 M tris-HCl buffer solution (pH 6.9) showed no change of rotatory parameters, $-a_0$ and $-b_0$, and of ATPase activity. On the contrary, in slow freezing it showed changes of every measured properties as shown in Figs. 7 and 8. The decrease in $-b_0$, the increase in $-a_0$ and the increase in viscosity, seen in Fig. 7,
Denaturation of Enzyme Protein by Freeze-Thawing and Freeze-Drying I.

Fig. 7. Effect of freezing temperature on the enzymatic activity and physico-chemical properties of frozen-thawed myosin
Upper: ○, $-b_0$; ●, $-a_0$
Lower: ○, ATPase activity; ●, Reduced viscosity
$\bar{\eta}_{sp}/c$: ○, value of $-\Delta O \cdot D_{270 m\mu}$
Solvent: 0.5 M KCl, 0.02 M Tris-HCl buffer (pH 6.9)
Cooling rate, about $-20^\circ C/min$

represent typical patterns of the unfolding of helical structure of the protein molecule. The difference spectra of the same specimen in an ultraviolet region were illustrated in Fig. 8. There was broad trough between 300 and 250 m\(\mu\) and the position of the minimum was near 270 m\(\mu\). Although this position was extremely shifted to the shorter wave length than that of the blue shift observed in ordinary protein denaturation, the values of the minima, $-\Delta O \cdot D_{270 m\mu}$, were increased like other parameters with the decrease of the freezing temperature. Ultracentrifugal analysis showed no occurrence of dissociation or association in freeze-thawed myosin. The ATPase activity was also decreased same as other physicochemical parameters depending on the freezing temperature.
The results indicated that a partial unfolding of the helix in the molecule was caused by freeze-thawing like by other denaturants. From the evidence that $-b_0$ of myosin decreased from 330 of the control value to 260 by the freezing at $-196^\circ C$, it can be estimated that freezing unfolded about 20% of helix, a value not so high as the values caused by other denaturant. In conclusion, it might be said that the secondary and tertiary structures of myosin are partially destroyed by freeze-thawing and the extent of the destruction increases with the decrease of the freezing temperature at the slow rate of cooling.

Meromyosin

As pointed before, freeze-thawing seemed to change the viscosity of G-actin less than that of F-actin or of myosin. The fact suggested some relationships between the shape of the protein molecule and the stability of the protein against freezing.

Myosin is dissociated by trypsin digestion into two subunits, H- and L-meromyosin. The former is of a rather globular type while the latter is a typical rod-like protein. The comparison of the effect of freeze-thawing on the two meromyosins will be useful for understanding the relationship.

Physico-chemical properties were measured of 0.8% H-meromyosin in 0.02 M tris-HCl buffer solution (pH 6.8) and 0.8% L-meromyosin in 0.5 M
Denaturation of Enzyme Protein by Freeze-Thawing and Freeze-Drying

Fig. 9. Effect of freezing temperature on the conformational parameters of frozen-thawed H-meromyosin

- $b_0$; ○, $-a_0$; +, ATPase activity; □, $-\Delta D_{285\text{m}u}$

Solvent: 0.02 M Tris-HCl buffer (pH 6.8)
Cooling rate, about $-20^\circ\text{C/min}$

Fig. 10. Effect of freezing temperature on the helix parameters of frozen-thawed L-meromyosin

- $b_0$; ○, $-a_0$; +, $-\Delta D_{285\text{m}u}$

Solvent: 0.5 M KCl, 0.02 M Tris-HCl buffer (pH 6.8)
Cooling rate, about $-20^\circ\text{C/min}$
KCl and 0.02 M tris-HCl solution (pH 6.8), both of which had been frozen slowly to a given temperature and thawed rapidly. Figures 9 and 10 showed the result on H- and L-meromyosin, respectively. The difference spectra of both meromyosin were illustrated in Fig. 11. There were troughs near 280–295 m\(\mu\), probably denaturation blue shift. All changes in helix parameters of \(-b_0\) and \(-a_0\), \(-\Delta O\cdot D_{285, \text{nm}}\) and enzymatic activity decreased with the lowering of freezing temperature, in the same way as found in myosin.

By freezing at \(-196^\circ\text{C}\), the value of \(-b_0\) of H-meromyosin decreased from the control value 240 to 150 and that of L-meromyosin from control

![Graph showing difference spectra of frozen-thawed H- and L-meromyosin](image-url)
Denaturation of Enzyme Protein by Freeze-Thawing and Freeze-Drying I

value 450 to 80, showing the unfolding of helix by 27% and 80%, respectively. Ultracentrifugal analysis showed that freeze-thawing caused neither dissociation nor association in both the protein.

The molecular conformation of L-meromyosin seemed to be more unstable than that of H-meromyosin against the process of freeze-thawing.

FREEZE-DRYING OF MYOSIN

Myosin of 1% in 0.5 M KCl solution was frozen under various freezing conditions with or without an additive and dried from the frozen state immediately by an oil rotary pump. After freeze-drying, it was resolved to make a solution of the final concentration, 1% protein, 0.5 M KCl and 0.02 M tris-HCl (pH 6.8). The freeze-dried myosin with KCl only as an additive was a little difficult to redissolve. Therefore, the specimens were used for measurement after 6 hours from the time of the addition of the solvent for complete redissolving.

The optical rotatory dispersion, the difference spectrum in an ultraviolet region and the ATPase activity of the freeze-dried myosin together with those of the control were shown in Table III, where a marked decrease of

<table>
<thead>
<tr>
<th>Sample</th>
<th>Medium</th>
<th>Freezing temp. (°C)</th>
<th>Helix Parameter $-b_0$</th>
<th>$-40 \cdot D_{278\text{ nm}}$</th>
<th>Relative ATPase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coctrol</td>
<td>0.5 M KCl</td>
<td>—</td>
<td>353</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.02 M Tris-HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>0.5 M KCl</td>
<td>— 80</td>
<td>138</td>
<td>0.56</td>
<td>30</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>—196</td>
<td>186</td>
<td>0.33</td>
<td>30</td>
</tr>
<tr>
<td>3.</td>
<td>0.5 M KCl</td>
<td>— 80</td>
<td>223</td>
<td>0.30</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>0.02 M Tris-HCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>—196</td>
<td>230</td>
<td>0.29</td>
<td>66</td>
</tr>
<tr>
<td>5.</td>
<td>0.5 M KCl</td>
<td>— 80</td>
<td>272</td>
<td>0.15</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>0.1 M Sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>0.5 M KCl</td>
<td>— 80</td>
<td>249</td>
<td>0.13</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>0.01 M Sucrose</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Protein concentration, 1%
a, Protein concentration reduced to 0.12%

$-b_0$, an increase of $-40 \cdot D_{278\text{ nm}}$, and a remarkable decrease of ATPase activity could be observed for the treated myosin without any buffer solution as an additive. Even with the buffer solution, the extent of denaturation was larger than that caused by freeze-thawing. About 60% and 30% of
helix were estimated to be disrupted by freeze-drying of myosin, without and with a buffer solution, respectively.

The extent of decrease of enzymatic activity was almost the same as that of $-b_0$ value. The change of $-b_0$ was greater by $-80^\circ$C freezing than by $-196^\circ$C freezing. Neither dissociation nor association of the molecules were observed in freeze-dried myosin by ultracentrifugal analysis.

The effects of some additives were shown in Table IV. In the presence of 0.2 M of some sugars or amino acids, the values of $-b_0$, $-D_{278}$, and the enzymatic activity were near the values for the control. But sodium pyrophosphate, which had been reported as a protectant for heat denaturation of myosin, accelerated denaturation like NaCl.

From these results, it was considered that freeze-drying accelerated the unfolding of conformation and the loss of ATPase activity of myosin more than freeze-thawing did, and sugars or amino acids protected myosin to a certain extent against denaturation by freeze-drying.

### IV. Discussion

In the present experiment, it was shown that freeze-thawing caused not only the reduction of enzymatic activity but also the partial unfolding of the helical structure in rod like proteins, such as myosin B, myosin and H- or L-meromyosin depending on the freezing conditions. It was also sug-
gested that the structure of F-actin was destroyed. Although both of the changes in molecular structure and enzymatic activity with freezing was not so remarkable as that with other physical & chemical denaturants, all of the changes occurred depending on the rate of cooling and the final freezing temperature. Freeze-drying of myosin more increased the extent of the denaturation than that of freeze-thawing.

Concerning the mechanism of denaturation by freezing, several hypotheses have been presented: (1) Mechanical damage by ice crystal; (2) denaturation by highly concentrated salts during freezing; (3) pH change during freezing; (4) aggregation by SH-SS conversion; (5) rupture of hydration water of protein molecule by ice formation.

The first is unplausible because of the large size of an ice crystal in comparison with a protein molecule; the second is questioned from the reversibility of the conformation of myosin by high concentration of KCl; the forth was excluded in the light of the result that the SH content of cod fish myosin did not change during storage in a freezing state.

The results of the present experiments are that no dissociation of protein occurred during freezing, that denaturation increased under the eutectic temperature of KCl and that no association took place during freezing. These also support the exclusion of the first, the second and the forth hypotheses and the result that denaturation without a buffer solution increased during freezing seems to exclude the third hypothesis.

On the other hand, the result that denaturation depended on freezing conditions suggests that the phase change of solvent water by freezing might play a direct role in denaturation by freezing, that is this result supports the fifth hypothesis.

On the basis of the observation that freezing between -40°C and -120°C caused a complete loss of ATPase activity of myosin, SHIKAMA assumed that the cubic ice structure having transitted from hexagonal in this temperature region might disturb the polyhedral structure of water around hydrophobic bonds in protein molecule.

Such a critical temperature region, however, was not observed in the present experiment, where curves representing relationship between the temperature and the extent of denaturation were rather uniformly declining, without any critical point. The reason probably be that the transition of crystal structure might not occur in the present experiment because of the slow rate of cooling due to a large amount of sample with high concentration of protein.

Hence, other mechanism should be considered of denaturation in the
present experiment. Since freezing of protein causes intramolecular conformational change, the denaturation of protein seems due to the destruction of hydrophobic bonds sustaining the protein structure and/or the break down of intramolecular hydrogen bonds in the protein molecule as a result of phase change of solvent water. Ice crystal formation in solvent water may place the water structure in order so completely that the environment around protein molecule becomes "non-aqueous" state, causing necessarily the destruction of hydrophobic bonds. This might cause some effect on the intramolecular hydrogen bonds. The break down of the hydrogen bonds may also be caused by the interaction of ice crystal with the hydration shell of a protein molecule. It is thought that the structure of hydration water has a regular arrangement of water molecule, different from liquid water, but the arrangement is not so regular when compared to pure ice crystals. In such situation, the interaction may change the arrangement of hydrogen bonds between ice and the hydration shell because all the hydrogen bonds are strongly correlated among them. The change propagates to the intramolecular hydrogen bonds in the protein, initiating the unfolding of the protein molecule. On this proposed assumption, the dependence of denaturation on the final freezing temperature may be explained by the amount of destructed hydrophobic or hydrogen bonds in the protein; the dependence on the cooling rate may be explained by the mode of disturbance of hydrophobic and hydrogen bonds.

As for the dependence on freezing condition, it is noted here that examples similar to the foregoing have been reported to the effect that the inactivation of lactic dehydrogenase depends on the cooling rate and that the denaturation of phycoerythrine depends on the final freezing temperature.

The extent of the decrease of helix content was larger in L-meromyosin than in H-meromyosin, and the change of viscosity was remarkably larger in F-actin than in G-actin, whereby both the facts suggested some relation between the shape of protein and its stability against freezing. The existence of such a relation is easily understood, if freezing affects primarily hydrophobic bonds and/or the hydration shell of a protein molecule as assumed above. Szent-Györgyi considered that myosin abounds in hydration water sustaining its helical structure. If his consideration is accepted, the unstability of myosin against freezing is also easily understood on this assumption.

In the present experiment, the freeze-drying of myosin accelerated the unfolding of protein more than freeze-drying did. This can be qualitatively understood, since freeze-drying dehydrates more completely than freeze-
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1. Denaturation by freeze-thawing does; the evidence can be qualitatively understood from the important role played by water in the conformation of protein.

The more detailed discussion on denaturation by freeze-drying will be given in the next paper together with the discussion of the protective effect of additives on denaturation by freeze-thawing and by freeze-drying.

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