STUDIES ON THE POSTERIOR SILK GLAND OF THE SILKWORM BOMBIX MORI

IV. Ultracentrifugal Analyses of Native Silk Proteins, Especially Fibroin Extracted from the Middle Silk Gland of the Mature Silkworm

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ABSTRACT

Ultracentrifugal analyses of the native silk proteins extracted from the various parts of the middle silk gland of the mature silkworm have revealed that there exist four components with S°M, values of 10S, 9-10S, 9S, and 4S in the extract. It is suggested that the fastest 10S component is the native fibroin synthesized in the posterior silk gland and transferred to the middle silk gland to be stored there, while the slower three components probably correspond to inner, middle, and outer sericins which were synthesized in the posterior, middle, and anterior portion of the middle silk gland, respectively. Native fibroin solution was prepared from the most posterior part of the middle silk gland. Ultracentrifugal analyses have shown that the solution contains considerable amounts of aggregates in addition to the main 10S component. Treatment with lithium bromide (LiBr), urea, or guanidine hydrochloride solution up to 6 M all have failed to dissociate the 10S component. From the sedimentation equilibrium analyses and partial specific volume of 0.716, the molecular weight of the 10S component of the native fibroin solution was found to be between 3.2 - 4.2 × 10^5, with a tendency to lie fairly close to 3.7 × 10^5.

INTRODUCTION

It is well known that the silk glands of the silkworm, Bombyx mori, synthesize at least two kinds of protein, fibroin and sericin; the former is synthesized in the posterior division of the silk gland (conventionally called posterior silk gland as used here) and is transferred to the middle division of the same gland or middle silk gland to be stored there as a fibroin core, while the latter is secreted in the middle silk gland and builds up three gelatinous sericin layers round the fibroin core (1, 2). This kind of conclusion, however, is based exclusively on microscopic observation of the tinctorial properties of the silk proteins in the gland lumina (3, 4), and no extensive physicochemical analysis has been carried out on native silk proteins extracted from the silk glands. In this report, the sedimentation properties of the native silk proteins which were extracted directly from various parts of the middle silk gland were analyzed and compared. This study shows not only how many kinds of protein exist in the lumen of the middle silk gland but also suggests the site of biosynthesis for each protein.

Physicochemical properties, such as ultra-
centrifugal heterogeneity, sedimentation, and diffusion coefficients, molecular weight, etc., of the fibroin have been the subject of a number of investigators (2, 5-11). Quite inconsistent and controversial results, however, have been reported. For example, the molecular weights for fibroin hitherto reported scatter from 33,000 to the order of 1,000,000 (2, 10). The fibroin solutions used for those studies usually were prepared from cocoons or raw silk by rather violent procedures such as boiling in alkaline soap solution (1, 2) or dissolution in alkaline cupriethylene diamine hydroxide solution (5). It is possible, therefore, that the fibroin suffered from more or less extensive molecular degradation during these procedures as discussed in detail by Lucas et al. (2). It appears desirable, therefore, to prepare native fibroin directly from the silk glands and to examine its physicochemical properties. We report here results of such experiments.

**MATERIALS AND METHODS**

**Silkworms**

The strains of the silkworms used for the extraction of silk proteins and the seasons of rearing are the following: Nichi 124 x Shi 124 (spring), Shungyoku x Gunpo (spring), Nichi 122 x Shi 124 (summer), and Nichi 115 x Shi 124 (summer). Such factors as strains and seasons of rearing do not seem to have serious effect on the physicochemical properties of the silk proteins. The fifth instar larvae were cultivated as described previously (12) and, when they arrived at full maturation, they were sacrificed for extraction of silk proteins.

**Extraction of Native Silk Proteins from Various Parts of the Middle Silk Gland**

Native silk proteins were extracted according to Shimizu et al. (1). The middle silk glands of both sides were dissected out and washed briefly in glass-distilled water. Immediately they were cut into five parts as shown in Fig. 1, and each part was put into a small Petri dish containing a small amount of distilled water. After several minutes in distilled water the luminal silk protein columns were softened and partially extruded out of the glandular lumina, and the swollen glandular tissues became easily separable by fine forceps from the silk protein columns. These Petri dishes with silk protein columns were then shaken very gently overnight in the cold (2-3°C). Vigorous shaking should be avoided strictly because it induced extensive surface denaturation and gelation of the silk proteins. The water extract was then collected by decantation, and a small amount of distilled water was added again to the dishes and the second extraction continued for several hours more. These procedures gave almost complete extraction of the silk proteins. The combined extracts were centrifuged for 60 min at 105,000 g, and the clear supernatant was collected by decantation and stored in the cold (2-3°C) until just before use.

**Extraction of Native Fibroin**

Only the most posterior part of the middle silk gland or part 5 of Fig. 1 was used to prepare native fibroin. The silk protein columns were prepared as described previously, and they were washed gently by distilled water several times. This washing serves to extract a very small amount of sericin which sticks to the surface of the fibroin column. The subsequent procedures are similar to those in the previous case. Extracts from the anterior and middle portions of the middle silk gland (parts 1-3) became turbid within 1 or 2 days, even in the cold, and then gradually separated into two layers: an upper turbid, and a lower transparent layer. In contrast to this, extracts from the posterior portion of the middle silk gland (parts 4, 5) and native fibroin solution are both much more stable in the cold and remain as clear solutions for as long as 2-3 wk. It is noted, however, that native fibroin solution became increasingly slightly
opalescent on standing in the cold, suggesting gradual formation of aggregates, and it gelled suddenly 2-4 wk after preparation. These solutions should be treated very gently, because fibroin gels quite easily; gelation is induced by gentle shaking, stirring, or even by pipetting.

**Sedimentation Analyses by the Schlieren Optical System**

Sedimentation analyses by the schlieren optical system were carried out at ~20°C and at a rotor speed of 59,780 rpm with a Spinco Model E analytical centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). Either 0.1 M carbonate-bicarbonate buffer (CB buffer), pH 9.0, alone or the same buffer containing 0.1 M NaCl (SCB buffer) was used as the solvent. Native fibroin gelled rapidly in pH 7.0 0.1 M phosphate buffer. Schlieren photographic plates were read on a Nikon comparator type 6CT (Nippon Kogaku K.K., Tokyo). Conversion to a standard state \( S_{20,w} \) was accomplished by the following equation (1) according to Svedberg and Petersen (13).

\[
S_{20,w} = S \frac{(1 - \bar{V}_p)_{20,w}}{(1 - \bar{V}_p)_{w}}
\]  

The standard sedimentation coefficient at an infinite dilution \( S_{20,w} \) was estimated by extrapolation to zero concentration.

**Sedimentation Analyses by the Interference Optical System**

In order to examine in more detail the sedimentation properties of the native fibroin solution, sedimentation analyses were also carried out by the interference optical system. The latter optical system makes it possible to detect even a small amount of aggregates, if present, and to analyze more dilute solution than does the schlieren optical system. Interference photographic plates were read on the same Nikon comparator.

**Equilibrium Ultracentrifugation**

The meniscus depletion method of Yphantis (14) was used exclusively for determination of the molecular weight of fibroin. The equilibrium centrifugation was carried out either in the cold (2-5°C) or at ~20°C for about 24 hr at a rotor speed of 9,945 rpm. Fibroin sometimes gelled during the run, especially when very dilute solution of fibroin was analyzed at 20°C, and was spun down to the bottom of the cell. Although fibroin is unstable in warm alkaline buffer as shown later, degradation of fibroin was not observable under the present experimental conditions except for some broadening of the sedimenting boundary. Apparent weight-average molecular weights were calculated according to equation (2),

\[
M_w(c) = \frac{2RT}{(1 - \bar{V}_p)\omega \rho} \left( \frac{d \ln c}{d(r^2)} \right)_w
\]  

where \( R \) is the gas constant, \( T \) is the absolute temperature, \( \bar{V} \) is the partial specific volume of the protein, \( \rho \) is the density of the solvent, \( \omega \) is the angular velocity, \( \left( \frac{d \ln c}{d(r^2)} \right)_w \) was evaluated from the expression given by Yphantis (14).

\[
\left( \frac{d \ln j}{d(r^2)} \right)_{r_s} = \frac{0.1}{2 n_A \Delta r} \left( 2 \ln j_s + \ln j_{s+1} - \ln j_{s-1} - 2 \ln j_{s-2} \right)
\]  

where \( \ln j_s \cdots \ln j_{s+2} \) are five data points spaced at equal increments \( \Delta r \). No fringe displacements less than 100 \( \mu \) were used.

**Determination of Partial Specific Volume \( \bar{V} \)**

The aqueous solution of fibroin was dialyzed overnight against cold 0.01 M CB buffer, pH 9.0, and then the density of fibroin solution at various concentration (0-14 mg/ml) was determined in this buffer by a pycnometer at a constant temperature of 20°C, and \( \bar{V} \) was calculated. A trial to obtain \( \bar{V} \) in distilled water was not successful, because the fibroin gelled during dialysis against cold distilled water.

**Determination of Chemical Composition and Concentration of the Fibroin Solution**

Fibroin solutions were dried by lyophilization and lipids were extracted and determined as described previously (12). RNA was extracted by Schneider's procedures (15), and the amount of RNA was determined by orcinol reaction (16). Determination of the lipids was kindly carried out by Dr. K. Saito, Department of Biochemistry of the Kansai Medical School. Since it was found that the fibroin solutions contain neither lipids nor RNA as described later, concentrations of fibroin solutions were determined by measurement of dry weight. An aliquot of a fibroin solution was treated with 5% (final) trichloroacetic acid (TCA), washed three times with 5% TCA, dehydrated by alcohol and by alcohol-ether (3:1), and finally the fibroin was washed with ether. The fibroin was then dried at 110°C for at least 5 hr before weighing.

**Electrophoretic Analyses**

In order to examine the electrophoretic homogeneity of the fibroin, electrophoretic analyses were
carried out by a Hitachi HTB-2A electrophoretic apparatus. The fibroin solutions were dialyzed against cold 0.1 M Veronal-Veronal acetate buffer of pH 8.6 containing 1.0 M urea and were analyzed in the same solution. Addition of urea seems essential for successful electrophoretic analyses of the fibroin solution; in the absence of urea, strange spikes or anomalies appeared frequently on the electrophoretic patterns. Probably gelation of fibroin during electrophoretic analyses is responsible for these anomalies.

RESULTS

Ultracentrifugal Analyses of Native Silk Proteins Extracted from Various Parts of the Middle Silk Gland

Fig. 2 a-e shows the sedimentation patterns of the native silk proteins that were observed at a concentration of ~5 mg/ml extracted from parts 1-5 of the middle silk gland, respectively. It is apparent that Fig. 2 e shows only a single peak, which will be called component 1. For comparison, the sedimentation pattern of the native fibroin run at the same concentration of 5 mg/ml is shown in Fig. 2 f. As the standard sedimentation coefficient at infinite dilution ($S_{20,w}$) and the sedimentation profile of component 1 of Fig. 2 e are quite similar to those of the native fibroin, it is quite likely that component 1 of Fig. 2 e corresponds to native fibroin. The sedimentation pattern of the extract from part 4 (Fig. 2 d) shows, in addition to the main peak of component 1, a small peak of component 2 which sediments a little slower than component 1 and, therefore, appears on the left shoulder of component 1. The sedimentation pattern of the extract from part 3 (Fig. 2 c) has two peaks with $S_{20,w}$ values of 10.0S and 8.9S, respectively. The faster 10S peak most probably corresponds to component 1. The slower peak was tentatively assumed, in this manuscript, to correspond to a new component 3, although the possibility exists that the slower peak corresponds to component 2 of Fig. 2 d as will be discussed later. A quite similar sedimentation pattern was obtained from part 2 of the middle silk gland as shown in Fig. 2 b. The only apparent difference seems to be an increase in the proportion of component 3 to component 1 in the pattern of Fig. 2 b. A new component 4, however, appears in the extract from part 1 (Fig. 2 a). This component has $S_{20,w} = 4.4S$ as calculated by extrapolation of two series of sedimentation runs.

It is to be noted here that the present experimental conditions are far from ideal for components 1-3; several ultracentrifugal anomalies were observed, such as a marked concentration dependence of the sedimentation coefficients, hypersharpening of the boundaries, and the Johnston-Ogston effect, etc. Fig. 3 a-f and Fig. 3 g-h show sedimentation patterns of serially diluted silk proteins extracted from part 1 and part 3, respectively. The former two anomalies are quite evident if Fig. 3 a-f are compared with each other. For example, component 1 and 3 sediment much more rapidly in dilute solution than in concentrated solution, and the profile of component 3 is much sharper in Fig. 3 a than in Fig. 3 b, c, or d. The Johnston-Ogston effect is quite apparent if Fig. 3 c is compared with Fig. 3 d and if Fig. 3 g is compared with Fig. 3 h. This effect is so marked in this system that the area of each component, even after correction for radial dilution, is not even qualitatively proportional to the concentration of each component. For example, although there is apparently almost as much component 3 as component 1 in Fig. 3 g, the sedimentation pattern of the same sample after a twofold dilution (Fig. 3 h) clearly shows that much more component 1 does exist in this extract than component 3.

In order to interpret these sedimentation patterns reasonably, therefore, it is essential to first correct for these ultracentrifugal anomalies. After correcting these effects by analyzing sedimentation patterns of serially diluted silk proteins, we suggest that component 1 in Fig. 2 a most probably corresponds to fibroin because the $S_{20,w}$ value of this component was about 10S either in 0.1 M CB or SCB buffer, pH 9.0, and this component constitutes approximately 70% of the total silk proteins in the extract from the part 1. In order to confirm this conclusion, a small amount of native fibroin was added to the extract from part 1 and the mixture was analyzed by ultracentrifugation. Comparison of Fig. 4 a with Fig. 4 b clearly shows that the amount of component 1 increased markedly after the addition of pure fibroin. In this experiment, the addition of a large amount of native fibroin caused an apparent increase of both components 1 and 3. This is certainly owing to the Johnston-Ogston effect.

The $S_{20,w}$ value of component 2 was found to be 9-10S. As to the existence of component 2, there are two possibilities: one is that it is an independ-
ent component, and the other is that it is identical, at least ultracentrifugally, with component 3. If the former possibility is correct, a peak of component 2 should be detected not only in the extract from part 4 but also from parts 3, 2, and 1. Our experiments have failed to show such a peak and, in this sense, the former possibility is not supported experimentally. It is possible, however,

**Figure 2** Sedimentation patterns of the silk proteins extracted from part 1 (2a), part 2 (2b), part 3 (2c), part 4 (2d) and part 5 (2e) of the middle silk gland, respectively. The concentration of silk proteins for Fig. 2a, c, d, and e was 5 mg/ml, while that for Fig. 2b was 6 mg/ml. For the purpose of comparison, native fibroin was also analyzed at a concentration of 5 mg/ml (Fig. 2f). Solvent: CB buffer, pH 9.0. All the sedimentation velocity analyses by the Schlieren optical system in this manuscript were carried out at a temperature of 20°C and at a speed of 59,780 rpm. Fig. 2e-f were taken 51 min, and all the other pictures 57 min, after attaining full speed, respectively.

**Figure 3** Sedimentation patterns of the serially diluted silk proteins extracted from part 1 (3a-f) and from part 3 (3g-h), respectively. Initial concentrations of silk proteins in Fig. 3a-f are 10, 7.5, 5.0, 2.5, 1.25, and 0.625 mg/ml, respectively, while those of silk proteins in Fig. 3g-h are 5.0 and 2.5 mg/ml, respectively. Solvent: SCB buffer, pH 9.0. All the pictures were taken 56 min after attaining speed.

**Figure 4a-b** Sedimentation patterns of the silk proteins extracted from part 1 before (Fig. 4a) and after (Fig. 4b) addition of a small amount of fibroin. 4a, silk protein extract from part 1 (5 mg/ml). 4b, a mixture of silk protein extract from part 1 (2.5 mg/ml) and fibroin (1.25 mg/ml). Solvent: SCA buffer, pH 9.0. Picture was taken 64 min after attaining speed.

**Figure 4c-d** Sedimentation patterns of the first (Fig. 4c) and second extract (Fig. 4d) from part 1 of the middle silk gland. 4c, the silk proteins were extracted from part 1 for 4 hr in the cold (3°C). 4d, the residues were further extracted for 20 hr in the cold. Solvent: CA buffer, pH 9.0. Picture was taken 57 min after attaining speed.
that component 2 does exist but was not detected simply because it was so small in amount, and furthermore its S value was so close to that of component 3, that the peak of component 2 overlapped with and, therefore, was concealed by component 3. At the present time, therefore, there is no decisive evidence to support either of the two possibilities, and this manuscript was written assuming that the former possibility is correct. As a matter of course, this assumption should be confirmed by future experiment.

From the S_{20\text{w}} value of component 3 (approximately 9S), it seems certain that component 3 of Fig. 2 a corresponds to component 3 of Fig. 2 b and c. Component 3 in the extract from part 2 is larger in amount than that in the extract from part 3 and, furthermore, the amount of component 3 in part 1 is approximately equal to that in part 2. This suggests that component 3 is secreted mainly in the middle portion of the middle silk gland (part 2 + part 3).

Finally, since component 4 is observed only in the extract from part 1, it is certain that component 4 is secreted only in the anterior portion of the middle silk gland.

We conclude, therefore, that components 2–4 are the other silk proteins, sericins, and they are called sericins 1–3, respectively. It is further suggested that sericins 1–3 are probably secreted in the posterior, middle, and anterior portion of the middle silk gland, respectively.

The above conclusion is also supported by the differential water extraction of silk proteins from part 1; that is, silk protein columns prepared from part 1 were extracted with water for several hours by gentle shaking in the cold and the first extract was collected as described above. A small amount of water was added to the residues and a second extraction was continued overnight, until complete dissolution of the remaining silk proteins. Both extracts were analyzed by ultracentrifugation as shown in Fig. 4 c and d, respectively. The first extract (Fig. 4 c) clearly contains more component 4 and 3 than component 1, while the second extract (Fig. 4 d) contains quite a large amount of component 1. This finding is consistent with the above conclusion because sericin is known to be more water soluble than fibroin.

As the extracts from parts 1–3 gel rapidly even in the cold, the physicochemical properties of the sericins have not been studied further.

Sedimentation Analyses of the Native Fibroin Solution by the Schlieren Optics

Since it has been confirmed that the water extract from part 5 of the middle silk gland contains exclusively fibroin, a number of silkworms
were sacrificed so as to prepare a large amount of fibroin by the methods described previously. The sedimentation analyses by the schlieren optical system showed only a single 10S peak, and usually neither any slower component nor any aggregate was observed under the present experimental conditions as illustrated in Fig. 5.

In the early stage of the present experiments, the 10S peak of the fibroin showed frequently skewness towards a smaller radius, suggesting the presence of smaller sedimenting species, either from heterogeneity or from dissociation. Such a skewness, however, disappeared as the method of preparation of the fibroin has been improved; as described in Materials and Methods, the fibroin was prepared only from the most posterior part of the middle silk glands, and the silk protein columns were washed well with distilled water. Probably the fibroin solutions prepared in the early stage had been contaminated with a small amount of sericin.

Sedimentation analyses of much more dilute fibroin solution down to 0.016% have shown essentially similar patterns, except for broadening of the peak (Fig. 6). In order to calculate the $S^0_{20,w}$ of this fibroin, $1/S^0_{20,w}$ was plotted against the concentration of fibroin as shown in Fig. 7. These plots give a straight line and, in the three series presented here, extrapolation gave the average value of 9.9S for $S^0_{20,w}$.

**Sedimentation Analyses of the Native Fibroin Solution by the Interference Optics**

Fig. 8 shows the sedimentation patterns by the interference optics of the native fibroin solution (0.063%). It is apparent that the fibroin solution contains considerable amounts of aggregates. They do not form any distinct boundary, but are distributed widely and evenly in the centrifugal

![Figure 7](image-url)  
**Figure 7** Concentration dependence of standard sedimentation coefficient ($S_{20,w}$) of native fibroin. Three series of experiments (O, □, and ●) were plotted together. Solvent: SCB buffer of pH 9.0. The concentration of fibroin is in gram per liter.

![Figure 8](image-url)  
**Figure 8** Sedimentation patterns by the interference optical system of native fibroin at an initial concentration of 0.0625 mg/ml. The patterns presented were taken 19.5 min (○), 35.5 min (●), 51.5 min (□), 67.5 min (■), and 83.5 min (▲) after attaining speed of 30740 rpm, respectively. $M$, position of meniscus, $B$, position of bottom. Solvent: SCB buffer, pH 9.0.
side of the 10S component. This probably explains why these aggregates were not detected by the schlieren optical system. It is also suggested that there is a trailing shoulder on the 10S boundary at this concentration or at a lower concentration of 0.031%.

In order to analyze more quantitatively the sedimentation profiles of the fibroin solution, the apparent integral distribution function $G^*(S)$ defined by the following equation (4) was calculated at various time points.

$$G^*(S) = \int_{S=0}^{S=\infty} dc \cdot \int_{t=0}^{t=t} \left( \frac{r}{r_m} \right)^3 dc$$

where $c_o$ is the initial concentration of fibroin, and $r_m$ is the distance from the center of rotation to the meniscus position. Then the values of $G^*(S)$ were plotted against $1/t$ to obtain integral distribution function $G(S)$ by extrapolation to infinite time. In this calculation, it was difficult to determine precisely the $c_o$ value from the fringe displacement at the plateau region because the plateau region disappeared rapidly in the beginning of the sedimentation analyses as the aggregates are spun down to the bottom of the cells. Therefore, $G(S) \times c_o$ was plotted against $S$ values as shown in Fig. 9. It is apparent that the 10S component is the main component of the fibroin solution. The larger side of the distribution curve shown by a broken line is not reliable at all, because heavier aggregates are continuously lost during the sedimentation analyses. The trailing to the smaller side of the distribution curve suggests the presence of smaller species, probably in reversible equilibrium with the main component.

**Effects of Various Agents on the Sedimentation Patterns of the Fibroin**

It is possible that the ~10S peak of the fibroin does not correspond to monomeric form of fibroin but to a polymeric form bound by forces other than covalent bond. In order to examine such a possibility, the effects of salts (LiBr and sodium chloride), ethylene diamine tetraacetate (EDTA), urea, and guanidine hydrochloride on the sedimentation pattern of the fibroin were observed.

Fig. 10 shows a series of sedimentation patterns of the fibroin analyzed in the presence of 1 M(a), 2 M(b), 3 M(c), 4 M(d), 5 M(e), and 6 M(f-h) LiBr, respectively. Concentrations of fibroin were 0.25% for a-f, 0.125% for g, and 0.0625% for h. No sign of dissociation of the 10S component was observed. Similarly, no dissociation of the 10S component was observed in the presence of 0-1 M NaCl. The $S$ value at infinite dilution of the 10S component in 6 M LiBr at 20°C ($S_{0,\infty,6\text{M LiBr}}$) was 3.2S. Addition of EDTA to final concentrations of 8 mM and 16 mM had no effect on the sedimentation patterns of the fibroin.

Effects of 1 M(a), 2 M(b), 3 M(c), 4 M(d), 5 M(e), and 6 M(f-h) urea and guanidine hydrochloride are shown in Figs. 11 and 12, respectively. Concentrations of fibroin were again 0.25% for a-f, 0.125% for g, and 0.0625% for h. As in the previous cases, dissociation of the 10S component was not observed in the presence of these denaturing agents. The $S$ value at infinite dilution of the 10S component in 6 M ura and 6 M guanidine hydrochloride at 20°C was 2.8 and 3.8S, respectively. The $S_{0,\infty}$ value calculated according to equation

\[ G(S) = \int_{S=0}^{S=\infty} dc \cdot \int_{t=0}^{t=t} \left( \frac{r}{r_m} \right)^3 dc \]
FIGURE 10 Sedimentation patterns of native fibroin in SCB buffer, pH 9.0, containing 1 M (Fig. 10 a), 2 M (10 b), 3 M (10 c), 4 M (10 d), 5 M (10 e), and 6 M (10 f-h) LiBr, respectively. Concentrations of fibroin are 2.5 mg/ml for 10a-f, 1.25 mg/ml for 10 g, and 0.625 mg/ml for 10 h. All pictures were taken 72 min after attaining speed.

FIGURE 11 Sedimentation patterns of native fibroin in SCB buffer, pH 9.0, containing 1 M (Fig. 11 a), 2 M (11 b), 3 M (11 c), 4 M (11 d), 5 M (11 e), and 6 M (11 f-h) urea, respectively. Concentrations of fibroin are 2.5 mg/ml for 11 a-f, 1.25 mg/ml for Fig. 11 g, and 0.625 mg/ml for Fig. 11 h. The exposures presented were taken 75 min (11 g-h), 72 min (11 c-f) and 74 min (11 a-b) after attaining speed, respectively.

FIGURE 12 Sedimentation patterns of native fibroin in SCB buffer, pH 9.0, containing 1 M (Fig. 12 a), 2 M (12 b), 3 M (12 c), 4 M (12 d), 5 M (12 e), and 6 M (12 f-h) guanidine hydrochloride, respectively. Concentrations of fibroin are 2.5 mg/ml for 12 a-f, 1.25 mg/ml for 12 g, and 0.625 mg/ml for 12 h. The exposures presented were taken 71 min (12 a-d), 72 min (12 g-h), and 74 min (12 e-f) after attaining speed, respectively.

(1), assuming that the equation is valid even in such concentrated urea and guanidine hydrochloride solutions, was 6.1 and 12.28, respectively. Data published by Kawahara and Tanford (17) were used for the viscosity and density corrections in these solutions.

Electrophoretic Analysis of the Native Fibroin

The electrophoretic patterns of the native fibroin in 0.1 M pH 8.6 Veronal-Veronal acetate buffer containing 1 M urea show only a single peak as illustrated in Fig. 13. The electrophoretic mobility of the fibroin calculated from ascending (Fig. 13 a) and descending boundaries (Fig. 13 b) was the same, $-2.8 \times 10^{-5}$ cm$^2$ per sec per volt at 5.5°C.

Chemical Composition and Partial Specific Volume ($V$) of the Native Fibroin Solution

The native fibroin solution contains neither detectable amounts of lipids nor RNA; lipids and RNA content were less than 0.1%. The amount of
protein in the native fibroin solution, therefore, could be determined reliably by the dry weight measurement as described previously.

The partial specific volume of the fibroin at 20°C was 0.716 in 0.01 M CB buffer of pH 9.0. This value was used for the calculation of molecular weight of fibroin as described in the following. No correction was applied for the possible effect of temperature. It was further assumed that the $\bar{V}$ value in 0.1 M SCB buffer of pH 9.0 is equal to that in 0.01 M CB buffer of pH 9.0.

**Molecular Weight Determination by an Equilibrium Centrifugation**

The graph of the logarithm of net fringe displacement against square of the radius does not give a straight line, but rather a line curved upwards towards the bottom of the cell. This non-linearity was more marked in the cold than at 20°C.

$M_w(r)$, calculated according to equations (2) and (3), was plotted as a function of concentration of fibroin as shown in Fig. 14 (at ~20°C). Al-
though the data show considerable scatter, it can be seen from Fig. 14 that the sedimentation equilibrium experiments yield the type of molecular weight vs. concentration relationships characteristic of a heterogeneous, partially reversible, aggregating system. The average molecular weight estimated by extrapolation to zero concentration of each series of the experiments is $3.7 \pm 0.5 \times 10^5$, which corresponds most probably to the molecular weight of the 10S component.

Similar relationships were observed in the cold as shown in Fig. 15. In this case, however, concentration dependence of $M_w(r)$ is so marked in some series that it is difficult to estimate by extrapolation to zero concentration the molecular weight of the smallest species present. It is quite apparent, however, that in such samples there exist considerable amounts of molecular species smaller than the 10S main component, i.e., smaller than $3.7 \times 10^5$ in molecular weight, which are presumably in chemical equilibrium with the 10S component. This problem will be discussed further in detail at the end of this paper.

The sedimentation equilibrium experiments reported in Figs. 14 and 15 were carried out at rather high initial concentration of 0.25–1.5 mg/ml. In order to calculate the molecular weights of the complete sample ($\bar{M}$) by linear extrapolation to the base of column as shown by Yphantis (14), it is necessary to perform the equilibrium experiment at a lower concentration. A sedimentation equilibrium experiment run at an initial concentration of 0.01% was not successful because fibroin gelled during the equilibrium run and was spun

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**Table**

| Exp. No. | Temperature | Channel | Initial concentration | Symbols |
|----------|-------------|---------|-----------------------|---------|
| 68-70    | 3.9°C       | A       | 0.025%                | □       |
|          |             | B       | 0.05%                 | △       |
|          |             | C       | 0.1%                  | ○       |
| 68-91    | 2.5°C       | A       | 0.025%                | ■       |
|          |             | B       | 0.05%                 | ▲       |
|          |             | C       | 0.1%                  | ●       |

**Figure 15** Sedimentation equilibrium experiments with native fibroin in SCB buffer, pH 9.0, in the cold. Two equilibrium experiments were plotted together:

PROTEIN CONCENTRATION

(FRINGE DISPLACEMENT IN 100 µ)

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FIGURE 16  Sedimentation patterns of native fibroin (6 mg/ml) kept in cold CB buffer, pH 9.0, for various periods. Fig. 16 a-b, 72 hr (16 a) and 96 hr (16 b) after dilution to 6 mg/ml. Fig. 16 c-d, kept in cold CB buffer at a concentration of 18 mg/ml for 96 hr (16 c) and 240 hr (16 d), respectively, and then diluted to 6 mg/ml just before the sedimentation analysis. The pictures shown were taken 56 min (16 a-b), 58 min (16 c), and 59 min (16 d) after attaining speed, respectively.

FIGURE 17  Sedimentation patterns of native fibroin (2.5 mg/ml) kept in cold SCB buffer, pH 9-11. Fig. 17 a-b, immediately after addition of SCB buffer, pH 9.0 and 11.0, respectively. Fig. 17 c-d, 18 hr after addition of SCB buffer, pH 10.0 and 11.0, respectively. Fig. 17 e-f, 10 days after addition of SCB buffer, pH 10.0 and 11.0, respectively. The exposures presented were taken 64 min (17 a, b, e, f) and 66 min (17 c-d) after attaining speed, respectively.

FIGURE 18  Sedimentation patterns of native fibroin (2.5 mg/ml) kept at 25°C in SCB buffer, pH 9.0, for 0 min (18 a), 30 min (18 b), 60 min (18 c), and 120 min (18 d), respectively. The photographs presented here were taken 58 min (18 a-b), 57 min (18 c-d) and 56 min (18 e-f) after attaining speed, respectively.

FIGURE 19  Sedimentation patterns of native fibroin (2.5 mg/ml) incubated at 37°C in SCB buffer, pH 9.0, for 0 min (19 a), 30 min (19 b), 60 min (19 c), and 120 min (19 d), respectively. The photographs presented here were taken 49 min (19 a-b) and 48 min (19 c-d) after attaining full speed, respectively.
down to the bottom of the cells. So the calculation of $M_w$ was tried in an equilibrium experiment run at 2.5°C at an initial concentration of 0.025% (channel A of Exp. No. 68-91 shown in Fig. 15—see legend). The value calculated was $\sim 7.7 \times 10^5$. Although this value is not very reliable because of the high initial concentration used, and the data may fluctuate from sample to sample, it is quite clear and indisputable that there are large amounts of species present that are significantly greater than $3.7 \times 10^5$ in molecular weight.

In order to find out whether the increase in the molecular weight with the concentration is caused by reversible association and/or irreversible aggregation, $M_w (r)$ was plotted against $r^2$ according to the procedure suggested by Yphantis (14). The scatter of the data, however, is just too large to make any definite conclusion on the present system. Probably reversible association and irreversible aggregation coexist in the fibroin solution, and the contribution of one of these two factors may be different case by case, depending not only on the experimental conditions (temperature, pH, salts, etc) but also on the previous history of each fibroin solution (shaking during preparation, period of storage in the cold, pipetting, usage of syringe for filling each channel of centerpiece, etc.).

**Stability and Degradation of Native Fibroin Solution**

The native fibroin prepared by the present method of water extraction was quite stable as long as it was kept in the cold, and, even partially gelled by prolonged aging in the cold, ultracentrifugal analyses showed that most of the fibroin still in solution sediments as a single peak of 10S. Only slight broadening of the 10S peak and appearance of aggregates were observed in the schlieren patterns.

Several series of experiments have shown clearly that the native fibroin is quite unstable in alkaline buffer and is degraded easily. The alkali degradation depends on various factors such as pH, temperature, and concentration of fibroin; degradation is faster in the more alkaline solution and is accelerated by rise in temperature and by dilution of the fibroin solution.

In one series of experiments, shown in Fig. 16 a-d, an aliquot of native fibroin was diluted to 6 mg/ml and 18 mg/ml in the CB buffer, pH 9.0, respectively, and these solutions were kept in the cold ($\sim 3°C$). The degradations of both solutions were followed by ultracentrifugal analyses at the same final concentration of 6 mg/ml. At 72 hr after dilution to 6 mg/ml, hardly any degradation of native fibroin was observed (Fig. 16 a). At 56 hr after dilution, however, the solution became slightly turbid (gelation completed next day) and a slow heterogeneous component appeared in the sedimentation pattern (Fig. 16 b). The more concentrated solution of fibroin (18 mg/ml) seems to be more stable because hardly any degradation was observable at 96 hr (Fig. 16 c). Even 6 days later or 10 days after addition of the alkaline buffer, degradation of the latter solution was not apparent except for appearance of some aggregates and for a broadening of the main boundary as shown in Fig. 16 d.

In another series of experiments, aliquots of dilute fibroin solution (3 mg/ml) were kept in cold SCB buffers, at pHs 9.0, 10.0, and 11.0. Fig. 17 a (pH 9.0) and b (pH 11.0), Fig. 17 c (pH 10.0), and 17 d (pH 11.0). Fig. 17 e (pH 10.0), and 17 f (pH 11.0) show sedimentation patterns of native fibroin immediately, 18 hr, and 4 days after transfer to the alkaline buffer, respectively. It is apparent that the degradation of fibroin is more rapid in the more alkaline solution.

Degradation of native fibroin was accelerated by temperature. At 25°C, fibroin in the SCB buffer of pH 10 (Fig. 18, upper series) and 11 (Fig. 18, lower series), was degraded considerably in 49 hr (Fig. 18 e-f). If the temperature was elevated to 37°C (Fig. 19), the fibroin was degraded much faster even in the SCB buffer at pH 9.0. Figs. 19 a and b, 10 c, and 19 d show sedimentation patterns immediately, and after incubation at 37°C for 30, 60, and 120 min, respectively.

**DISCUSSION**

There has been much evidence suggesting that sericin is not a homogeneous protein, but a mixture of several components. From the tinctorial properties of the silk proteins in the middle silk gland, Yamanouchi (3) and Machida (4) have shown that there exist three kind of sericins which cover the fibroin core concentrically: the inner, middle, and outer sericin layers. Kikkawa (18) and Oba (19) have confirmed this finding and have further suggested that the sericins in the inner, middle, and outer layers are secreted exclusively in the posterior, middle, and anterior portion of the middle silk gland, respectively.
The present sedimentation analyses have shown that there exist three sericins in the extracts from the middle silk gland; sericins 1–3, though the existence of sercin 1 is still questionable. Further, it was suggested that sericins 1–3 are probably secreted from the anterior, middle, and posterior portion of the middle silk gland, respectively. This estimation is consistent with the above morphological suggestion.

There have been also a number of lines of biochemical evidence which suggest heterogeneity of sercin molecules. For example, Sheehan and Johnson (20) fractionated sericin into two parts by fractional precipitation with ammonium sulfate. Later, Mosher (21) and Bryant (22) concluded that there are three distinct sericins (A–C), of which sercin C is in the innermost layer, in intimate contact with the fibroin core. It is tempting to suppose that the sericins 1–3 correspond to sercin C, B, and A, respectively. Of course, since different methods of preparation of the sericins were used in these studies, this correspondence should be tested by future experiment.

Sedimentation analyses of fibroin were reported first by Holmes and Smith (6) who examined a solution of fibroin prepared with cupriethylene-diamine hydroxide solution by Coleman and Howitt's technique (5). They obtained evidence for only one component whose mean sedimentation coefficient was 2.6S. A similar value of 2.6S for the sedimentation coefficient of the main fraction in a solution of fibroin prepared by a similar technique was also obtained by Hayashi and Oda (8). Mercer (7) has shown the existence of 1.8, 1.5, and 1.0S components in alkaline extract (1% NaHCO₃) of the posterior silk gland. The present experiments, however, have clearly shown that the main component of the native fibroin is an ultracentrifugally homogeneous molecule whose standard sedimentation coefficient at infinite dilution (S₀) is 10S. The fibroin solutions also contain considerable amounts of heavier aggregates probably formed by reversible and/or irreversible aggregation of the 10S component. The low values of the sedimentation coefficient of fibroin reported by previous authors (6–8) are probably due to the degradation of fibroin molecules during preparation procedures, as will be discussed later.

It is interesting to examine whether the 10S component of the fibroin is a monomeric form of fibroin or is in a polymeric form bound by forces other than covalent bond. As described previously, extensive dilution of the solution, addition of monovalent salts such as LiBr and NaCl, or treatment with EDTA, urea, and guanidine hydrochloride has failed to dissociate the 10S component into smaller species. Therefore, we conclude tentatively that the 10S component is the monomeric species of the native fibroin molecules.

The molecular weight of fibroin has been determined by various methods. The data hitherto reported scatter over quite a wide range: 33,000 (5) by osmotic pressure measurement; 60,000 (6) by sedimentation-viscosity measurement; 250,000–450,000 (average 300,000) (10), 290,000 (11), and 1,000,000 (7) by light-scattering measurement; 278,000 (23) by the low-angle x-ray-scattering method; 84,000 (24) and 200,000–300,000 (25) by amino acid analysis. The fibroin solutions used for the previous studies were prepared by rather violent procedures, and it is probable that the fibroins may have suffered more or less extensive degradation during these procedures. This estimation is supported by our experiments which clearly showed the instability of native fibroin in warm alkaline buffer; even at pH 9.0, fibroin was degraded rapidly if the temperature was kept at 37°C.

In this experiment, native fibroin was extracted directly from the middle silk gland by very gentle procedures as described previously. The molecular weight of the monomeric species present, which most probably corresponds to the 10S component, was found to be between 3.2 and 4.2 × 10⁴, with a tendency to lie fairly close to 3.7 × 10⁴. This value is at the upper limit of the previous values of the fibroin which was prepared from raw silk or from cocoons without using cupriethylene-diamine hydroxide (10, 11, 23, 25). From these results, it is suggested that silk fibroin is secreted as a macromolecule ~3.7 × 10⁴ in molecular weight and is probably incorporated into silk fiber without a marked change in molecular weight.

The present experiments have shown that native fibroin is unstable in warm alkaline buffer, and several signs of degradation appeared in the sedimentation patterns of the native fibroin solution when treated with warm alkaline buffer: (a) broadening of the 10S boundary (Fig. 16 d); (b) decrease in the area of the 10S boundary in proportion to the aggregation of the 10S component (Fig. 18 e–f, Fig. 19 d); (c) slowing or decrease in the sedimentation velocity of the 10S compo-
component in the more alkaline solution (compare Fig. 17 with Fig. 18 and Fig. 19 with Fig. 20); and (d) appearance of a slower component as shown in Figs. 16 and 17. The third sign, or the slowing of the 10S component, is probably due to the unfolding of the fibroin molecules in the warm alkaline buffer. The fourth sign, or the appearance of a slow component, is quite interesting and could be interpreted in two ways. One is that this phenomenon represents simply the conformational change of the native fibroin molecules, as suggested in the previous case. The other possibility is that the native fibroin molecules are dissociated into subunits in warm alkaline buffer. It has been reported that some disulfide bonds of certain protein molecules are unstable in warm alkaline solution (26, 27). Recently we examined, therefore, the effects of sulfhydryl compounds such as β-mercaptoethanol and dithiothreitol upon theIOS component of the native fibroin and found that the 10S component is dissociated reversibly to the 6.8S component, ~1.7 × 10⁴ in molecular weight (28). This result suggests that each native fibroin molecule is composed of the two subunits, approximately equal in molecular weight, which are connected by one or by several disulfide bonds.

As described previously, the presence of a small amount of molecular species that are significantly smaller than the 10S component and are probably in reversible equilibrium with the 10S component has been suggested by the sedimentation velocity analyses with the interference optics as well as by the sedimentation equilibrium analyses. It was difficult, however, to find a reasonable interpretation for these observations. We have now shown that the 10S component is dissociated reversibly to the 6.8S component by treatment with the sulfhydryl compounds. Since all the sedimentation analyses in this paper were carried out in a weakly alkaline buffer of pH 9.0 in which disulfide bond(s) of the native fibroin is (are) likely to be unstable, it is quite probable that a small amount of the 10S component was dissociated reversibly to the 6.8S component in dilute solution, and was detected by the sedimentation velocity as well as by the sedimentation equilibrium analyses. Dissociation of the native fibroin by treatment with sulfhydryl compounds will be reported in detail in the following paper (28).

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