Structure of Micelles Formed by Monodisperse Hexa-(γ-benzyl-L-glutamate) in Solution

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Synopsis

The angular dependence of light scattering and the concentration dependence of the relative viscosity have been measured in solutions of *o*-nitrophenylthio-hexa-(γ-benzyl-L-glutamate) ethylamide in ethylene dichloride. Both the reduced intensity of scattered light and the reduced viscosity of the solution suddenly increase above a certain critical concentration, below which both of them remain low and constant. The Debye plot of light scattering indicates that primary micelles having an aggregation number 48 are formed at the critical micelle concentration and that secondary micelles, each consisting of 294 molecules, then appear in increasing amounts with increasing concentration beyond the critical micelle concentration. The secondary micelle is rodlike and has a length of 1170 Å, if it is rigid. An analysis of the reduced viscosity leads to the intrinsic viscosity for the primary micelle, 0.360 dL g^{-1}, and to that of the secondary micelle, 1.28 dL g^{-1}. If the secondary micelle is represented by a prolate ellipsoid, it should have an axial ratio of 47. If the polypeptide chains are extended in the micelle, the observed aggregation number and axial ratio of the secondary micelle can well accommodate the intermolecularly hydrogen-bonded in-register β-structure of antiparallel chains. In the primary micelle, some folded polypeptide chains are involved, and an intermolecularly hydrogen-bonded out-of-register structure would form a rather open network.

INTRODUCTION

It has been shown that some monodisperse oligopeptides can form large aggregates or micelles in solution above a certain critical concentration,[1,2] and that some others undergo a conformational change mainly related to the formation of β-structure above a critical concentration.[3] The concomitant occurrence of both phenomena has recently been observed by means of ir absorption spectra[4] and low-angle laser light scattering[5] on *o*-nitrophenylthio-hexa-(γ-benzyl-L-glutamate) ethylamide in dioxane and ethylene dichloride solutions. In dioxane the hexapeptide forms small micelles, each consisting of about 15 molecules, when its concentration exceeds a critical concentration, 0.38 g dL^{-1}. In ethylene dichloride, however, it forms small primary micelles at a critical micelle concentration, 0.075 g dL^{-1}, and these primary micelles further associate into secondary micelles at higher concentrations. The aggregation numbers of the small and large micelles were found to be 53 and 330, respectively. In both solutions the micelle formation accompanies hydrogen bonding between peptide groups.

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In order to elucidate the structure and equilibrium of these two kinds of oligopeptide micelles in solution, we have measured the angular dependence of light scattering and the relative viscosity of solutions of *o*-nitrophenylthio-hexa-(γ-benzyl-L-glutamate) ethylamide dissolved in ethylene dichloride. The homopeptide has a molecular weight of 1514.

EXPERIMENTAL

Materials

The sample of *o*-nitrophenylthio-hexa-(γ-benzyl-L-glutamate) ethylamide was the same as previously prepared and used.[4,5] Ethylene dichloride was redistilled over CaH_2 and used immediately. The concentration of solutions was determined either from the weight of dissolved sample or by measuring the absorbance of solutions. For the latter procedure, an observed value of the molar extinction coefficient, $\epsilon_{376} = 3822$ L mol^{-1} cm^{-1}, was taken at concentrations lower than 0.03 g dL^{-1}.

Light Scattering

The angular dependence of light scattering was measured on a Shimadzu light scattering photometer, PG-21, using the unpolarized green wavelength, 546 nm, of a mercury lamp. The blue wavelength of the mercury lamp was absorbed by the yellow solutions of the hexapeptide and could not be used for the measurement. A cylindrical cell, which required about 35 cm^3 of solution for the measurement, was put in a cell housing, and water of constant temperature was circulated through the brass housing to keep the temperature of solution or solvent at 25 ± 0.05°C. The temperature was measured by dipping a thermistor tip directly into the solution or solvent.

The solutions were prepared by dissolving the sample into the solvent or by mixing a solution with the solvent or another solution and then kept overnight. The solutions and solvent were filtered through a Millipore filter FGLP (47 mmϕ) having pore size of 0.20 μm under a pressure gently applied by a manual pneumatic pump. The filtration was usually repeated more than five times, when the solution or solvent became dust-free. The concentration of solution was then determined spectrophotometrically.

The apparatus was calibrated at 25°C in the same way as previously described,[6] by using benzene as a standard having $R_{90} = 16.3 \times 10^{-6}$ cm^{-1}.[7] It was confirmed that the hexapeptide did not fluoresce in ethylene dichloride solution when it was irradiated at 376 nm.

The refractive index increment was measured on a Shimadzu differential refractometer DR-3, Brice type, at the 546 nm wavelength of a mercury lamp at 25 ± 0.05°C. The apparatus was calibrated by the use of aqueous solutions of NaCl.[8] The refractive index increment of the solutions of the hexapeptide in ethylene dichloride was found to be 0.106_6 cm^3 g^{-1}.

Relative Viscosity

The relative viscosity of the solutions was measured by means of an Ubbelohde viscometer capable of fivefold dilution and having a flow time 85 s for ethylene dichloride at 25°C. The relative viscosity of a solution was obtained as the ratio of flow times for the solution and the solvent, without applying correction for density difference.

The flow time of a solution exhibited some change with time after it was prepared, especially when its concentration was high, and the equilibrium value was obtained after it was kept overnight. The flow time was reproducible, irrespective of whether the solution had been prepared by dilution of a solution with the solvent or by its concentration due to evaporation of solvent by evacuation in a desiccator with a water aspirator.

RESULTS

Light Scattering

Figure 1 shows the variations of reduced intensity, R_{90}, and dissymmetry, z_{45}, with hexapeptide concentration, c (g dL^{-1}). The scattering intensity suddenly increases above 0.075 g dL^{-1}, which can be identified with the critical micelle concentration, c_0. That is, the hexapeptide forms aggregates that can be called micelles. The convex curvature downward means that the primary micelles formed at the critical micelle concentration associate together to form larger aggregates, i.e., secondary micelles, with further increases in concentration. The observed aspects are in complete agreement with those previously found.[5]

The dissymmetry also increases above the critical micelle concentration and reaches a constant value 1.22_7. This increase in dissymmetry indicates that the primary micelles associate together to form the secondary micelles and that the secondary micelle has a dimension larger than 273 Å or $1/20$ of the wavelength of light. The constant dissymmetry attained means that the secondary micelle has a definite size and does not grow with increasing concentration.

Figure 2 shows the angular dependence of light scattering from the micellar solutions of the hexapeptide. When the solution is dilute, the light scattering is independent of scattering angle at low angles but becomes dependent on it at high angles. At an intermediate concentration the angular dependence is negative and is anomalous at low angles. On increasing the concentration further, the linearly dependent part occurs at a lower angle and spreads over a wider region. Above 0.200 g dL^{-1}, the angular dependence becomes positive and normal, giving a uniform slope.

The dual nature of the angular dependence of light scattering with the micellar solution would be related to the presence of two kinds of micelles, one being smaller and formed at the critical micelle concentration, and the other being larger and formed at higher concentrations. Similar behavior of angular dependence was observed with the micellar solutions of sodium

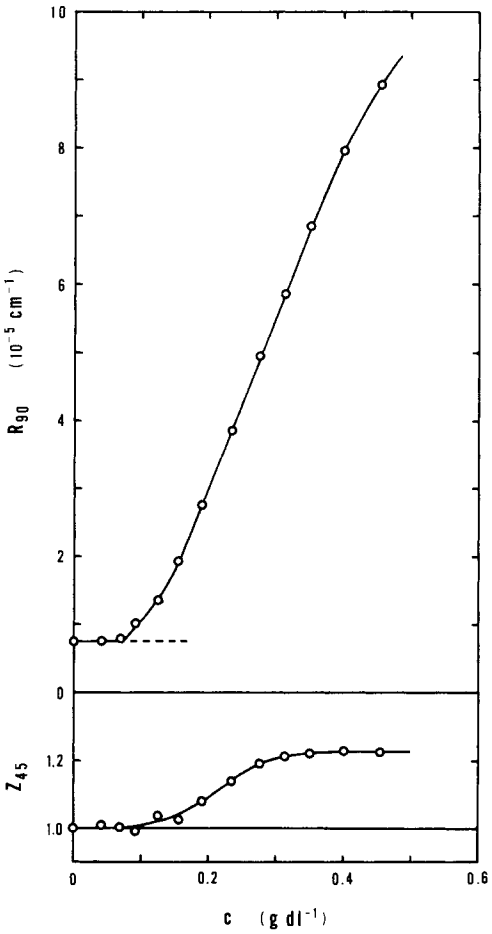

Fig. 1. The variations of reduced intensity and angular dissymmetry of light scattering with hexapeptide concentration. Top: reduced intensity, R_{90}; bottom: dissymmetry, z_{45}.

dodecyl sulfate in concentrated sodium halide solutions.[6,9] Although quantitative explanation for the mechanism of this behavior must await further investigation, the effect of anisotropic units in the micelles would be responsible for it.[10–14] The negative angular dependence found at an intermediate concentration would also have the same origin.

The Debye plot of light scattering at the 0° direction is obtained by simple extrapolation, as shown in Fig. 3. The Debye plot in the 90° direction is also included in Fig. 3. In the following, we assume that the effect of anisotropy is negligible in the 0° direction.

From Fig. 3 it is seen that the primary micelles having a moleular weight of M_m = 73,000, or an aggregation number m = 48, are formed at the critical micelle concentration, and that with increasing concentration, the larger secondary micelles are also brought about. We may put forward as the simplest mechanism the two-step micellization such as expressed by

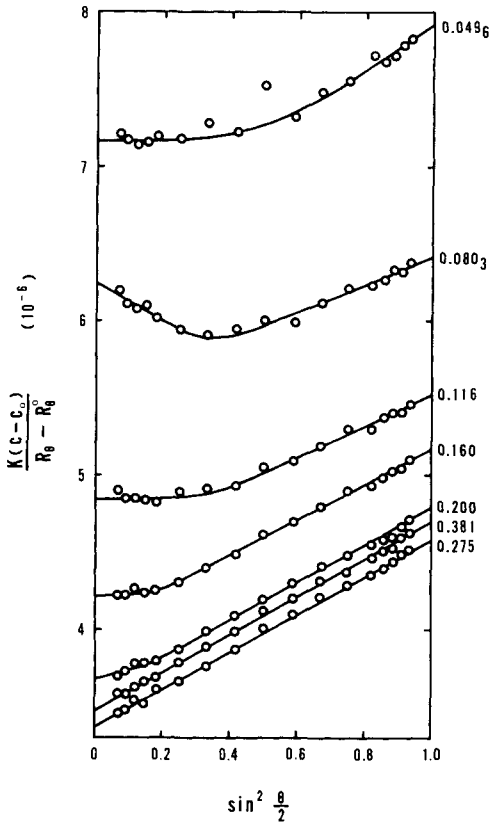

Fig. 2. Angular dependence of reciprocal envelope of light scattering from micellar solutions. Micelle concentrations, $c - c_0$ (g dL^{-1}), are given at the right side of curves.

$$mA \rightleftharpoons A_m \tag{1}$$

$$nA_m \rightleftharpoons A_{mn} \tag{2}$$

where A is the monomer and A_m and A_{mn} are the primary and secondary micelles, respectively. Then the weight-average molecular weight, $\overline{M}_w$, of micelles at a given concentration, c, which can be obtained from the reciprocal value of the Debye plot after correcting for the second virial coefficient, is represented by

$$\overline{M}_w = \frac{M_m c_m + M_{mn} c_{mn}}{c - c_1} \tag{3}$$

where c_1 is the concentration of monomer, which is substantially equal to c_0, and c_m and c_{mn} are the concentrations of primary and secondary micelles having molecular weights, M_m and M_{mn}, respectively. (Do not confuse $\overline{M}_w$ here with M_w in the previous paper,[5] where we included the monomer in addition to the two kinds of micelles.) In the present system, the value of light-scattering second virial coefficient, $2B$, is assumed to be 1.0×10^{-4}

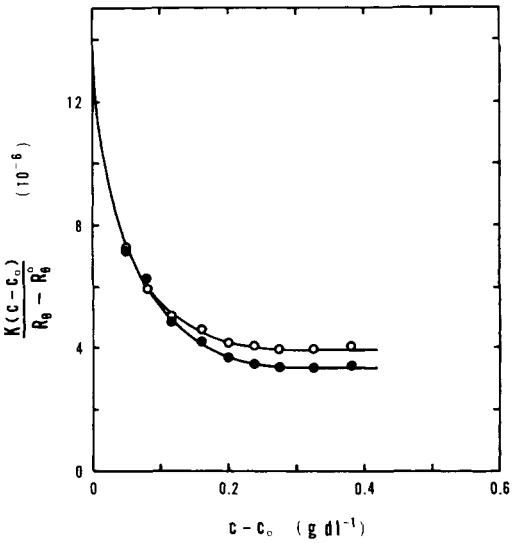

Fig. 3. Debye plots for the hexapeptide solutions at the 0° and 90° directions. ●, 0°; ○, 90°.

$cm^3\ g^{-1}$, which will prove to be appropriate for the micelle size and shape obtained.

In order to estimate the molecular weight of the secondary micelle, Eq. (3) is rearranged into

$$\overline{M}_w = M_{mn} - (M_{mn} - M_m)c_m/(c - c_1) \tag{4}$$

If the total concentration is far beyond the critical micelle concentration, c_m may be almost constant, and $\overline{M}_w$ is linearly dependent on $1/(c - c_0)$. From such a plot it is found that the secondary micelle has a molecular weight, M_{mn} = 445,000, or an aggregation number, mn = 294.

Before proceeding further, we examine the presence of two kinds of micelles by observing the monodispersity of micelles at the critical micelle concentration and at the concentrated extremum. While the light scattering directly gives the weight-average molecular weight, $\overline{M}_w$, at a given concentration, the number-average molecular weight of micelles can be calculated by

$$\overline{M}_n = \frac{c - c_0}{\int_0^{c-c_0} d(c - c_0)/\overline{M}_w} \tag{5}$$

at a given concentration.[15] Then an index of polydispersity, $\overline{M}_w/\overline{M}_n$, of the micelle can be derived as a function of concentration. Figure 4 shows such a plot and indicates the presence of a single kind of micelle at each of the concentration extrema, and the equilibrium between the two micelles at the concentration range examined.

The concentrations of the primary and secondary micelles can then be evaluated from the weight-average molecular weight by

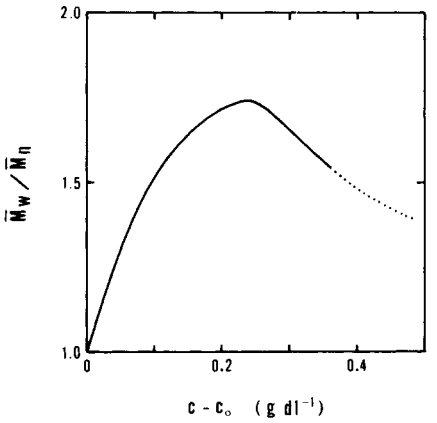

Fig. 4. Variation of polydispersity index, $\overline{M}_w/\overline{M}_n$, of micelle with its concentration.

$$c_m = \frac{M_{mn} - \overline{M}_w}{M_{mn} - M_m}(c - c_0) \tag{6a}$$

$$c_{mn} = \frac{\overline{M}_w - M_m}{M_{mn} - M_m}(c - c_0) \tag{6b}$$

From the initial slope of the angular dependence of light scattering at higher concentrations, the root-mean-square light-scattering average radius of gyration, $(\overline{R_G^2})_{ls}^{1/2}$, of the micelles can be obtained. The mean-square radius is represented by

$$(\overline{R_G^2})_{ls} = \frac{M_m R_{G,m}^2 c_m + M_{mn} R_{G,mn}^2 c_{mn}}{\overline{M}_w (c - c_0)} \tag{7}$$

where $R_{G,m}$ and $R_{G,mn}$ are the radii of gyration of primary and secondary micelles, respectively.[16] Equation (7) is rearranged into

$$\overline{M}_w(\overline{R_G^2})_{ls} = M_m R_{G,m}^2 + (M_{mn} R_{G,mn}^2 - M_m R_{G,m}^2)\frac{c_{mn}}{c - c_0} \tag{8}$$

Equation (8) indicates that $\overline{M}_w(\overline{R_G^2})_{ls}$ is linear against $c_{mn}/(c - c_0)$. Since $M_m R_{G,m}^2$ is so small that it may be put zero, the slope of the straight line passing the origin should give the value of $M_{mn} R_{G,mn}^2$. In this way the radius of gyration of the secondary micelle, $R_{G,mn}$, is obtained as 338 Å.

Considering the estimated value of molecular weight, the secondary micelle can be fit most adequately to a rodlike shape, rather than to a sphere or a random coil. Assuming a rigid rod, the length of the secondary micelle is 1170 Å. It is also noted that the observed dissymmetry value leads to a length, 1040 Å, of a rigid rod.[17]

The excluded-volume effect of the rod having a length of 1170 Å and a radius of 10 Å is found to give the second virial coefficient, $B = 4 \times 10^{-5}$ cm^3 g^{-1}, for its dilute solution,[18] which is close to the value assumed above.

Table I summarizes the data on the primary and secondary micelles obtained from light scattering.

TABLE I
Micelle Size and Shape Derived from Light Scattering

Micelle	Molecular Weight	Aggregation Number	Radius of Gyration (Å)
Primary	73,000	$48._2$	(<273)
Secondary	445,000	294	338

Relative Viscosity

Figure 5 shows the reduced viscosity of the hexapeptide solution as a function of concentration. It is remarkable to see that a critical concentration exists, above which the viscosity suddenly increases. The critical micelle concentration, c_0, is 0.084 g dL^{-1}, below which the reduced viscosity remains equal to the intrinsic viscosity, $[\eta] = 0.039$ dL g^{-1}.

In general, the reduced viscosity of solutions of solute capable of forming micelles should be constant at concentrations where no micelles are yet formed. Nevertheless, the relative or reduced viscosity of aqueous solutions of surfactant has never exhibited such behavior, but the reduced viscosity has already increased with increasing concentration at low concentrations and has given some kind of break at the critical micelle concentration.[19-23] It was recently pointed out[23] that such a discontinuous change in reduced viscosity of surfactant solutions should have been caused by the effect of surface tension in the capillary viscometer; the surface tension of surfactant solutions strongly decreases with increasing concentration up to the critical micelle concentration. The present observation for the hexapeptide solution, which is free from such an effect of surface tension, has definitely confirmed this supposition. On the other hand, if the effect of surface tension could be corrected in the viscometry for aqueous solutions of surfactant, we should have such behavior as observed here.

In order to obtain the reduced viscosity of micellar solution, the relative viscosity of a solution referred to the solution of the critical micelle con-

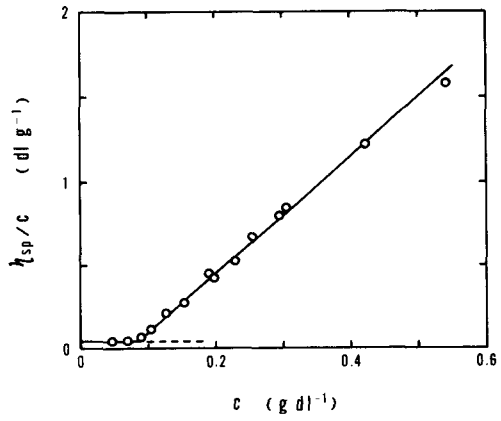

Fig. 5. Reduced viscosity of the hexapeptide solutions.

centration, $\eta_{rel,cmc}$, is calculated, and then the reduced viscosity of micellar solution, $(\eta_{rel,cmc} - 1)/(c - c_0)$, is readily obtained as a function of micellar concentration. Figure 6 shows the reduced viscosity of the micellar solution of the hexapeptide.

A straight line can be drawn, and, if the reduced viscosity is the weight-average of reduced viscosities of the two kinds of micelles, it can be represented by

$$\frac{\eta_{rel,cmc} - 1}{c - c_0} = \left(\frac{\eta_{sp,m}}{c_m}\right)\frac{c_m}{c - c_0} + \left(\frac{\eta_{sp,mn}}{c_{mn}}\right)\frac{c_{mn}}{c - c_0} \tag{9}$$

where $\eta_{sp,m}$ and $\eta_{sp,mn}$ are the specific viscosities of (hypothetical) micellar solution containing only the primary or secondary micelles, respectively. The extrapolation of the straight line to infinite dilution leads to the intrinsic viscosity of the primary micelle, $[\eta]_m$, which is found to be 0.360 dL g^{-1}.

To estimate the intrinsic viscosity of the secondary micelle, it is assumed that the reduced viscosity of the solution of primary micelle is equal to its intrinsic viscosity, since the micelle is small and its concentration dilute, and that the reduced viscosity of the solution of secondary micelle is linearly dependent on concentration,

$$\eta_{sp,m}/c_m = [\eta]_m \tag{10a}$$

$$\eta_{sp,mn}/c_{mn} = [\eta]_{mn} + k'_{mn}[\eta]^2_{mn}c_{mn} \tag{10b}$$

where $[\eta]_{mn}$ is the intrinsic viscosity of the secondary micelle and k'_{mn} is its Huggins constant.

Equation (9) is rearranged by introducing Eqs. (10) and converted into the equation for the reduced viscosity of the solution of secondary micelle:

$$\frac{\eta_{rel,cmc} - 1 - [\eta]_m c_m}{c_{mn}} = [\eta]_{mn} + k'_{mn}[\eta]^2_{mn}c_{mn} \tag{11}$$

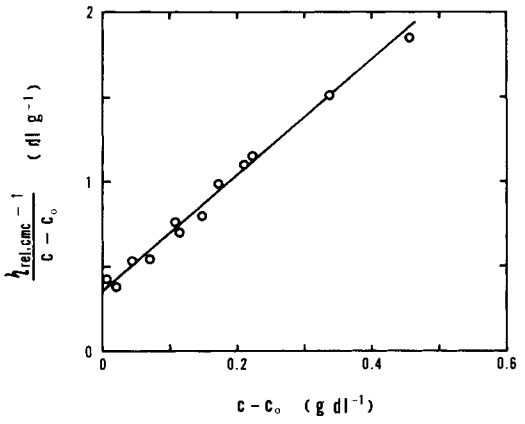

Fig. 6. Reduced viscosity of micellar solutions.

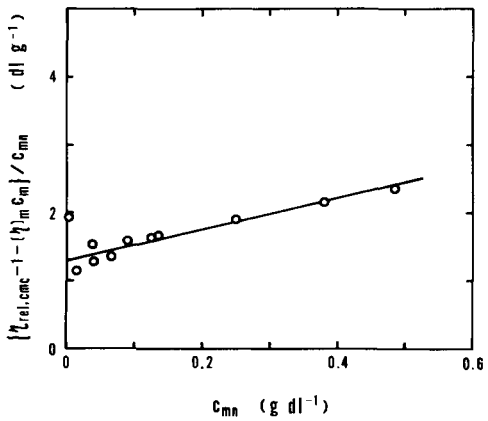

Fig. 7. Reduced viscosity of solutions containing only secondary micelles.

Figure 7 gives the plot of the left-hand side of Eq. (11) against c_{mn}, which can be drawn by a straight line, as given by Eq. (11). The intrinsic viscosity of the secondary micelles, $[\eta]_{mn}$, is estimated from the intercept to be 1.28 dL g^{-1}, and from the slope the Huggins constant, k'_{mn} = 1.41 is obtained.

Light-scattering results indicate that the secondary micelle is rodlike, and therefore, it can be approximated by a prolate ellipsoidal shape. Using the Simha equation,[24,25] together with the observed value of partial specific volume, 0.812 cm^3 g^{-1}, for low-molecular-weight poly(γ-benzyl-L-glutamate) hexylamide, $[A]/[I]$ = 4,[26,27] dissolved in dioxane, the axial ratio of the rigid ellipsoid is found to be 47.

If the primary micelle could be approximated by an ellipsoid, it should be prolate, and its axial ratio would be 22. For both the primary and secondary micelles, no oblate ellipsoidal shape corresponding to the observed aggregation numbers is possible.

Table II summarizes the data derived from the relative viscosity of the hexapeptide solutions.

DISCUSSION

Based on the observed data for the micelle size and shape, we will build the possible structure of the primary and secondary micelles, assuming the conformation of hexapeptide molecules in the micelles. (We revise the

TABLE II
Micellar Parameters Derived from Relative Viscosities

Micelle	Intrinsic Viscosity (dL g^{-1})	Axial Ratio of Prolate Ellipsoid
Primary	0.360	22
Secondary	1.28	47

models proposed previously,[5] which were built on a knowledge of the aggregation number and the content of β-structure or the fraction of hydrogen-bonded residues.)

We first consider the structure of the secondary micelle. It is postulated that all the hexapeptide chains in the secondary micelle are extended and a single chain has the following dimensions:

32 Å in the extended main-chain direction,

4.74 Å in the hydrogen-bond direction,[28]

15.48 Å in the side-chain direction.[28]

The side-chain spacing was taken from x-ray diffraction data and would not represent the length of an extended side chain nor that of a side chain in solution. However, it will not be far from an average length of the side chain in solution.

Ir spectral studies[4,5] revealed that almost all the peptide groups are hydrogen-bonded in solutions containing sufficient amounts of secondary micelle. Consequently, we may construct a model of the secondary micelle, in which the extended hexapeptide chains are hydrogen-bonded intermolecularly to form the in-register β-structure of antiparallel chains.

When the hexapeptide molecule is represented by a rectangular parallelopiped having three edges given above and 294 hexapeptide molecules are held together by the hydrogen bonds, then a rodlike structure having a length of 1390 Å is built as the secondary micelle. If the long, rodlike parallelopiped is substituted by a prolate ellipsoid having the equal length and volume, its axial ratio is 45, which is quite close to the observed value. Thus we propose a structure for the secondary micelle illustrated in Fig. 8. The β-sheet of the hexapeptide formed in ethylene dichloride solutions looks like a long rod. It is also noted that no stacking of the β-sheets is involved in the secondary micelle. The rather rigid structure of the secondary micelle is in conformity with the low magnitude of standard free energy of secondary association, -160 cal mol^{-1}, previously obtained[5] because the enthalpy gain due to the hydrogen bonding, about -6000 cal mol^{-1}, is supplemented by the large entropy loss.

We can imagine an alternative structure that conforms to the data observed here. It consists of a stacked pile of 98 β-sheets, each sheet composed of 3 hexapeptide molecules held together by hydrogen bonds. However, the fraction of hydrogen-bonded residues is unacceptably low when compared with the ir spectral data.

On the other hand, we do not have sufficient information on the shape of the primary micelle and its state of hydrogen bonding. If the hexapeptide chains are extended in the primary micelle, 48 hexapeptide molecules held together intermolecularly could form a structure having a prolate ellipsoidal shape, but its axial ratio proves to be too low, when compared with the observed value.

If we postulate that the hexapeptide molecules have a β-turn at the middle portion and have the intramolecularly hydrogen-bonded β-structure at both ends, we may construct a model for the primary micelle, to which

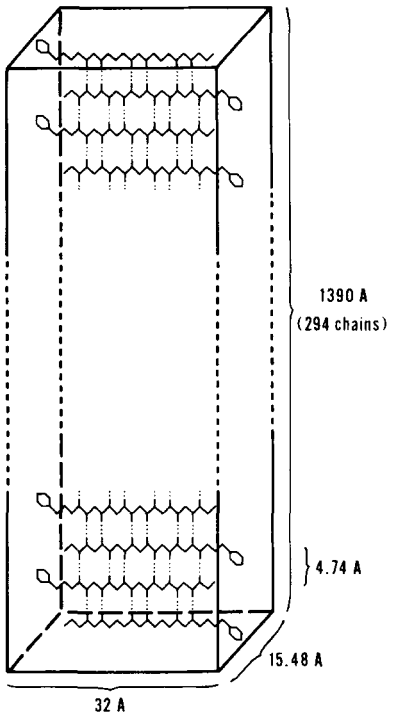

Fig. 8. Model proposed for the secondary micelle of the hexapeptide in ethylene dichloride solutions.

a prolate ellipsoid having the observed axial ratio is hydrodynamically equivalent. In such a conformation the hexapeptide molecule has the following dimensions:

24 Å in the main-chain direction,

9.48 Å in the hydrogen-bond direction,

15.48 Å in the side-chain direction.

If 48 hexapeptide molecules are held together by intermolecular hydrogen bonds in an antiparallel way and in register, a rodlike parallelopiped having a length of 455 Å is found. The hydrodynamically equivalent prolate ellipsoid has an axial ratio of 17, which is somewhat smaller than the observed value. The ellipsoid has a radius of gyration of 102 Å, which is certainly smaller than $1/20$ of the wavelength of the light. However, such a rigid structure is not compatible with the ir spectral data,[4,5] which suggest the presence of small fractions of hydrogen-bonded residues in the primary micelle. Consequently, we propose for the primary micelle a more open, hydrogen-bonded network structure in which folded hexapeptide chains are involved.

High-resolution proton magnetic resonance can give some detailed information on hydrogen-bonded states of peptide groups, and such studies have been performed on various oligopeptides in solutions.[29–32] Recent

work on Boc-oligo-L-norvaline methyl ester and Boc-oligo-L-methionine methyl ester in deuterochloroform solutions revealed the states of these peptide chains in solution, whether they are intramolecularly hydrogen-bonded and folded or intermolecularly held together and extended.[31,32] These results indicated that these oligopeptides higher than tripeptides have an increasing tendency to have the in-register β-structure with increasing concentration, while they are folded and associated together when the concentration is low. These conclusions are consistent with the present observation.

Vacuum-uv CD on solid films of Boc-oligopeptide methyl ester having alkyl side chains showed that hexa and heptapeptides are in the β-structure of antiparallel chains, when the side chain is small as for Ala, but with bulky side chains, they take up the β-structure, in which both the parallel and antiparallel chains are present.[33-35] In the present series of works, it was shown that the hexapeptide of γ-benzyl-L-glutamate has the β-structure of antiparallel chains in ethylene dichloride solution, as well as in solid state, despite having the bulky side chain. Although it is uncertain whether the parallel chains are completely absent from the β-structure, the deviation would be due to the polar nature of side chain of the hexa(γ-benzyl-L-glutamate).

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