Biochimica et Biophysica Acta, 1023 (1990) 254–265
Elsevier

BBAMEM 74773

Purification of octyl β-D-glucopyranoside and re-estimation of its micellar size

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(Received 6 October 1989)

Key words: Octyl β-D-glucopyranoside; Detergent purification; Micellar size; Protein solubilization; Membrane reconstitution

The commercial non-ionic detergent octyl β-D-glucopyranoside is often contaminated by significant amounts of UV absorbing and/or ionic compounds that can associate with membrane proteins. Such impurities can be monitored by several techniques (i.e., spectrophotometry, size exclusion chromatography, and pH, conductivity, and surface tension measurements) and can be removed using mixed-bed ion exchange chromatography. High performance size exclusion chromatography, dynamic light scattering, and ultracentrifugation have been used to re-estimate the size of micelles of octyl β-D-glucopyranoside since previously published data varied over a wide range. Aggregation numbers were 27 to 100 for micellar molecular weights 8000 to 29 000. Direct physical methods that do not perturbate the sample indicated a large size for the micelles (hydrodynamic radius 23 ± 3 Å; M_r 22 000 ± 3000; aggregation number 75 ± 10 for a 34 mM aqueous solution). In contrast the chromatographic micellar size appeared to be smaller (hydrodynamic radius 15 ± 1 Å; M_r 8000 ± 1000; aggregation number 27). This underestimation may be the result of adsorption and/or alteration of the micelles.

Introduction

During the last ten years the non-ionic detergent 1-*O*-*n*-octyl β-D-glucopyranoside has proven to be useful in the study of hydrophobic proteins. It efficiently solubilizes most membrane proteins and is compatible with most enzymatic activities. It has been used in the final step of the functional reconstitution of membrane proteins solubilized by stronger detergents and therefore has been studied when incorporated in mixed micelles with lipids (see, for example, Ref. 1). The detergent has been added in the mobile phase during fractionation of hydrophobic proteins, either by low pressure or by high performance liquid chromatography (see, for example, Ref. 2). Finally octyl glucoside has been used successfully for crystallization of several membrane proteins and membrane associated-protein complexes (see, for example, Refs. 3 and 4). It can also have a beneficial effect on the growth of crystals of soluble proteins [5]. In contrast to some other common detergents, it is a chemically well defined compound of small size (M_r 292). It is very soluble in aqueous solutions and is easily removable by dialysis. Its critical micellization concentration is about 20–25 mM [6]. It is commercially available as a white powder typically 98–99% pure. Beside peroxides and octanol which are often controlled by the manufacturers, other contaminants which can bind to membrane proteins, occur frequently in octyl glucoside. Such low levels of contamination cannot easily be detected by standard thin-layer chromatography but they can be monitored by spectrophotometry, by size exclusion chromatography or by measurement of pH and/or conductivity of a detergent solution. In addition, surface tension measurements can indicate the presence of surface-active impurities.

Although numerous studies have been devoted to octyl glucoside, its micellar size remains unclear. Published values for aggregation number or molecular weight of the micelles cover a wide range. Lässer and Elias [7] have reported aggregation numbers of 83 and 84 from sedimentation equilibrium and light scattering measurements, respectively. In the same series of studies, a hydrodynamic radius of 41 Å was obtained from viscosity measurements [8]. Using gel filtration chromatography, Rosevear et al. [9] and Grabo [10] have found a small micellar size (M_r 8000, aggregation number = 27) and an apparent hydrodynamic radius of 15 Å [11]. An aggregation number of 38 was obtained by

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extrapolation from the values obtained by measuring resonance energy-transfer efficiency between two fluorescent lipid probes present in trace amounts in mixed micelles [12]. (An aggregation number of 100 has also been reported without experimental details [13].)

The crystallization of membrane proteins requires very pure detergent and an accurate estimation of the size of the detergent micelles. A purification technique based on ion exchange chromatography which removes the above contaminants has been developed. Because of discrepancies concerning the size of the micelles, experiments have been performed to clarify existing data. High performance size exclusion chromatography, dynamic light scattering and ultracentrifugation methods were used to estimate the micellar size of purified octyl glucoside. The results of these investigations are presented here. Part of this work has been presented as a poster at the Third International Conference on the Crystallization of Biological Macromolecules (College Park, Maryland, 13–19 August, 1989).

Materials

Chemicals

Octyl β-D-glucopyranoside was purchased from Boehringer (Mannheim, F.R.G.) (Cat. No. 737674), from Calbiochem Behring Diagnostics (La Jolla, CA) (Cat. No. 494459), from Serva (Heidelberg, F.R.G.) (Cat. No. 31055), from Sigma (St. Louis, MO) (Cat. No. 0-8001), and from Fluka (Buchs, Switzerland) (Cat. No. 75083). The lot numbers of the analyzed samples are indicated where mentioned.

Water was purified through an Ultrapure cartridge kit from Millipore Corp. (Bedford, MA). Ethanol, methanol, sulfuric acid and phenol were from Fisher Scientific Company (Fair Lawn, NJ). Pharmalytes were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Bistris buffer (bis[2-hydroxymethyl]imino-tris[hydroxymethyl]methane) and Tris buffer (tris[hydroxymethyl]aminomethane) were from Sigma (St. Louis, MO). Other chemicals obtained from Fisher Scientific Company (ACS grade) were used without further purification. Solutions were made with freshly deionized water and were filtered over 0.22 μm nylon filters (Rainin, Emeryville, CA).

Proteins

Bacteriorhodopsin was solubilized from *Halobacterium halobium* purple membrane as described by Muccio and DeLucas (1985) [2]. The molecular weight and apparent hydrodynamic radius for proteins (used for calibration of the size exclusion columns) are given as used for the calculations. Bovine thyroglobulin (660000; 85 Å), hen ovalbumin (43000; 30.5 Å), and horse myoglobin (17000; 19 Å) were obtained from Bio-Rad (Richmond, CA; Cat. No. 151-1901). Bovine pancreas ribonuclease A (13700; 16.5 Å), chymotrypsinogen A (25000; 21 Å) and bovine serum albumin (67000; 35.5 Å) were obtained from Pharmacia (Piscataway, NJ; Cat. No. 17-0442-01). Human transferrin (81000; 30.5 Å) was obtained from Technimed Corp. (Fort Lauderdale, FL; Cat. No. 070351) and bovine lung trypsin inhibitor (6500; 12 Å) from Serva (Heidelberg, F.R.G.). Bovine proteins, heart cytochrome *c* (13400; 17 Å), insulin beta chain (3500; 8 Å), lung trypsin inhibitor (6500; 12 Å), and ubiquitin from red blood cells (8500; 13 Å) were obtained from Sigma (St Louis, MO; Cat. No. C-2037, I-6383, and U-6253, respectively).

Chromatographic material

The mixed-bed chromatographic support Rexyn I-300 (H-OH), a mixture of strong acid cation exchanger in hydrogen form and of strong base anion exchanger in hydroxyl form, was obtained from Fisher Scientific Company (Cat. No. R208-500). The analytical size exclusion HPLC columns Bio-Sil TSK 125 were purchased from Bio-Rad (Richmond, CA) (Cat. No. 125-0060). Their characteristics were: size 7.5 × 300 mm; volume 13.3 ml; particle size 10 μm; pore size 125 Å; useful M_r 5000–100000.

Methods

Size exclusion chromatographic analysis of octyl glucoside

For analysis of the purity of the detergent a Bio-Sil TSK 125 size exclusion column was equilibrated with one of following mobile phases: (i) water; (ii) a solution of 1% (w/v) octyl β-D-glucopyranoside (lot No. 710235 from Calbiochem Behring Diagnostics) in water; (iii) a solution containing 0.1 M KCl and 10 mM Bistris buffer adjusted at pH 6.8 with HCl. The flow rate was 0.6 ml/min for all experiments. Samples containing 50 mg detergent freshly dissolved in 500 μl water (= 10% (w/v) solution) were injected. The absorbance at 280 nm was recorded.

Size exclusion chromatography of detergent micelles was performed after calibration of the column in a buffer solution containing 0.1 M sodium sulfate and 0.02 M sodium phosphate (pH 6.8). The partition coefficient K_d was calculated so that

$$K_d = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume, V_0 the void volume and V_t the total volume of the column. It is generally accepted that the hydrodynamic radius (R_h) is the dominant parameter in size exclusion chromatography since it takes into account mass and shape characteristics which are reflected in the frictional coefficient and in the diffusion coefficient. Values of R_h were plotted versus values of the inverse error function of $(1 - K_d)$

as described by Nalecz et al. [14]. Void volume and total volume of the packed column were obtained from the elution of thyroglobulin and vitamin B-12, respectively. Values of the micellar molecular weight were obtained from the plot of R_h versus M_r for reference proteins and were corrected for the specific volume of the molecules.

Three mobile phases were used for the chromatography of octyl glucoside micelles: (a) water; (b) 0.2 M sodium chloride and 0.02 M Bistris-HCl buffer (pH 6.5); (c) 0.1 M sodium sulfate with 0.02 M sodium phosphate (pH 6.8). In order to stabilize the micelles, mobile phases contained 34 mM octyl glucoside. Absorbance of the eluant was monitored at 280 nm. Octyl glucoside was detected in the fractions using the colorimetric glucoside assay as described by Dubois et al. [15]. To 10-μl fraction aliquots, 1 ml of a 5% (v/v) phenol solution in water and 2 ml of concentrated sulfuric acid was added. This solution was mixed and allowed to cool. Absorbance of the orange colored product was read at 490 nm after 30 min. Absorbance was proportional to the amount of octyl glucoside in the range 5–150 μg and stable for at least 12 h.

Samples containing 10 to 100 mg octyl glucoside dissolved in 500 μl mobile phase or various volumes (100 to 500 μl) of a 10% (w/v) solution were analyzed by size exclusion chromatography. The flow rate was 0.6 ml/min under standard conditions; other flow rates in the range 0.15 to 1.2 ml/min were used to study the effect on elution profiles.

Purification of octyl glucoside by ion exchange chromatography

Octyl glucoside was purified in one step by chromatography on a mixed-bed strong ion exchange resin Rexyn I-300 (H-OH form). For purification of 5 g of detergent, the size (length × i.d.) of the column was 150 mm × 10 mm (volume 12 ml). The column was prepared in water and washed with 10 bed volumes of ethanol followed by 50 bed volumes of water at a flow rate of 1 ml/min. The detergent was dissolved in water at a concentration of 10% (w/v) and allowed to flow through the column at a flow rate of 0.2 ml/min. At flow rates higher than 0.5 ml/min significant amounts of contaminants were found in the fractions. Once the detergent was loaded, the column was washed with water and the eluate was collected until two times the volume of the sample was reached. The eluate was then lyophilized and the purified detergent stored in brown glass vials at −20 °C.

Surface tension measurements

The apparent surface tension of detergent solutions was measured with a manual surface tensiometer (Model 20, Cat. No. 14-812, Fisher Scientific Company, Pittsburg, PA). The instrument uses the ring removal method described by Lecomte du Noüy [16]. In a glass vial of 50 mm i.d., known volumes (5 to 200 μl in one step) of a freshly prepared 10% (w/v) detergent solution were added progressively to 20 ml of water. The solution was mixed with a Pasteur pipet. Duplicate measurements of apparent surface tension at 22 °C (read directly in dyn/cm) displayed a variance of ±0.5 dyn/cm. The critical micellization concentration corresponds to the detergent concentration for which an inflection occurs in the graph of apparent surface tension versus logarithm of the detergent concentration.

Preparative isoelectric focusing

The behavior of the membrane protein bacteriorhodopsin in a pH gradient was studied in a Rotofor preparative isoelectric focusing cell (Bio-Rad, Richmond, CA). The experiments were done in the cold room and the cell was thermostated at 4 °C by water circulation. The cell was filled with 50 ml solution containing 500 mg octyl glucoside (final concentration 1% (w/v) = 34 mM) and ampholytes (1.25 ml Pharmalyte 2.5–5.0 and 1.25 ml Pharmalyte 5.0–8.0). The pH gradient was prepared by a prerun of 1 h at 10 W. The sample (2 mg protein in 1 ml solution containing 10 mM sodium phosphate buffer at pH 7.0 and 2% (w/v) octyl glucoside) was loaded in the chamber located in the middle of the cell. Focusing was done for 3 h at a constant power of 10 W. Due to sensitivity of the protein to light, all steps were done under dim light.

Dynamic light scattering

Dynamic light scattering was used to determine the translational diffusion coefficient D_t of the micelles. D_t was obtained by analysis of fluctuations in scattered light intensity $I(t)$ detected by a photomultiplier tube positioned at a fixed angle. The fluctuations were analyzed by means of the autocorrelation function $G(\tau)$ of $I(t)$ given by

$$G(\tau) \equiv \lim_{T \to \infty} \frac{1}{2T} \int_{-t}^{T} I(t) I(t-\tau)\, \mathrm{d}t$$

where τ is the signal delay time. Experiments were performed using a monochromatic laser light source ($\lambda = 488$ nm) (Ar^+ laser, Spectra-Physics, Piscataway, NJ), a photomultiplier tube and a 72 channel digital correlator (Brookhaven Instruments Corp., Holtsville, NY). The instrumentation program BI2030 controlled the correlator and BI-PCS (Brookhaven Inst. Corp.) was used to perform the calculations. Scattering angle and sampling time were varied to optimize the measured $G(\tau)$ for each sample. The theoretical form of the normalized $G(\tau)$ for light scattered from a monodisperse solution of spherical particles is given by

$$G(\tau) = 1 + b\mathrm{e}^{-2D_t q^2 \tau}$$

where b is a constant determined by the optical setup of the experimental system. The scattering vector q is given by

$$q = (4\pi/\lambda)\sin(\theta/2)$$

where $\lambda = \lambda_0/n$ is the wavelength in the solvent, θ the scattering angle, and D_t the translational diffusion coefficient. The hydrodynamic radius of the particles, i.e., the radius of the equivalent sphere is calculated from the Stokes-Einstein relation [17]

$$R_h = kT/(6\pi\eta D_t)$$

where k is the Boltzman constant, T the Kelvin temperature, and η the viscosity of the solvent. Detergent solutions (in water or 0.15 M NaCl) were filtered twice over 0.22 μm Millex filters (Millipore Corp., Bedford, MA) and stored for 20 h at room temperature before beginning the experiment.

Ultracentrifugation

Measurements of the sedimentation velocity of octyl glucoside micelles were performed in an analytical ultracentrifuge Model E (Spinco Division, Beckman Instruments, Palo Alto, CA). Aqueous solutions containing 34, 68 or 170 mM octyl glucoside were analyzed. Optimal results, regarding the sharpness of the peak and the centrifugation duration, were obtained with the 68 mM detergent solution using a single sector cell in a titanium rotor (type An-H) at 60000 rpm (20 °C). Schlieren patterns were photographed at intervals of 16 min. Calculation of the sedimentation velocity was performed as described by Schachman [18]. By combining the data from diffusion coefficient and sedimentation velocity, the M_r of the particles can be obtained using the Svedberg equation [19]

$$M_r = sRT/D_t(1 - \bar{v}\rho)$$

where s is the sedimentation velocity, R is the gas constant ($8.314 \cdot 10^7$ erg/mol per K), T the absolute temperature, D_t the diffusion coefficient (measured under the same conditions as s), $\bar{v}$ the partial specific volume of the detergent, and ρ the density of the solvent.

Miscellaneous

Conductivity measurements were done at 22 °C with a Radiometer conductivity meter CDM3 (Copenhagen, Denmark). Viscosity measurements were performed using an Ostwald kinematic viscosimeter (Cannon-Fenske type). Refractive index measurements were done with an Abbe refractometer. Density of solutions and partial specific volume of octyl glucoside were measured by pycnometry. A partial specific volume of 0.86 ml/g was found for octyl glucoside as previously reported [20].

Results

Detection of contaminants and purification of octyl glucoside

Table I compares some properties of 1% (w/v) detergent solutions prepared from several batches of octyl glucoside commercialy available (some of which were purified in the laboratory). For a molecule which is non-ionic and does not contain any chemical group which absorbs in UV (in the wavelength range 250–350 nm), measurements of solution pH, of conductivity or absorbance can be used to assess purity.

As can be seen a 1% (w/v) solution of commercial octyl glucoside can have an acidic or an alkaline pH (below 6 or above 8) indicating the presence of ions. The conductivity of the detergent solution can be very low (as expected for a non ionic molecule) but it can also be high (above 50 microSiemens (μS) equivalent to 0.5 mM NaCl) due to the presence of ionic impurities. Such a high conductivity reflects the presence of up to 0.5 mM impurities in a 34 mM detergent solution which corresponds to a contamination level of 1 to 2%.

UV-absorbing contaminants can be visualized by spectrophotometry as illustrated in Fig. 1. When impurities were found their maximum absorbance wavelength was approx. 275 nm. As shown in Table I, the absorbance can be higher than 0.02 indicating the presence of impurities since the detergent itself does not absorb at this wavelength. The UV absorbing contaminants can be separated from detergent micelles by size exclusion chromatography (Fig. 2). The impurities have a retention time close to the retention time of octyl glucoside monomers, indicating that they are small molecular weight molecules ($M_r < 2000$) (peak 3 in Fig. 2). It is also possible for contaminants to form mixed micelles with detergent as implied by absorbance peaks having a shorter retention time (major peak 1 and minor peak 2 in Fig. 2). However, these absorbance peaks could also be due to a change in refractive index or in turbidity due to the presence of micelles. Fractions of peak 3 containing UV-absorbing impurities of the sample analyzed in Fig. 2 were collected. When water was the mobile phase, their conductivity was 30 μS. A positive reaction was obtained in the colorimetric glucoside assay but is was due to the presence of detergent monomers (from peak C in Fig. 2). The analysis of purified detergent samples on a size exclusion column equilibrated with a 1% (w/v) aqueous solution of contaminated octyl glucoside produced a negative absorbance peak (in place of peak 3) which confirmed that impurities were removed by purification (result not shown).

TABLE I

Comparison of some physical chemical properties of several batches of commercial and purified octyl-β-D-glucoside

Company and lot number	Indicated purity [a] (%)	Properties of a 1% (w/v) aqueous solution [b]			CMC in water [c] (mM)
		pH	conductivity (μs)	A_{275nm}	
Boehringer					
No. 11350824	99	6.0	19	0.060	(x)
No. 11350831	99	6.1	18	0.030	(x)
Purified lot					
No. 11350824 [d]		6.2–6.7	6	< 0.005	18–20
Calbiochem					
No. 210131	> 99	6.8	8	0.005	18–21
No. 710235	> 99	5.2–5.3	7	0.050	18–22
No. 810198	100	5.4	7	0.005	18–21
Purified lot					
No. 710235 [d]		6.1–6.6	7	< 0.005	18–20
Fluka					
No. 275988-488	> 97	6.4–7.0	15	0.030	19–21
Serva No. C8	98	9.4–9.9	58–75	0.020	20–23
Sigma					
No. 58F5010	> 98	9.0–9.8	62–70	0.020	24–26

[a] According to the catalogue of the corresponding company or as given onto the label of the product.
[b] 10 ml of a 1% (w/v) detergent solution were prepared by dissolving 100 mg octyl glucoside in 10 ml freshly deionized water. pH and conductivity were measured at 22° C.
[c] The critical micellization concentration (CMC) was determined at 22° C as described under Methods. (x) indicates that it could not be estimated accurately (see also Fig. 3 and text). Experimental values are ±2 mM.
[d] Purified as described under Methods.

Surface tension measurements have also proven to be very sensitive to the presence of impurities in some batches of octyl glucoside as illustrated by one example in Fig. 3. The presence of surface-active impurities was detected by a minimum of the surface tension vs. concentration curve in the region before the critical micellization concentration was reached. Thus the value of the critical micellization concentration (CMC) was sensitive to the presence of contaminants (see Table I). The

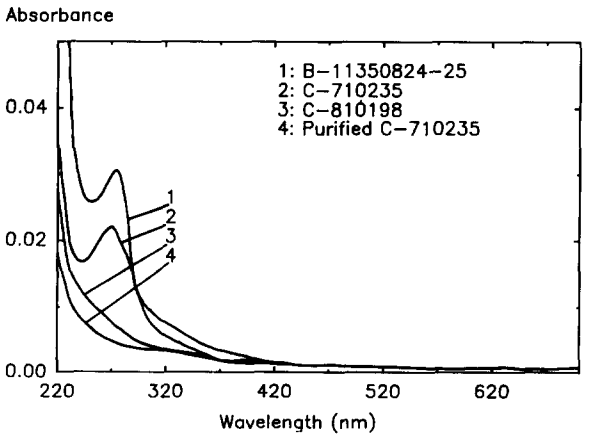

Fig. 1. Absorbance spectra of four freshly prepared solutions of octyl glucoside. Solutions at 1%w/v were prepared by dissolving 30 mg of each detergent in 3 ml freshly deionized water. After stirring the solutions were allowed to sit for 1 h before spectra were read against water. Letters before lot numbers indicate Boehringer (B) and Calbiochem Corp. (C). Spectrum 4 was obtained with octyl glucoside purified by mixed-bed ion exchange chromatography.

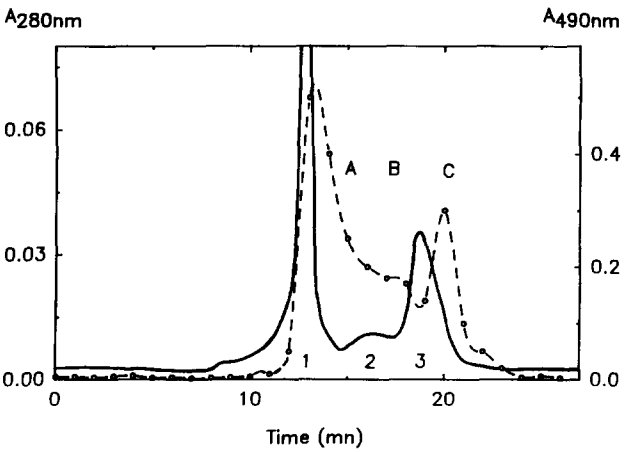

Fig. 2. High performance size exclusion chromatography profiles of octyl glucoside. The Bio-Sil TSK 125 column was equilibrated in a buffer containing 100 mM KCl and 10 mM Tris-HCl adjusted at pH 7.8. The flow rate was 0.6 ml/min. A sample of 50 mg detergent (lot C 710235) solubilized in 500 μl buffer was injected. The solid line corresponds to absorbance at 280 nm. Fractions of 0.2 ml were collected. Peak 1 corresponds to detergent micelles. After purification over a mixed-bed ion exchange column peaks 2 and 3 were absent. (○) Absorbance at 490 nm corresponding to the result of the phenol/sulfuric acid assay for glucosides.

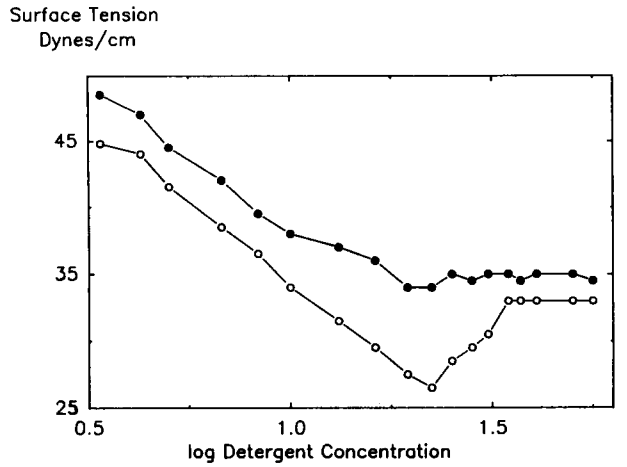

Fig. 3. Comparison of the surface tension behavior of a commercial and a purified sample of octyl glucoside. (○) Change in surface tension occurring near the critical micellization concentration of a commercial detergent (lot B 11350824-25). (●) Measurements done after purification of the same detergent show that surface active impurities have been removed.

occurrence of foam when stirring aqueous solutions containing octyl glucoside at concentrations (about 1 mM) far below the CMC was also observed. This provided an easy way to detect surface active impurities because foam occurs only near the CMC and never at such low detergent concentrations with pure octyl glucoside unless salt has been added.

The presence of impurities in octyl glucoside became a problem when the membrane protein bacteriorhodopsin was solubilized in the presence of this detergent and subsequently subjected to preparative isoelectric focusing in a Rotofor cell (Fig. 4). In the presence of some commercial detergents the protein peak appears

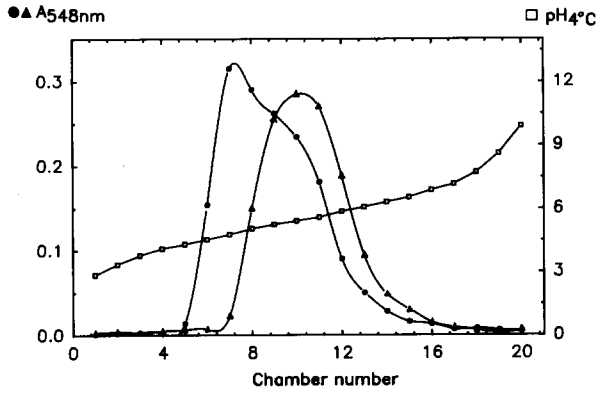

Fig. 4. Preparative isoelectric focusing of bacteriorhodopsin in the presence of octyl β-D-glucopyranoside. Absorbance profiles at 548 nm obtained during two independent isoelectric focusing separations have been superposed. ●, Bacteriorhodopsin solubilized and analyzed in the presence of octyl glucoside lot 11350824-25 from Boehringer. ▲, Similar experiment done with the same detergent lot after purification over the mixed-bed ion exchange column. □, pH gradient. See Methods for details.

asymmetric. The higher part of the absorbance peak at 548 nm (this wavelength corresponds to the absorbance maximum for the chromophore bound to the protein in the native conformation) contained mostly denatured protein. The apparently higher value was due to light scattering resulting from the suspended protein aggregates. Such aggregates of 'bleached' protein sedimentated rapidly (in less than 30 min) when fractions were allowed to sit on the bench. The other part of the protein peak located in the more alkaline region of the pH gradient (between pH 5.0 and 6.5) was identified as soluble native protein. It did not sediment in the centrifuge and showed the typical absorption peak at 548 nm. Irreversible denaturation occured consistently in a region of the gradient where the pH was between 4.3 and 4.8. The loss of protein represented 25 to 50% of the sample. It was found to be related to the detergent batch. Interestingly the contaminants appear to bind strongly to the protein, as the complex is stable in the electrical field. For those experimental conditions (2 mg protein in 50 ml solution at 1% (w/v) octyl glucoside pure at 99%), calculation of the contaminant/protein stoichiometry introduced above showed that there could be up to 100 molecules of contaminant per molecule of bacteriorhodopsin if there were only a single type of impurity. The shift of bacteriorhodopsin toward the acidic pH was also observed for larger protein samples (e.g., 3 to 8 mg). The proportion of protein aggregated decreased when the protein amount was increased in the presence of a constant amount of detergent in the Rotofor cell. Several experiments were conducted to understand if the impurities affected bacteriorhodopsin only during the course of the isoelectric focusing. The protein was solubilized either in contaminated or repurified detergent and subsequently fractionated by isoelectric focusing in either type of detergent. A shift toward low pH and precipitation of denatured protein occured only when the protein had been solubilized in the presence of impurities. This suggested that impurities were already bound to the protein before the isoelectric focusing. Nevertheless no significant change in the absorbance spectrum of bacteriorhodopsin was detected when it was incubated in the presence of contaminated detergent (result not shown).

Micellar size of octyl glucoside

Fig. 5 shows high performance size exclusion chromatography elution profiles obtained with four samples of purified octyl glucoside. When 10 mg of detergent (dissolved in 100 μl mobile phase) was loaded onto the column, the detergent detected by the colorimetric glucoside assay eluted as a symmetric peak. The elution volume corresponding to the highest absorbance was 8.4 ± 0.1 ml. When the sample had the same concentration but was two times larger (20 mg/200 μl) the elution peak was followed by a significant shoulder. The

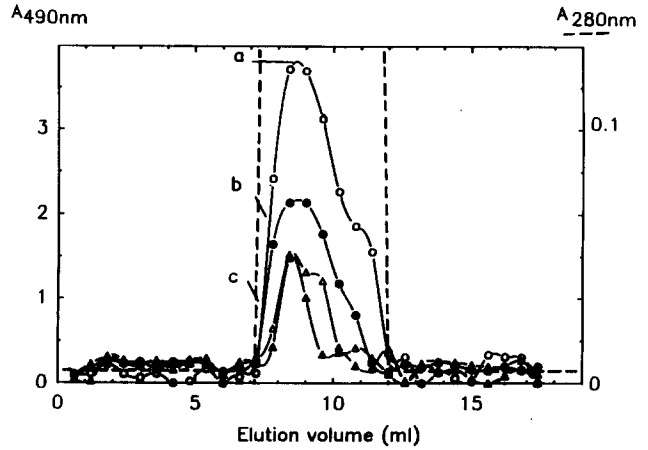

Fig. 5. Elution profiles of micelles and monomers of purified octyl glucoside in high performance size exclusion chromatography. The analytical Bio-Sil TSK-125 column was equilibrated with a solution containing 0.2 M sodium chloride and 0.02 M Bistris-HCl buffer (pH 6.8). Purified octyl glucoside samples were: ○, 100 mg dissolved in 500 μl mobile phase; ●, 50 mg in 500 μl; △, 20 mg in 200 μl; ▲, 10 mg in 100 μl. Flow rate was 0.6 ml/min and fractions of 1 min were collected. Detergent was detected by the colorimetric method as in [15]. Void volume was 4.9 ml and partition coefficient $K_d = 1$ for $V_t = 9.9$ ml. Values for R_h were following: (a) 15 ± 1 Å; (b) 21 ± 2 Å; (c) 23 ± 2 Å. Broken line gives the absorbance profile (at 280 nm) corresponding to the analysis of the sample containing 100 mg octyl glucoside dissolved in 500 μl mobile phase.

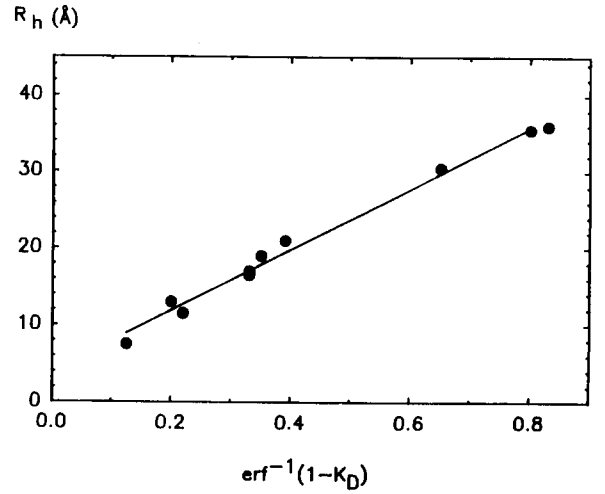

Fig. 6. Plot of the apparent hydrodynamic radius R_h vs. $\text{erf}^{-1}(1 - K_d)$ used as calibration curve for the analytical Bio-Sil TSK 125 column. Values of K_d for the reference proteins were calculated from their elution volume and the corresponding values of $\text{erf}^{-1}(1 - K_d)$ were from the table in Ref. 15.

same detergent amounts (10 or 20 mg) dissolved in a larger volume (500 μl) gave peaks identical to those in Fig. 1 (result not shown). Sample concentration (340 mM in the first two cases and 68 and 136 mM, respectively, in the latter cases) was not an important parameter for the formation of micelles or for the chromatographic resolution provided the concentration was at least three times above the critical micellization concentration. A dilution factor of 3 had to be taken into account as was found for reference proteins (result not shown).

When the sample amount was increased to 50 mg (dissolved in 500 μl) the peak height increased (at the higher absorbance $V_e = 8.6 \pm 0.1$ ml) but also broadened and was followed by a small shoulder. In these experiments the void volume was 4.9 ml and K_d was equal to 1 for an elution volume $V_t = 9.9 \pm 0.1$ ml. Therefore molecules eluted later in the chromatographic run were retarded on the column (see discussion). When detergent concentration in the sample was increased (i.e., 100 mg/500 μl, or 680 mM) the peak height was again increased and the shoulder was extended behind the total volume (Fig. 1).

All four chromatograms in Fig. 5 displayed a maximal absorbance for the same elution volume (average $V_e = 8.5 \pm 0.1$ ml). The gaussian shape of the peaks (obtained when small samples were chromatographed) was suitable for elution volume measurements. Calculation of the value of $\text{erf}^{-1}(1 - K_d)$ gave 0.28 ± 2. On the calibration curve obtained with globular proteins (Fig. 6) this value corresponded to an apparent hydrodynamic radius $R_h = 15 \pm 1$ Å. This result is in good agreement with values ($R_h = 15$ Å) reported earlier [10,11] using gel filtration on dextran (Sephadex) or on crosslinked polyacrylamide-agarose (AcA Ultrogel) gels.

Analysis of larger detergent samples (i.e., 50 or 100 mg in 500 μl) displayed absorbance peaks with a non-gaussian shape. Elution patterns were identical for samples containing varying amounts of detergent with the exception that peaks broadened on their trailing edge (Fig. 5) (the highest absorbance consistently corre-

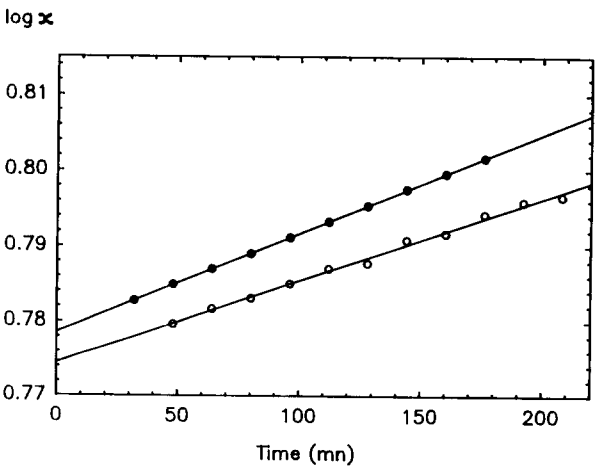

Fig. 7. Sedimentation rate of purified octyl glucoside micelles. Plots of log x vs. centrifugation time for (○) 34 mM and (●) 68 mM aqueous detergent solutions at rotor speeds 56000 and 60000 rpm, respectively (at 20 °C). x is the distance (in cm) of the maximum of the Schlieren peak to the rotor axis. Linear regression gave $Y = 1.313 \cdot 10^{-4} X + 7.786 \cdot 10^{-1}$ ($r = 0.997$) and $Y = 1.113 \cdot 10^{-4} X + 7.742 \cdot 10^{-1}$ ($r = 0.999$) for the plots.

sponded to the same elution volume as that measured for smaller samples). This suggests that a maximal size for the micelles was reached in the leading edge of the peaks. Chromatographic separations done after equilibration of the column with water or with various low ionic strength mobile phases (at various pH values between 6 and 7.5) showed this phenomenon in a reproducible way. Identical elution volumes were displayed for various mobile phase. A sample volume of 500 µl may not be negligible when compared to the size of the column (total volume 9.9 ml; ratio of sample volume to total volume = 5%). In this case, a more accurate way to measure the elution volume would be to use the volume corresponding to the half-height of the beginning of the peak. For $V_e = 7.7 \pm 0.1$ ml a R_h value of 21 ± 2 Å was obtained. The largest R_h value which could be found for the micelles in this series of experiments was 23 ± 3 Å for a V_e of 7.3 ± 0.1 ml (Fig. 5).

More convincing evidence for large particle size was given by absorbance profiles (at 280 nm) recorded during the chromatography. Peaks with a very steep frontal edge were obtained independently of the amount of detergent analyzed (one example is shown in Fig. 5). Such profiles do not correspond to an absorbance (since the purified detergent does not absorb) but to light scattering due to the micelles. For detergent samples containing 10 mg/500 µl, 20 mg/500 µl, or 50 mg/500 µl elution volumes of frontal elution edge were as follows: $V_e = 7.6 \pm 0.1$ ml; 7.4 ± 0.1 ml, and 7.3 ± 0.1 ml, respectively. For the two last elution volumes the R_h values would be 21 ± 2 Å and 23 ± 3 Å, respectively (Fig. 6). Corresponding molecular weights (19000 and 24000, respectively) were obtained from the plot of R_h for proteins of known molecular weight, after correction for partial specific volume of the particles.

Results from the dynamic light scattering experi-

TABLE II

Size parameters for octyl glucoside micelles

Abbreviations: n.g., not given; D_t, translational diffusion coefficient; S, sedimentation velocity.

Method and sample composition [a]	T(°C)	R_h (Å)	M_r	Aggregation number	References
Static light scattering					
24–41 mM	20		21000	68	Lässer and Elias (1972)
24–41 mM	30		26000	84	
24–41 mM	50		22000	72	
Dynamic light scattering [b]					
34 mM	20	23 ± 3	($D_t = 0.90 \cdot 10^{-6}$ cm$^2 \cdot$s^{-1})		this paper
68 mM	20	28 ± 3	($D_t = 0.70 \cdot 10^{-6}$ cm$^2 \cdot$s^{-1})		
170 mM	20	31 ± 3	($D_t = 0.57 \cdot 10^{-6}$ cm$^2 \cdot$s^{-1})		
Sedimentation equilibrium					
15–50 mM	30		24000 [c]	83 ± 3	Lässer and Elias (1972)
Sedimentation velocity					
34 mM	20	($S = 1.22 \cdot 10^{-13}$ s)			this paper
68 mM	20	($S = 1.28 \cdot 10^{-13}$ s)			
Combination of sedimentation velocity and diffusion data [d]					
34 mM	20		22000 ± 3000	75 ± 10	this paper
68 mM	20		30000 ± 3000	103 ± 10	
Viscosimetry					
30–400 mM	20	27			Waterson et al. (1972)
30–400 mM	30	46			
30–400 mM	50	42			
Size exclusion chromatography					
n.g.	20		8000		Rosevear et al. (1980)
n.g.		15	8000	27	VanAken et al. (1986)
34 mM	22	13–14	($D_t = 1.5 \cdot 10^{-6}$ cm$^2 \cdot$s^{-1})		Grabo (1982)
[e]	20	15 ± 1	8000 ± 1000	27 ± 4	this paper

[a] Octyl glucoside concentration in water.
[b] R_h values were calculated from Stokes-Einstein equation.
[c] Calculated from aggregation number.
[d] A value of 0.86 ml/g was used for the partial specific volume for octyl glucoside. The densities of the 34 mM and 68 mM solution were 1.001 and 1.004, respectively, at 20 °C.
[e] Elution volume was measured at maximum of the absorbance peak (see text).

ments indicate that the diffusion coefficient was decreased with an increase in detergent concentration (Table II). Calculated hydrodynamic radii were in the range 23 to 31 Å. Sedimentation velocities obtained by ultracentrifugation of 34 and 68 mM octyl glucoside solutions (Fig. 7) and molecular weight values calculated from velocity and diffusion data are given in Table II. They also indicated a large micellar size (M_r 25 000) and showed an increase in size with detergent concentration.

Discussion

Purification of octyl glucoside

It has been reported several times for detergents other than octyl glucoside that commercial detergents often contain traces of impurities [21–27]. Such impurities can be remainders from synthesis of the detergent (contaminants of the ingredients or intermediary products) or transformation products which form during storage of the pure detergent. In molecular biology small quantities of macromolecules are often utilized and such chemical impurities are not negligible. The following illustrates what could exist in a real experimental situation. If octyl glucoside (pure at 99% (w/w)) contains a single contaminant (of the same molecular weight) and is used at final concentration of 1% (w/v) (= 34 mm) in a solution together with a protein of M_r 60 000 present at 10 mg/ml (final concentration = 0.17 mM), the stoichiometry equals two molecules of contaminant for one protein molecule. This stoichiometry will be higher if the molecular weight of the protein is smaller or if the molecular weight of the contaminant is lower. It will also increase when protein concentration decreases. Contaminants can also bind to the protein when the contact time is long. Impurities reported to occur in several detergents are peroxides which form and decompose in the presence of light, sulfhydryl oxidizing agents, aldehydes, and carbonyl compounds [22,24,25,27]. These impurities can introduce errors in various enzymatic assays, e.g., when formation or consumption of reducing agents is measured.

The described purification technique (ion exchange chromatography on a mixed-bed column and lyophilization) is currently used for purification of samples of 5 g with a yield of about 90% (in weight). The characteristics of the purified detergent are listed in Table I. The purified octyl glucoside is free of ionic impurities: the pH of a 1% (w/v) aqueous solution is close to neutrality and its conductivity is very low. (A conductivity of 7 μS seems to be a limit for a 1% (w/v) solution.) Moreover the absorbance at 275 nm of the detergent solution is negligible. Fig. 1 shows an absence of absorbance between 220 and 700 nm. This was verified by the detection of an absorbance peak at 275 nm for the contaminant isolated by size exclusion chromatography in water. The fractions corresponding to this peak had also a high conductivity (when chromatography was done in water) suggesting that it contained ionic impurities (but ionic impurities could also be incorporated in detergent micelles). The repurified detergent does not contain any low molecular weight UV-absorbing molecules (result not shown). Only the first peak (peak 1) corresponding to detergent micelles was found. Therefore ion exchange chromatography removed both the ionic and the UV-absorbing impurities suggesting that both properties belong to the same compounds. The purity of the detergent is also demonstrated by a plot of surface tension versus log of the detergent concentration (Fig. 3). It does not exhibit any decrease of surface tension before the critical micellization concentration is reached.

Further verification that the contaminants were removed was demonstrated by isoelectric focusing experiments of bacteriorhodopsin (Fig. 4). In the presence of purified detergent the protein was distributed in a symmetric peak with a maximal absorbance located in the pH gradient around pH 5.5 instead of pH 4.9. Interestingly, no denaturation occured and no protein was present at a pH below 4.5 (note: no attempt was made to identify the impurities present in octyl glucoside). Variation from batch to batch was observed in the commercial detergent as shown in Fig. 1 and Table I.

Other purification methods have been described for detergents including: (i) selective absorption of impurities by foam extraction or emulsion extraction [21], (ii) chromatography over octadecylsilanized silica (in the case of sodium dodecylsulfate [26]), (iii) treatment of aqueous detergent solutions with either $NaHSO_3$ or $SnCl_2$ followed by an extraction procedure [24], (iv) elimination of sulfhydryl oxidizing contaminants by reaction with sodium borohydride followed by silica gel column chromatography and passage through a mixed-bed ion-exchange resin [25], or (v) anion exchange chromatography [28]. For octyl β-D-glucopyranoside preparations (and other alkyl glucosides or maltosides) the octanol-detergent mixture obtained at the end of the synthesis is usually applied to an anion exchange column (Dowex in OH form in Refs. 9, 11 and 28) to separate the alcohol from the detergent during elution by methanol (It separates also the anomers of octyl glucoside). An alternative purification technique uses chromatography of the acetylated detergent derivative on silicic acid. These steps may not be efficient when done on a large scale because the resins have limited resolution and may not retain all ionic compounds. Additional chromatography on a mixed-bed ion exchanger was necessary to eliminate the last traces of contaminants. An anion (DEAE-Sephacel) or a cation exchanger (RSO_3^--H^+ resin) alone was not sufficient to achieve this (results not shown).

For bacteriorhodopsin the presence of trace impuri-

ties in octyl glucoside was critical at another level. Such chemical impurities affected crystallization of the protein. Crystallization experiments lead to reproducible formation of cubic crystals (as described by Michel and Oesterhelt [4] and Michel [29]) when performed in the presence of some detergent batches. With other batches no crystals were obtained under the same conditions in parallel experiments. In the presence of purified octyl glucoside larger crystals (0.1 mm instead of 0.02 mm edge) were obtained. Unfortunately these crystals were too small for a crystallographic analysis. Similar observations related to nucleation and growth of proteins crystals have been reported for chemical impurities found in other compounds used for crystallization of proteins (e.g., polyethylene glycol used as a precipitant) [30].

Purification of commercial octyl glucoside according to a standard procedure is necessary to ensure a product of reproducible quality. The user should also be aware of the instability of octyl glucoside in aqueous solutions. Hydrolysis can occur under improper storage conditions (lyophilization and storage of the dry powder under inert gas is recommended) or during protein purification and crystallization. The presence of *n*-octanol, remaining from synthesis or as the product of hydrolysis, can be detected by its penetrating aromatic odor (some commercial octyl glucoside 99% pure batches have a strong octanol smell).

Size of octyl glucoside micelles

The results reported here for octyl glucoside micelles generate several concerns regarding the validity of methods presently used to estimate particle size.

The concept emerging from the studies of amphiphiles is that micelles are aggregates which can form spontaneously in aqueous solution as a consequence of the hydrophobic effect [31]. Micelle formation occurs only above a given concentration range (known as the critical micellization concentration) and at temperatures above the critical micellization temperature. Below the critical concentration only monomers are present in solution, above this concentration monomer aggregates coexist with free monomers. Micelles have a dynamic structure (for reviews, see Refs. 32 and 33). Two dynamic processes have been distinguished: one is due to the continuous exchange of monomers between the micelles and the bulk, and the other is related to the lifetime of the micelles (this corresponds to their formation and breakdown [33]). Because of this characteristic, micelles do not possess a precise shape such as a rigid particle would and populations of micelles are polydisperse. The gross shape of micelles might simulate a small sphere, a disk, an oblate or prolate ellipsoid or a long cylinder [31]. The aggregation and continuous exchange of monomers results in a rough surface of the micelles. Micelle size and/or shape can also vary with various factors including amphiphile concentration, ionic strength, or the presence of other amphiphiles.

Numerous methods are available to measure the size of particles. Some physical methods, including equilibrium sedimentation and light scattering, do not perturbate the sample during the measurements. Indirect methods either mechanically perturbate the solution or use foreign compounds introduced into the micelles to serve as probes. Several examples of successful applications of the chromatographic methods for the determination of the size of detergent micelles have been reported (see Ref. 34, and references therein). Classical and high performance size exclusion chromatography have also been demonstrated to be a valuable technique for the estimation of size and for the separation of phospholipid membrane vesicles and protein-coated vesicles [35]. Dubin and co-workers [34] have shown that size exclusion chromatography gives values of the particular hydrodynamic radius which agree with those obtained from quasi-elastic light scattering in the case of mixed micelles of Triton X-100 and sodium dodecyl sulfate. This demonstration has suggested a priori that chromatography could give a good estimation of the size of the micelles of octyl glucoside.

In the case of octyl glucoside several methods have been applied to estimate the size of the micelles. Experimental results from literature and from present work are compiled in Table II. It appears that physical methods are consistent with a large micellar size (R_h 23 Å, M_r 22000) while chromatography indicates a smaller one (R_h 15 Å, M_r 8000). For this reason, the validity of results from size exclusion chromatography as an indication of micellar size is questionable, at least for the peculiar case of octyl glucoside. Light scattering and ultracentrifugation methods can be considered as nonperturbating since the composition of the sample is not altered during the measurements. In liquid chromatography, particles are subjected to mechanical perturbations resulting from turbulence in the mobile phase flowing through the column. As a consequence, the elution of the micelles could be retarded by interactions with the gel matrix or the size and/or the shape of micelles could be altered by the sieving effect. A small size (R_h 15 Å; M_r 8000; aggregation number 27) was found when interpretation was based upon the assumption that average micellar size would correspond to the central part of the symmetrical elution peak as it is the case for more rigid particles like proteins, nucleic acids, or other polymers. The peculiar behavior of octyl glucoside in chromatography suggests that micelles could have a large size but have become smaller during the chromatography. The elution peaks displayed non-gaussian shapes and a maximal size seemed to be reached because larger samples had identical frontal elution volumes. When the leading edge of the elution peak was considered, a micellar size comparable to that found by

the other methods was obtained. Nevertheless those results cannot be compared because it has been shown that micellar size increased with detergent concentration (from 34 to 170 mM) and because very high detergent concentrations (e.g., 340 mM or 680 nm) had to be loaded onto the column in order to reach a micellar size comparable to that found by other methods. This behavior was interpreted as an alteration of the micellar structure during chromatography and/or as a retardation of the micelles by adsorption. As a consequence, the micellar size found by this method was considered to be underestimated. This interpretation was also supported by the results of light scattering analyses done on chromatography fractions. A particle size similar (within the limits of experimental error) to that present before the chromatography was found in fractions of the eluted detergent.

Some geometrical considerations concerning the shape of the large micelles can be drawn from values of the diffusion coefficient and the R_h from chromatography. Since the length of the octyl glucoside molecule is 15 Å (9.3 Å for the acyl chain length calculated as in [31] with a length equal to 80% of the fully extended chain), the smallest micelles would be spherical with $R_h = 15$ Å, an average M_r of 8000, and an aggregation number of 27 monomers per micelle. If such small micelles had an elongated shape they should elute earlier in chromatography, as this is the case when elongated proteins are compared to globular proteins of identical molecular weight (see, for example, Ref. 36).

For the large micelles (with $R_h = 23$ Å) calculation of the frictional coefficient suggests that the oblate ellipsoid (i.e., disk-like) shape for the octyl glucoside micelles is more probable than a sphere since R_h would be larger than the length of the octyl glucoside molecule. In ultracentrifugation, particle size and shape dominate the sedimentation rate. The Svedberg equation is based on the assumption that the frictional resistance encountered by a particle in sedimentation is equal to that encountered in diffusion. Velocity and diffusion coefficient appear in the numerator and denominator, respectively, and therefore the effect of shape is cancelled if both were measured under the same experimental conditions (e.g., temperature, detergent concentration, solvent viscosity).

A model of micelles with octyl glucoside molecules in alternating orientation, filled with water, has been postulated by Watterson and co-workers [8] to account for the large R_h (41 Å) obtained from kinematic viscosity measurements as well as from the second virial coefficient. In the latter case the assumption that octyl glucoside micelles were spherical could have contributed to an overestimation of the hydrodynamic radius. Interestingly the reported viscosity measurements done at 20 °C gave $R_h = 27$ Å (Table II) which is similar to the value obtained by other techniques. Finally it should be mentioned that the presence of impurities could lead to the formation of mixed micelles, alter the micellar structure or affect experimental results in some as yet undetermined way.

Acknowledgements

We thank Dr. Ralph E. Schrohenloher (Clinical Immunology/Rheumatology Laboratory) for help in the ultracentrifugation analyses. We are grateful to Drs. Barry Ganong (Department of Biochemistry) and Jimmy Mays (Chemistry Department) for use of their equipement and stimulating discussion. The technical assistance of Mr. Dwight Moore in crystallization experiments is acknowledged. We thank also Drs. R. Michael Garavito, Daniel Picot and Qing Chen Meng for fruitful discussion and the referees for helpful comments on the manuscript. This work was supported in part by C.N.R.S. (France), N.A.T.O. and N.I.H. (Grant 1/RO/DK39832/02).

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