Determination of the Molecular Weight and the Hydrodynamic Properties of a Polypeptide from the Thylakoid Membrane by Sedimentation, Diffusion and Binding Measurements in Dodecyl Sulphate Solutions

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Dedicated to Prof. Dr. W. Menke *on the Occasion of His 65th Birthday*

Membrane Polypeptide, Molecular Weight, Dodecyl Sulphate

The molecular weight and hydrodynamic properties of a polypeptide isolated from the lamellar system of *Antirrhinum* chloroplasts were determined in sodium dodecyl sulphate solution by measurement of sedimentation velocity, diffusion and effective partial specific volume. The polypeptide fraction exhibits a molecular weight of 25 000 which agrees with the apparent molecular weight found by polyacrylamide gel electrophoresis. The molecular weight of the polypeptide-sodium dodecyl sulphate micelle was 54 000, with a friction ratio of 1.6 which indicates an effective asymmetric hydrodynamic shape. For binding measurements self-diffusion equilibrium dialysis with dodecyl [^{35}S] sulphate was used. In this case, dialysis equilibrium was reached within about 10 hours, in contrast to the dialysis with initial concentration differences which requires much longer times. A binding value of $\delta_D = 1.15$ g sodium dodecyl sulphate per g polypeptide was obtained which corresponds to a molar binding ratio of 100 mol dodecyl sulphate bound per mol of polypeptide. After the removal of dodecyl sulphate the polypeptide is present in an aggregated state. In phosphate buffers of pH 6.8 and 7.5 the aggregates preponderantly have sedimentation coefficients of 11.7 and 6.8 Svedberg units respectively. Assuming equivalent spheres the molecular weights were calculated to be 340 000 and 150 000.

Molecular weights of polypeptides are often determined by dodecyl sulphate polyacrylamide gel electrophoresis [1]. This method is based on the fact that different polypeptides bind approximately the same amounts of dodecyl sulphate and that the hydrodynamic properties of the polypeptide-dodecyl sulphate micelles are a function of the length of the unfolded molecule [2–4]. This has been demonstrated for soluble proteins with known molecular weights in numerous cases. However, for membrane proteins, which are not soluble in the absence of detergent, the verification of these observations was not possible. Thus, until now only apparent molecular weights are known from membrane proteins. However, recently Tanford *et al.* [5] have shown that the real molecular weight of polypeptides can also be determined in the presence of dodecyl sulphate by application of the theory of multicomponent systems [6, 7] for the sedimentation equilibrium. In the present paper we report on the determination of the molecular weight of a polypeptide fraction from the lamellar system of chloroplasts which was achieved by sedimentation velocity and diffusion analysis. Moreover, the amount of sodium dodecyl sulphate which is bound to this polypeptide was determined by self-diffusion * equilibrium dialysis using dodecyl [^{35}S] sulphate.

Materials and Methods

Isolation of the polypeptide fraction 24 000. Stroma-freed chloroplasts from *Antirrhinum majus* [8] corresponding to the dry weight of 700 mg are dissolved in 100 ml 0.01 M sodium phosphate buffer pH 7.2, containing 2.2% sodium dodecyl sulphate and 1% mercaptoethanol. By means of gel filtration a fraction is separated from this mixture of polypeptides which exhibits in the dodecyl sulphate polyacrylamide gel electrophoresis [2, 9] a molecular weight of 24 000. Gel filtration is first carried out with Sepharose 6B (Pharmacia) and subsequently

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* The term self-diffusion is used if in a multicomponent system without any concentration differences the diffusion of a component is followed.

with Sephadex G-150 (Pharmacia) according to Menke *et al.* [10]. The elution buffer is 0.01 M sodium phosphate buffer pH 7.2, containing per liter 2.5 g sodium dodecyl sulphate and 1 ml mercaptoethanol. In order to remove the lipids the preparation is precipitated with acetone. After being dried the precipitate is dissolved in such an amount of 0.01 M sodium phosphate buffer pH 7.2, dodecyl sulphate and mercaptoethanol as to give a final concentration of 1% protein and mercaptoethanol and 2.2% sodium dodecyl sulphate. In order to remove aggregates from this solution an additional gel filtration on Sephadex G-150 is done. Those fractions are isolated which exhibit in the dodecyl sulphate polyacrylamide gel electrophoresis the apparent molecular weight of 24 000. For the determination of the molecular weight in the ultracentrifuge and for the determination of dodecyl sulphate binding by equilibrium dialysis the purified polypeptide fraction 24 000 is transferred by a 48 hours dialysis into a dodecyl sulphate containing sodium chloride solution, adjusted to pH 7 by means of 0.1 M NaOH. The dodecyl sulphate free polypeptide fraction is prepared according to the method of Weber and Kuter [11]. The exchange of the solvent is done by diafiltration. The protein concentration is determined by the procedure of Lowry *et al.* [12]. The calibration is done by dry weight determination of a corresponding purified polypeptide preparation from chloroplasts.

Densities and partial specific volumes are determined using a precision densimeter Model DMA 02 (Paar), according to Kratky, Leopold and Stabinger [13]. Temperatures are maintained constant to $20.00 \pm < 0.01$ °C by double ultrathermostating. Calibration is performed with water ($\varrho = 0.99821$ g/cm³, Table I) [14a].

Sedimentation velocity (24 000 and 30 000 rpm) and *diffusion measurements* are carried out at 20 °C in double sector synthetic boundary cells using a Beckman Model E analytical ultracentrifuge, equipped with a photoelectric scanner at a wavelength of 280 nm. The solvents used are sodium dodecyl sulphate solution (0.05 M NaCl, 0.25% sodium dodecyl sulphate, pH 7) and 0.01 M sodium phosphate buffers (pH 6.8 and 7.5).

Binding measurements of dodecyl sulphate to the polypeptide are made by self-diffusion equilibrium dialysis, using radioactive sodium dodecyl [^{35}S] sulphate purchased from Amersham Radiochemical Centre (U.K.). Dialyses are carried out in a dialyzing system, Model Dianorm-GD, as described by Weder, Schildknecht and Kesselring [15]. Teflon cells composed of two compartments containing either 0.2 or 1.5 ml solution and separated by a Visking membrane, are rotated at 10 rpm. The polypeptide (concentration about 1 mg/cm³) dissolved in unlabelled sodium dodecyl sulphate solution is dialyzed at 20.0 ± 0.05 °C against the same concentration namely 0.25 per cent by weight but containing dodecyl [^{35}S] sulphate. The samples are counted in a Packard Liquid Scintillation Spectrometer Model 3375. Quenching of radioactivity in the samples is determined by the usual two channel method with an external standard. Conversion of the observed counts to decays per minute and to concentration units is obtained as usual by comparison with radioactive standard samples. Dialysis with initial concentration differences was carried out as usual [15–19]. In this method an aqueous solution of 0.05 M NaCl on one side of the semipermeable membrane, either with or without polypeptide, was dialyzed against a 0.5% by dry weight solution of [^{35}S]-labelled or unlabelled dodecyl sulphate in the same solvent on the other side of the dialysis cell. In the case of unlabelled dodecyl sulphate, which is only used in the absence of polypeptide as a control the methylen blue method by Reynolds *et al.* was used [17, 18].

Viscosity determinations of the solvents are carried out in a suspended level Ubbelohde viscometer (Schott, Model I). Temperature is maintained constant at 20.00 ± 0.01 °C by an ultrathermostate (Haake). Kinetic energy corrections are applied. Calibration measurements are carried out with water ($\eta = 1.005$ centipoise, Table I) [14b]. The properties of the aqueous solvent mixtures are shown in Table I where ϱ_s is the density in g/cm³ and η_s the viscosity in centipoise.

Table I. Properties of the aqueous solvent mixtures at 20 °C.

Aqueous solvent	pH	ϱ_s [g/cm³]	η_s [centipoise]
H_2O	7	0.99821 [14a]	1.005 [14b]
0.05 M NaCl	7	1.00029 ±0.00001	—
Sodium dodecyl sulphate (0.25%), 0.05 M NaCl	7	1.00066 ±0.00001	1.022 ±0.001
Sodium dodecyl sulphate (0.25%), 0.09 M NaCl	7	1.00176 ±0.00001	—
0.01 M Sodium phosphate buffer	6.8	0.99934 ±0.00001	1.011 ±0.001
0.01 M Sodium phosphate buffer	7.5	0.99948 ±0.00001	1.012 ±0.001

Results and Discussion

Eisenberg [20] has approached the transport problem in multicomponent systems, defining the com-

ponents by reference to dialysis equilibrium [7, 21]. Assuming that a. the solution ahead of the boundary is of constant concentration with regard to the protein (c in g/cm³; $dc = 0$), and that b. the diffusible (electrolyte) components are essentially at equilibrium over the cell ($d\mu = 0$; μ = chemical potential of any diffusible component), the molecular weight that is measured is the molecular weight of pure protein, excluding bound detergent and other solvent components. It is related to the sedimentation (S_0 in s) and diffusion coefficients (D_0 in cm²/sec) in the initial state of infinite dilution of the experimentally determined concentration by the relation

$$\bar{M} = \frac{R\,T\,S_0}{D_0[\partial\varrho/\partial c]_\mu} \qquad (1)$$

where $R = 8.315 \times 10^7$ erg/degree mol is the molar gas constant and T the absolute temperature. $[\partial\varrho/\partial c]$ is the density increment which is related to the apparent specific volume $\Phi' \equiv (1 - \Delta\varrho/c)/\varrho_s$, extrapolated to zero concentration according to Casassa and Eisenberg [7]

$$[\partial\varrho/\partial c]_\mu = \lim_{c\to 0}(\partial\varrho/\partial c)_\mu = 1 - \Phi_0'\,\varrho_s \qquad (2)$$

($\Delta\varrho = \varrho - \varrho_s$; ϱ = solution density; Φ_0' = effective partial specific volume of the protein). Therefore, Eqn (1) is the equivalent of the Svedberg equation in multicomponent systems [6, 7].

The quantity directly determined by the sedimentation and diffusion coefficients is the buoyant weight $\bar{M}(1 - \Phi_0'\,\varrho_s)_\mu = \bar{M}[\partial\varrho/\partial c]_\mu$. If the right-hand side is substituted according to Casassa and Eisenberg [7] and Tanford *et al.* [5] by $\bar{M}(1 - \Phi_0'\,\varrho_s)_\mu = \bar{M}\{(1 - \bar{v}_P\,\varrho_s) + \delta_D(1 - \bar{v}_D\,\varrho_s)\}$, it follows

$$\Phi_0' = \bar{v}_P - \delta_D\left(\frac{1}{\varrho_s} - \bar{v}_D\right). \qquad (3)$$

Here $\bar{v}_P$ is the true partial specific volume of the protein and $\bar{v}_D$ the partial specific volume of sodium dodecyl sulphate bound to the protein. δ_D designates the amount of binding expressed on a weight basis rather than a molar basis as g of sodium dodecyl sulphate per g protein which can be determined by equilibrium dialysis binding measurements. If M_D (288.5) is the molecular weight of the detergent and $\bar{\nu}$ its average number of moles bound per mole of protein, then $\bar{\nu}$ is equal to $\delta_D\bar{M}/M_D$. With regard to Eqn (3) it is assumed that binding of sodium chloride may be omitted at low salt concentrations [7].

The value $\bar{v}_D$ for sodium dodecyl sulphate in aqueous solutions may be determined from measurements of the solution density ϱ as a function of the dodecyl sulphate concentration (c_D in g/cm³). Provided that $\bar{v}_D$ is not significantly altered by association with the protein, the $\bar{v}_D$-value for an isolated sodium dodecyl sulphate molecule can be obtained in the absence of protein from the slope of the experimental curve extrapolated to the limit at $c_D = 0$, according to $\lim_{c_D\to 0}(\partial\varrho/\partial c_D) = (1 - \bar{v}_D\,\varrho_s)$. Corresponding considerations are valid for a protein in the absence of dodecyl sulphate. If the amount of bound dodecyl sulphate is the same in media of different density and if there are no other preferential interactions, according to Tanford *et al.* [5] the procedure can be used to calculate the molecular weight $M^* = \bar{M}(1 + \delta_D)$ of the entire micelle consisting of protein and sodium dodecyl sulphate. The corresponding partial specific volume is $\bar{v}^* = (\bar{v}_P + \delta_D\,\bar{v}_D)/(1 + \delta_D)$.

The experimental results are summarized in Table II. All ultracentrifugal measurements in sodium dodecyl sulphate solutions were carried out above the critical micelle concentration ($cmc = 0.1$ per cent of sodium dodecyl sulphate). Fig. 1 shows a logarithmic plot of the radial distance r (in cm) of the moving boundary of the polypeptide from the centre of rotation during sedimentation *versus* time (t in min) and Fig. 2 the time course of the apparent diffusion coefficient. Sedimentation (S_c in s) and diffusion coefficients (D_c in cm²/s) were calculated from the intercepts at zero time (polypeptide concentration of the samples $c = 0.47$ mg/cm³). In Figs 3 – 5, the sedimentation coefficients, the diffusion coefficients and the densities (in g/cm³) were plotted against the polypeptide concentration. All graphs are linear at low values of time and concen-

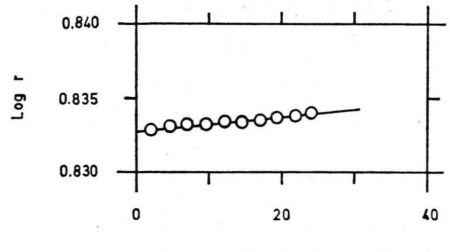

Fig. 1. Plot of log r *vs* time. r is the distance in cm of the boundary from the axis of rotation. The solution contained 0.47 mg/cm³ polypeptide, 0.25% dodecyl sulphate and 0.05 mol/l NaCl.

Table II. Determination of molecular weights and hydrodynamic properties. Deviations are given as mean error of the average value.

Solvent	Sodium dodecyl sulphate (0.25%), 0.05 M NaCl; pH 7	0.01 M Sodium phosphate buffer; pH 6.8	0.01 M Sodium phosphate buffer; pH 7.5	0.05 M NaCl; pH 7
$\overline{M}$	25200 ± 900	340000 [a]	150000 [a]	—
M^*	54000 ± 3000	—	—	—
$S_0 \times 10^{13}$ [s]	2.50 ± 0.04	11.70 ± 0.02	6.81 ± 0.01	—
$D_0 \times 10^7$ [cm²/s]	7.04 ± 0.06	—	—	—
Φ_0' [cm³/g]	0.656 ± 0.006	—	—	—
$\bar{v}_P$ [cm³/g]	—	0.81 [b]	0.81 [b]	0.811 ± 0.006
$\bar{v}^*$ [cm³/g]	0.84	—	—	—
$\bar{v}_D$ [cm³/g]	0.864 ± 0.002 [c]	—	—	—
δ_D [g sodium dodecyl sulphate/g polypeptide]	1.15 ± 0.03	—	—	—
$\bar{\nu}$ [mol sodium dodecyl] sulphate/mol polypeptide]	100 ± 6	—	—	—
cmc [mg/cm³]	1.0	—	—	—
f/f_K	1.60 ± 0.08	1 [a]	1 [a]	—

[a] Calculated under the assumption that the particles are spherically shaped. [b] Estimated value. [c] $c > cmc$. $\overline{M}$ = molecular weight of the polypeptide. M^* = molecular weight of the polypeptide-dodecyl sulphate micelle. S_0 = sedimentation coefficient. D_0 = diffusion coefficient. Φ_0' = effective partial specific volume of the polypeptide. $\bar{v}_P$ = partial specific volume of the polypeptide. $\bar{v}^*$ = partial specific volume of the polypeptide-dodecyl sulphate micelle. $\bar{v}_D$ = partial specific volume of the dodecyl sulphate. δ_D = g sodium dodecyl sulphate bound per g polypeptide. $\bar{\nu}$ = mol sodium dodecyl sulphate bound per mol polypeptide. *cmc* = critical micelle concentration. f/f_K = frictional ratio.

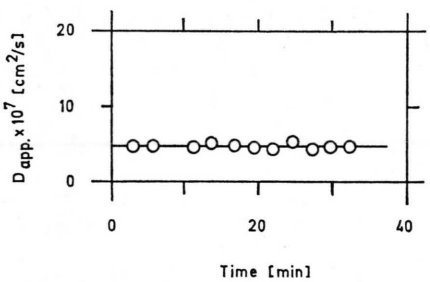

Fig. 2. Plot of the apparent diffusion coefficient *vs* time. The solution is the same as in Fig. 1.

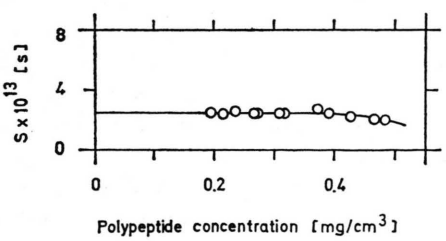

Fig. 3. Plot of the sedimentation coefficient *vs* concentration of the polypeptide.

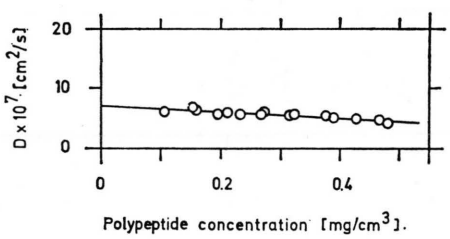

Fig. 4. Dependence of the diffusion coefficient on the polypeptide concentration.

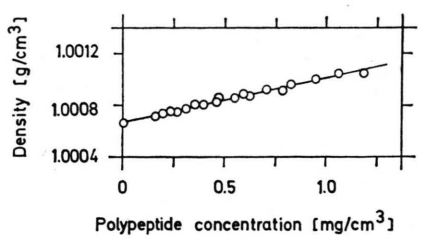

Fig. 5. Dependence of the solution density on the polypeptide concentration. The solution contained 0.25% dodecyl sulphate and 0.05 mol/l NaCl.

tration. Therefore, self-aggregation or thermodynamic perturbation can be disregarded. The values $S_0 = 2.5 \times 10^{-13}$ s, $D_0 = 7.0 \times 10^{-7}$ cm²/s and $\Phi_0' = 0.656$ cm³/g were obtained by the least-squares analysis from the intercepts and the limit value of the slope of the density at infinite dilution. Hence, a molecular weight of the polypeptide of $\overline{M} = 25\,000$ results from Eqn (1).

This result is controlled by a second method, because it depends on small differences of the effective partial specific volume Φ_0'. According to Eqn (3), the effective partial specific volume is related to the

partial specific volume of the polypeptide, the partial specific volume as well as the binding value of detergent and the density of the solvent. By experimental determination of the values mentioned, one can independently calculate Φ_0'. The partial specific volume $\bar{v}_P$ was found to be 0.811 cm³/g as determined by extrapolation of the slope of the density curve to infinite dilution (Fig. 6). In this case the

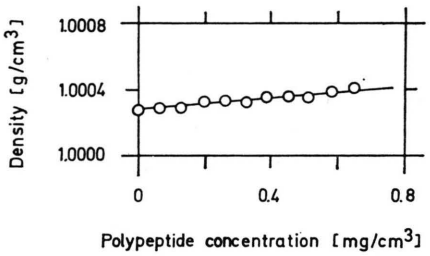

Fig. 6. Dependence of the solution density on the polypeptide concentration. The solution contained 0.05 mol/l NaCl but contained no dodecyl sulphate.

polypeptide was dissolved in 0.05 M NaCl in aqueous solution. For the 0.25 per cent sodium dodecyl sulphate solution with 0.05 M NaCl a partial specific volume of $\bar{v}_D = 0.864$ cm³/g was obtained. In Fig. 7

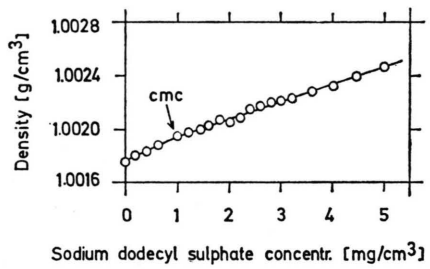

Fig. 7. Dependence of the solution density on the concentration of dodecyl sulphate containing 0.09 M NaCl below and above the critical micelle concentration.

the density of aqueous solutions of sodium dodecyl sulphate with 0.09 M NaCl is shown as a function of the concentration. Plotting shows that the density curve of sodium dodecyl sulphate undergoes a change in slope at $cmc = 1.0$ mg/cm³, where the partial specific volume in the micellar state above the critical micelle concentration is several per cent larger than it is below this concentration: a. $c_D < cmc$, $\bar{v}_D = 0.819 \pm 0.006$ cm³/g; b. $c_D > cmc$, $\bar{v}_D = 0.866 \pm 0.002$ cm³/g. These results agree with the data of Tanford *et al.* within the error limits [5]. Because of difficulties which may arise below the critical micelle concentration all molecular weight determinations in dodecyl sulphate solutions were carried out at 0.25% of detergent.

The binding values of sodium dodecyl sulphate per g polypeptide were measured by self-diffusion. Decays per min and per unit volume of sodium dodecyl [^{35}S] sulphate on the two sides of the membrane were converted into the corresponding concentrations and plotted in Figs 8 and 9 against

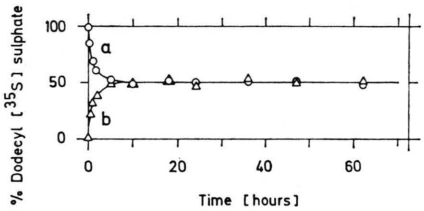

Fig. 8. Time dependence of the diffusion of dodecyl [^{35}S] sulphate through the membrane. Both compartments contained 0.25% dodecyl sulphate in 0.05 M NaCl. Compartment a of the dialysis cell contained at the time onset the radioactivity (—○—). Compartment b contained no label at zero time (—△—).

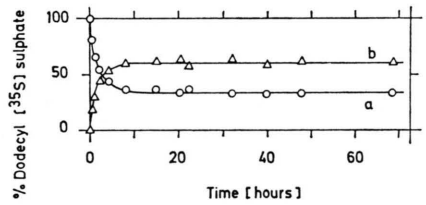

Fig. 9. Same experiment as in Fig. 8 but here the compartment b which did not contain the radioactivity contained in addition the polypeptide (1 mg/cm³).

dialysis time. Fig. 8 shows the time course of the control experiment without polypeptide. After the dialysis time of about 10 hours steady state conditions were reached at 50% of the applied dodecyl [^{35}S] sulphate which demonstrates that no adsorption of dodecyl sulphate to the membrane occurs. In the presence of polypeptide (Fig. 9) the double plot shows that more sodium dodecyl sulphate is in the compartment where the polypeptide is (curve b) than in the compartment without polypeptide (curve a). Steady state values were used for the calculation of the binding values δ_D. 1 g polypeptide binds 1.15 g dodecyl sulphate which corresponds to 100 molecules dodecyl sulphate per polypeptide. This is in agreement with the data reported by Tanford *et al.* [5]. If the dialysis is started with the dodecyl sulphate in only one compartment equilibrium is not reached after 60 hours (Figs 10 and 11).

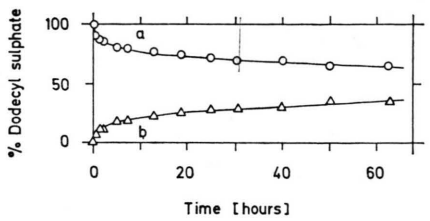

Fig. 10. Time dependence of the diffusion of unlabelled dodecyl sulphate through the membrane. Compartment a contained 0.5% dodecyl sulphate in 0.05 M NaCl (—○—). Compartment b contained at the time onset only 0.05 M NaCl (—△—).

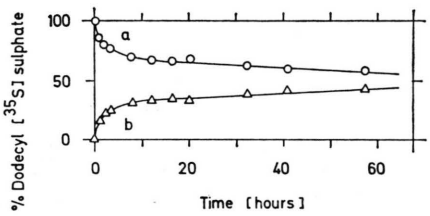

Fig. 11. Same experiment as in Fig. 10. Compartment a contained 0.5% dodecyl sulphate in 0.05 M NaCl which was labelled with dodecyl [^{35}S] sulphate (—○—). Compartment b contained the polypeptide at a concentration of 1 mg/cm^3 in 0.05 M NaCl at the onset of time (—△—).

These results yield with Eqn (3) $\Phi_0' = 0.655$ cm^3/g for the effective partial specific volume and $\bar{v}_P = 0.812$ cm^3/g for the true partial specific volume of the polypeptide. These values agree with the data obtained by density measurements (Table II) and confirm the multicomponent thermodynamic theory [6, 7]. With the binding value δ_D the molecular weight of the polypeptide – sodium dodecyl sulphate micelle was calculated to be $M^* = 54\,000$. For the corresponding partial specific volume $\bar{v}^*$ a value of 0.84 cm^3/g was obtained. If this value is used in the Svedberg equation, the same molecular weight of the polypeptide – dodecyl sulphate micelle is found as by calculation from the molecular weight of the polypeptide and the amount of dodecyl sulphate bound to the polypeptide. By use of S_0 and D_0 the frictional ratio was calculated to be $f/f_k = 1.60$ which points to an asymmetric shape.

After removal of dodecyl sulphate sedimentation velocity measurements were carried out in 0.01 M phophate buffers of pH 6.8 and 7.5. The values of the sedimentation coefficients obtained at a polypeptide concentration of 0.24 mg/cm^3 are 11.7 and 6.8 Svedberg units. This concentration is so low that the sedimentation coefficients do not differ significantly from the values at the limit $c = 0$. If equivalent spheres are assumed for the hydrodynamic particles, molecular weights of 340 000 and 150 000 are obtained respectively. The corresponding Stokes radii are 48 and 37 Å. From these data it results that the polypeptides aggregate in the absence of dodecyl sulphate. The aggregation is higher at pH 6.8 than at pH 7.5. It must be borne in mind that the above listed molecular weights represent the weight of the predominant aggregational state.

Summarizing we can say, that it appears possible to determine the molecular weights of membrane polypeptides in the presence of dodecyl sulphate by ultracentrifugation. In the present case the obtained molecular weight is within the error limits identical to that obtained by dodecyl sulphate polyacrylamide gel electrophoresis. Due to the gain in time the self-diffusion method was found to be superior for the determination of the dodecyl sulphate binding to the usual equilibrium dialysis with initial concentration differences in the two compartments [19, 22]. This difference is obviously due to a membrane effect.

For the sake of completeness it should be mentioned that already earlier investigations had shown, that the molecular weight of the membrane proteins of the chloroplasts is approximately 25 000 [23–26]. This result is remarkable, because it is known now that this molecular weight is that of a mixture of polypeptides, with molecular weights between 66 000 and 10 000 [9, 10]. However, the polypeptide fraction with the molecular weight 25 000 is by far the major component in comparison to the other polypeptides present.

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