

Title	Use of Dodecyl Sulfates in Separation and Analysis of Proteins : Assessment and Development of New Techniques
Author(s)	Kubo, Kanenobu
Citation	大阪大学, 1986, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/35531
rights	
Note	

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Use of Dodecyl Sulfates in Separation and Analysis of Proteins:

Assessment and Development of New Techniques

A doctoral dissertation submitted to Biochemistry Course, Faculty of Science, Osaka University, Osaka 565

(1986)

Kanenobu Kubo

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CURRICULUM VITAE

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履歴書 (CURRICULUM VITAE)

氏	名	久保兼信	(Kanenobu Kubo)					
生年月	日	1949年1月23日						
本籍坦	<u>b</u>	大阪府八尾市	市沼3丁目47番地					
現住所	ŕ	大阪府八尾市	市沼3丁目47番地					

略歴

1973年	3月	近畿大	学薬学部薬	学科卒	業			
1973年	4月	近畿大	学薬学部	実験助) 手	(薬品物	勿理化学研 究	室)
1976年	4月		同	助手				
1980年	4月	大阪大	学蛋白質研	f 究所	共同有	F究員	(研究期間	1年)
1981年1	0月	大阪大	:学蛋白質矿	f 究所	共同矿	F究員	(研究期間	6ヶ月)
		現在に	至る					

著作 (PUBLICATIONS)

原報 (Original Reports)

- Kubo,K., Isemura,T. & Takagi,T. (1975) J.Biochem. <u>78</u>, 349-354 : Electrophoretic properties of sodium dodecyl sulfate and related changes in its concentration in SDSpolyacrylamide gel electrophoresis.
- 2. Takagi,T., Kubo,K., Asakura,J. & Isemura,T. (1975) J.Biochem. <u>78</u>, 1297-1300 : Retarding effect of dodecyl alcohol on polyacrylamide gel electrophoresis of SDS micelles and SDS-protein polypeptide complexes.
- 3. Takagi, T., Kubo,K. & Isemura,T. (1977) Anal.Biochem. <u>79</u>, 104-109 : Simple visualization of protein bands in SDS-polyacrylamide gel electrophoresis by the insoluble complex formation between SDS and a cationic surfactant.
- 4. Isemura, T. & Kubo, K. (1979) Acta medica Kinki Univ. <u>4</u>,
 369-374 : Thermoreversible sol-gel transformation of collagen solution.
- 5. Kubo,K., Isemura,T. & Takagi,T. (1979) Anal.Biochem. <u>92</u>, 243-247 : Electrophoretic behavior of micellar and monomeric sodium dodecyl sulfate in polyacrylamide gel electrophoresis with reference to those of SDS-protein complexes.

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- Takagi,T. & Kubo,K. (1979) Biochim.Biophys.Acta <u>578</u>, 68-75 : Sodium dodecyl sulfate-protein polypeptide complexes in 8 M urea with special reference to sodium dodecyl sulfatepolyacrylamide gel electrophoresis.
- 7. Takagi,T., Kubo,K. & Isemura,T. (1980) Biochim.Biophys.Acta 623, 271-279 : Solubilization of oil-soluble dyes by sodium dodecyl sulfate-protein polypeptide complexes with reference to SDS-polyacrylamide gel electrophoresis.
- 8. Kubo,K., Isemura,T. & Takagi,T. (1980) Anal.Biochem. <u>105</u>, 188-191 : Model study on the fate of lipophilic materials incorporated into sodium dodecyl sulfate-protein polypeptide complexes in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
- 9. Kubo,K., Isemura,T. & Takagi,T. (1982) Biochim.Biophys.Acta <u>703</u>, 180-186 : Interaction between the α1 chain of rat tail collagen and sodium dodecyl sulfate with reference to its behavior in SDS-polyacrylamide gel electrophoresis.
- 10. Kubo,K. & Takagi,T. (1984) Collagen Rel.Res. <u>4</u>, 201-208 : The α 1(I) and α 2(I) chains of collagen separate in sodium dodecyl sulfate-polyacrylamide gel electrophoresis due to differences in sodium dodecyl sulfate binding capacities.

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- 11. Kubo,K., Okabe,N. & Takagi,T. (1985) Collagen Rel.Res. <u>5</u>, 149-156 : Separation of cross-linked products of rat tail tendon collagen from monomeric components by gel chromatography in the presence of Tris dodecyl sulfate at 4°C.
- 12. Kubo,K. & Takagi,T. (1986) Anal.Biochem. <u>156</u>, <u>in press</u>: Binding of lithium dodecyl sulfate to polyacrylamide gel at 4^oC perturbs electrophoresis of proteins.
- 13. Kubo,K. & Takagi,T. (1986) J.Biochem. <u>99</u>, <u>in press</u>: Marked effect of cations on the separation of spinach thylakoid chlorophyll-proteins on polyacrylamide gel electrophoresis at 0°C in the presence of alkanolammonium dodecyl sulfates.

総説 (Reviews)

- 1. 高木俊夫、 久保兼信 (1975) 生化学 <u>47</u>,206-215: SDS-ポリアクリルアミドゲル電気泳動(SDSに重点をおいた考察)
- 高木俊夫、 白浜啓四郎、 辻井薫、 久保兼信 (1976)
 蛋白質 核酸 酵素 21,811-829:SDS-蛋白質ポリペプチド複合体 (SDS-ポリアクリルアミドゲル電気泳動法と関連して)
- 3. 久保兼信、 高木俊夫 (1985) 生化学 <u>57</u>,117-120: ドデシル硫酸リチウム存在下でのポリアクリルアミドゲル電気泳動(問題点と効用)

著書 (Book)

 久保兼信、 高木俊夫 (1986) 生化学実験講座・続 第2巻 タンパク質(今掘ら編), 東京化学同人, 印刷中
 "SDS-ポリアクリルアミドゲル電気泳動に適用できる特殊な検出方法"

Preface

Nowadays research in the field of biochemistry depends heavily on separation techniques. Among them, polyacrylamide gel elecrophoresis is the most favorite of biochemists. In 1965, Summers et al. introduced the use of sodium dodecyl sulfate (SDS) as an additive to the buffer solution in polyacrylamide gel electrophoresis. The modification introduced primarily following merits : 1) SDS binds to a protein in a level of gram-to-gram, and thus the interchain interaction is totally abolished ; and 2) all protein polypeptides acquire negative charge and thus can be analyzed in a single electrophoretic run to positive electrode. Shapiro et al. (1967) further noticed that the lower is the molecular weight of a protein polypeptide the higher is its electrophoretic mobility. They proposed that polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) can be used to estimate molecular weights of proteins. Weber and Osborn (1969) assessed reliability of the technique and established a standard procedure.

Entity being electrophoresed in SDS-PAGE is a complex formed between SDS and protein polypeptide. Its nature has been studied by several groups, and each of them proposed a model for the complex (Reynolds & Tanford, 1970; Shirahama et al., 1974; Wright et al., 1975; Mattice et al., 1980). Among the models proposed, the present author is in favor of the model proposed by Shirahama et al. (1974). This is because various phenomena observed in

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the present study could be best interpreted based on the above model.

SDS-PAGE has been and is being applied to various objects. In every phase of application, new phenomena are observed. Behind each of such a phenomenon, interesting facts are concealed and are mostly worthy of investigation. Outcome of such an investigation will be not only interesting of itself but also contribute to the understanding of the basis of SDS-PAGE and further to develop the technique.

The present author has carried out a series of studies on SDS-PAGE from the viewpoint of "Learn from phenomena, and make clear facts behind them". Recently I have also carried out a study on the effect of substitution of sodium ion in SDS for another cation. The results obtained indicates that new frotiers can be opened for polyacrylamide gel electrophoresis by the manipulation mentioned above. In this thesis, I have compiled the results of the studies described above. Electrophporetic Properties of Sodium Dodecyl Sulfate and Related Changes in Its Concentration in SDS-polyacrylamide Gel Electrophoresis

I. SUMMARY

Sodium dodecyl sulfate (SDS) contained in a protein sample solution migrates in SDS-polyacrylamide gel electrophoresis as a band with a mobility higher than those of protein bands. Behind this band, which is mostly composed of SDS micelles, SDS concentration is raised uniformly in a gel column as a result of the retardation effect of the gel matrix on SDS micelles. Electrophoretic patterns of SDS were obtained when SDS was omitted from various portions of the gel electrophoretic system.

II. INTRODUCTION

Sodium dodecyl sulfate (SDS) undoubtedly plays a key role in SDS-polyacrylamide gel electrophoresis (Weber & Osborn, 1969) which is now extensively used for analytical and preparative purposes. Nevertheless, the behavior of SDS in the electrophoresis is not well understood. In a study by Tsujii and Takagi (1975a) an anionic aromatic surfactant was used in place of SDS in polyacrylamide gel electrophoresis, and its distribution in a gel column was examined by UV-scanning. It was found that micelles

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derived from excess surfactant in a sample solution migrate as a distinct band, and the concentration of surfactant behind this band maintains a higher level than the initial value.

This chapter describes the finding of similar phenomena in SDS-polyacrylamide gel electrophoresis. All the observed phenomena could be interpreted in termes of the electrophoretic properties of SDS in polyacrylamide gel.

III. EXPERIMENTAL PROCEDURE

Sodiuum dodecyl sulfate (SDS) designated as SPS-4 was obtained from Nakarai Chemicals, and used without further purification. The critical micelle concentration was determined to be 8.1 mM in water and 0.95 mM in 0.12 M sodium phosphate buffer, pH 7.2, at 25°C by the conductance and drop weight methods respectively. The former value is in good agreement with those reported for SDS preparations of high purity (Mukerjee & Mysels, 1971). The above buffer was used exclusively in the present study.

Electrophoresis was carried out at room temperature (near 25°C) in a gel (0.5 X 7.5 cm) containing 10 % acrylamide and 0.27 % N,N'-methylenebisacrylamide. Polymerization was initiated by ammonium persulfate and N,N,N',N'-tetramethylethylenediamine. In preliminary experiments, the distribution of SDS in a gel column after electrophoresis was visualized by storage of the gel column after electrophoresis in a refrigerator overnight, as illustrated in Fig.1.

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The concentration of SDS in a gel column was determined as follows. The gel column was cut into small discs each with a length of 2 mm using a scalpel. Each disc was put into a test tube containing 5 ml of distilled water, and kept for 4-6 hr at room temperature. Extraction for this period was confirmed to give a correct value with a gel containing SDS of known concentration. Crushing the gels did not give better results. Α 1 ml aliquot of the extract was added to a test tube containing 1 ml of 0.007 % methylene blue in 1 % sodium sulfate and 5 ml of chloroform. The test tube was shaken vigorously for 1 min with a flash mixer. The chloroform layer containing extracted SDS-methylene blue complex was transferred into an absorption cuvette after filtration through an absorbent cotton plug in a small funnel to remove water droplets. The absorbancy at 650 nm was measured. The concentration of SDS was read from а calibration curve.

IV. RESULTS

Figure 1 shows the distribution of sodium dodecyl sulfate (SDS) in polyacrylamide columns, visualized by chilling after electrophoresis. Except for the absence of proteins in the 1 % SDS solution layered on the gel, the electrophoretic runs were carried out according to the standard procedure for SDS-polyacrylamide gel electrophoresis (Weber & osborn, 1969).

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Fig.1. Visualization of the distribution of SDS by chilling gel columns after electrophoresis. The gel and electrode buffer contained 0.1 % SDS. A current of 8 mA per tube was applied for 3 hr. a) 20 μ l of 1.0 % SDS in 0.01 M sodium phosphate buffer, pH 7.2, was layered on the gel. For b) and c), malachite green (0.0025 %) and bromophenol blue (0.01 %) were added to the 1 % SDS solution, respectively. Arrows indicate the positions of the bands of SDS micelles (a), malachite green (b), and bromophenol blue (c).

The degree of whitening due to crystallization of SDS may be taken to reflect the concentration of SDS in the columns. The column (a) can be divided into three parts with respect to SDS concentration. The degree of whitening near the posiyive electrode tray was the same as that observed with a gel column not subjected to electrophoresis. At the position indicated was observed. In other parts of the gel column, the degree of whitening was found to be uniformly raised as a result of electrophoresis.

Malachite green and bromophenol blue were added to the sample solutions in columns (b) and (c), respectively. It is well-known that these dyes are electrophoresed in advance of protein bands in SDS-polyacrylamide gel electrophoresis, and they are generally used as marker dyes. It was found that malachite green was electrophoresed with the same mobility as the white band, and bromophenol blue was behind the white band. These results clearly indicate that the white band has a higher electrophoretic mobility than any SDS-protein polypeptide complexes.

determine the distribution of SDS quantitatively, То SDS content in the gel column was analyzed after electrophoresis. results obtained. Figure 2 illustrates the The starting conditions were variously modified with respect to SDS. Figure results obtained for an electrophoretic 2a shows the run identical to that of column (a) in Fig.1. Clearly the photograph (Fig.1) faithfully reflects the distribution of SDS in the gel. We have found that all the features in the distribution of SDS shown in Fig.2 could also be detected qualitatively by means of

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Fig.2. Distribution of SDS in polyacrylamide gel after electrophoresis under various initial conditions.

Gel, 10 %; 8 mA/tube, 3 hr. The sample solution (20 μ l in 0.01 M, ક SDS of 1 7.2, sodium phosphate Hα buffer) was layered on a gel for a), c), e-1), and The buffer solution in f). the negative electrode tray contained 0.1 % SDS for a), b), c) and d-1); 0.01 % SDS for d-2); and none for e) and f). The polyacrylamide qel contained 0.1 ક્ર SDS

for a), b) and e); and none for c), d) and f). The positive electrode tray was always filled with the buffer used for the negative electrode tray. The presence or absence of SDS in the positive electrode buffer, however, was found to have no effect on the final distribution in a gel in any of the electrophoretic runs shown in Fig.2. Figure 2a includes all data points to illustrate the extent of scattering of the measured values.

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visualization by chilling.

Fgure 2b shows that the increase of SDS concentration occurs even in the absence of added 1 % SDS solution. As is clear from Figure 2c and d (curve 1), the absence of SDS in the gel at the start of electrophoresis has no major effect on the migration of the white band and the uniform increase of SDS concentration. Figure 2d (curve 2) shows that the increase of SDS concentration does not occur when the initial concentration is 0.01 %. Figure 2e shows the results obtained when SDS was omitted from the buffer solution used to fill the trays. Figure 2f shows the electrophoretic pattern of SDS when it was added only in the sample solution. It should be noted that SDS is electrophoresed as a sharp band with only slight tailing.

A series of experiments similar to those shown in Fig. 2d was carried out. The amount of SDS added to the electrode trays was varied. Figure 3 shows plots of SDS concentration, in the portion of the gel column where the increase of SDS concentration was observed, <u>versus</u> that in the buffer solution used to fill the electrode trays in the electrophoretic run.

V. DISCUSSION

Micelle Band In SDS-polyacrylamide gel electrophoresis, proteins are generally dissolved in 1 % (35 mM) sodium dodecyl sulfate (SDS). SDS present in the sample solution in excess of the critical micelle concentration (0.95 mM under the condition

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Fig.3. Plots of SDS concentration in the portion of the gels near the negative electrode tray <u>versus</u> that in the buffer solution used to fill the trays. The gels contained no SDS at the start of electrophoresis.

used in the present experiments) naturally exists as micelles. shown by Stigter and Mysels (1955), an SDS micelle is As electrophoresed to the positive electrode as an entity. Tsujii & Takagi (1975a) showed that the presence of a micelle band was observed in polyacrylamide gel electrophoresis in which SDS had been replaced by an aromatic surfactant. Based on experimental obtained with SDS micelles and SDS-protein polypeptide data complexes, the presence of a micelle band with a mobility higher than any of the complexes was predicted for SDS-polyacrylamide gel electrophoresis (Tsujii & Takagi, 1975a; Shirahama et al., 1974). This band, observed as a white band in Fig.1 and as a sharp peak in Fig.2, has the expected properties, and can be assigned unambiguously as the micelle band.

The presence of the micelle band has attracted the attention of only a few groups (Wallace et al., 1974; Stoklosa and Latz, 1974), and has been treated only as a "surplus" band. We believe, however, that the micelle band plays an important role, and should be utilized in SDS-polyacrylamide gel electrophoresis. applied to biomembranes, lipids the and When method was glucosamine were found to be electrophoresed in advance of bromophenol blue, used as a marker dye (Lopes & Siekevitz, 1973; Gamberg, 1971). As pointed out by Tanford (1973), it seems that these compounds are actually electrophoresed not as an isolated entity but in a state solubilized or adsorbed on SDS micelles. micelle band plays a role in removing Presumably the contaminating compounds, which might otherwise interfere with the normal behavior of protein bands, from a sample solution.

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As is clear from Figs.1(a) and 2a, SDS micelles migrate as a sharp band. The micelle band is, therefore, expected to be an excellent marker. Actually the band acts as a marker when cationic dyes, such as malachite green, are used. The dye was found to be bound to the micelles, and electrophoresed en bloc, as shown in Fig.1(b). Acridine orange, a cationic dye, has been shown to be bound to micelles of SDS and to become strongly fluorescent (Flanagan & Ainsworth, 1968). Our preliminary experiments showed that the band in Fig.1 became strongly fluorescent following the addition of a trace amount of the dye to the 1 % SDS solution layered on the gel, and could be used as an excellent marker in SDS-polyacrylamide gel electrophoresis.

An anionic dye, bromophenol blue, frequently used in SDS-polyacrylamide gel electrophoresis was found to be electrophoresed independently behind the micelle band, as shown in It has been found that protein bands with relatively Fig.1(c). high electrophoretic mobility may overlap the band of bromophenol blue. Use of a cationic dye as a marker is therefore recommended, because no protein band is expected to be electrophoresed with mobility comparable to that of SDS micelles, as mentioned by Tsujii & Takagi (1975a), and the SDS micelles form a sharper band than bromophenol blue, as is clear from Fig.1.

As shown in Fig.2a, c, e (curve 1), and f, the SDS micelle band was found to be electrophoresed as a sharp peak under various conditions. When the micelle band migrates through a medium with an SDS concentration above the critical micelle concentration, the micelles do not dissociate and the presence of a sharp band (Fig.2a) is expected. SDS micelles are expected to be dissociated, when they are put into a medium containing SDS in a concentration below the critical micelle concentration. Observation of a sharp micelle band in each of the present experiments shown in Fig.2c, e (curve 1), and f seems to be a consequence of the peculiar situation that the micelles and single SDS ions have almost the same electrophoretic mobility in the present experimental condition, as can be seen from the two curves in Fig.2d. The micelle band in Fig.2f is slightly tailed. This indicate that the micelle has a mobility slightly higher than that of the single ion. However, the situation will be different when a gel has acrylamide concentration other than 10 %.

Why Does the Concentration of SDS Increase ? The increase in the SDS concentration in the region behind the micelle band (Fig.2a and c) can be interpreted as follows. Following the micelle band, SDS in the buffer solution of the negative electrode tray intrudes into the gel. As shown in Fig.3, no increase in the SDS concentration was observed when its concentration was less than 0.8 mM, roughly equal to the critical micelle concentration of SDS (0.95 mM). This clearly indicates that single SDS ions are not involved in the increase in the SDS concentration. Under the conditions of the present experiments, an SDS micelle is spherical and has a Stokes' radius of about 24 A (Stigter & Mysels, 1955). This is comparable to the size of a globular protein with a molecular weight of about 32,000 (Rodbard & Chrambach, 1970; Hedrick & Smith, 1968). The SDS micelles,

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therefore, must be retarded as soon as they intrude into the gel. The electrophoretic mobility in a gel, U_g , can be correlated with the free electrophoretic mobility, U_0 , according to the following equation (Rodbard & Chrambach, 1970).

$\log U_{q} = \log U_{0} - K_{R}T$ (1)

The retardation coefficient, $K_{
m R}$, for a micelle with the above size is estimated to be 0.053 from plots of the value of $\ensuremath{\kappa_{\mathrm{R}}}$ versus the Stokes' radii of various proteins (illustrated in Ref. of Rodbard & Chrambach, 1970). In the 10 % acrylamide gel used in the present experiments, the SDS micelles, thus, are expected to be retarded and to have an electrophoretic mobility of 30 % of that in the absence of the gel matrix, according to Eq.1. In the case of the electrophoretic run shown in Fig.2a (total concentration, ca.3 mM) it is expected that only SDS micelles (2.0 mM) will be retarded in the gel, and the total concentration of SDS in the gel will increase from the initial value of 3.0 to 7.7 mM. The expected increase (2.6 times) of the SDS concentration agrees fairly well with the observed 2.3-fold increase. Because the uniform increase of SDS concentration is caused by SDS micelles initially present in the buffer of the upper trough, the same phenomenon can be observed when the preceeding micelle band is absent, as in Fig.2b and d.

VI. CONCLUSION

Surprisingly, only one report has been previously concerning the distribution of SDS in polyacrylamide gel using S³⁵-labeled SDS (Shapiro & Mizel, 1969), despite the widespread use of SDS-polyacrylamide gel electrophoresis. The results obtained in the present study clearly indicate that the distribution of SDS in the gel is significantly influenced by the electrophoretic and hydrodynamic properties of SDS micelles. It is believed that the data obtained here will be valuable whenever SDS-polyacrylamide gel electrophoresis is applies.

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 Electrophoretic Behavior of Micellar and Monomeric Sodium Dodecyl Sulfate in Polyacrylamide Gel Electrophoresis with Reference to Those of SDS-Protein Complexes

I. SUMMARY

Electrophoretic behavior of sodium dodecyl sulfate (SDS) in polyacrylamide gels has been examined at various gel concentrations. Micellar SDS is subject to significant molecular sieving from the gels while monomeric SDS is virtually free from the effect. The two forms are in a rapid equilibrium with each other. The gel concentration, therefore, has a significant effect on the electrophoresis of SDS added in SDS-polyacrylamide gel electrophoresis. The simplified procedure of Stoklosa and Latz (1974), in which SDS is added only in sample solutions, has been criticized based on the results obtained.

II. INTRODUDTION

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Weber & Osborn, 1969) is now extensively used, and various modified procedures have been described. Among them, the simplified procedure proposed by Stoklosa and Latz (1974) attracted our attention, since it indicated that it is necessary to add SDS only to sample solutions. In the previous study by

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Kubo et al. (1975), it was demonstrated that the SDS in equilibrium with SDS-protein complexes during electrophoresis is supplied from the upper buffer compartment alone. Elimination of SDS from gels and the lower buffer compartment, therefore, will not affect the electrophoresis. However, it appeared that elimination of SDS from the upper buffer compartment would have serious consequences.

SDS-protein complexes, which are the electrophoresing entities in the protein bands, are probably in a rapid equilibrium with free SDS in the surrounding medium by analogy to the detection of such behavior in the complex between a polypeptide derived from bovine serum albumin and a surfactant analogous to SDS, using NMR spectroscopy (Tsujii & Takaqi, 1975b). Such an SDS-protein complex must dissociate when it is removed from free SDS as the result of electrophoresis. There is reason to believe that anomalous results may be obtained with the procedure of Stoklosa and Latz (1974). Nelles and Bamburg (1976) reported that the presence of a small amount of SDS in the upper buffer compatment is necessary to obtain decent results using SDS-polyacrylamide gel electrophoresis. Stoklosa and Latz (1975) later reported that the amount of SDS added to a sample solution has a crucial effect on the electrophoretic behavior of protein bands in their Since information on the electrophoretic behavior of procedure. micelles and monomers of SDS in polyacrylamide gels is lacking, the present study was initiated.

III. EXPERIMENTAL PROCEDURE

Sodium dodecyl sulfate designated as SPS-4 was obtained from Nakarai Chemicals. It was analyzed by gas chromatography after hydrolysis. The result showed that 96.9 % was dodecyl sulfate. The major contaminant was tetradecyl sulfate. A cationic surfactant, alkylbenzyldimethylammonium chloride (alkyl, C₈H₁₇-C₁₈H₃₇) was obtained from Kao-Atlas Company Ltd. as a 50 % aqueous solution known as Sanisol C and used without further purification. Hen's egg white lysozyme which had been reduced and carboxamidomethylated (RCAM) was used (Shirahama et al., 1974).

Polyacrylamide gel electrophoresis was carried out essentially according to the procedure of Weber and Osborn (1969), except that the amount of SDS added to the upper buffer compartment was changed as indicated and SDS was not present in either the lower buffer compartment or the gels. Electrophoresis was carried out at 25° C using a thermostated apparatus. Sodium phosphate buffer, 0.1 M, pH 7.0, was used to prepare the gels and to fill the upper and lower compartments. In the preparation of the gels of various acrylamide concentrations, the weight ratio of acrylamide to N,N'-methylenebisacrylamide was kept constant (100 : 2.7). Gel concentrations will be expressed in terms of the total acrylamide concentration (w/v).

Gels were stained for proteins with 0.025 % Coomassie brilliant blue R in a mixture of isopropanol-acetic acid-water (volume ratio, 25 : 10 : 65). Distribution of SDS in a gel was visible as a white zone by the method of Takagi et al. (1977).

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It utilizes the insoluble complex formation between SDS in a gel and the cationic surfactant, alkylbenzyldimethylammonium chloride, diffused into the gel. Gels, in which the location of protein or SDS had been visible, were also scanned with a Toyo Model DMU-33C densitometer. Most of the results were, however, illustrated as photographs, because they can directly demonstrate what occurred in a gel. All photographs of the gels except the left one in Fig.5 were taken with gels immersed in test tubes filled with water, and, thus, laterally expanded. The left photograph of Fig.5 shows the usual form of the gels.

IV. RESULTS

electrophoretic behavior of monomers and micelles The of sodium dodecyl sulfate was examined in the absence of protein Their distribution in the polyacrylamide gel can be samples. clearly detected by the visualization technique (Takagi et al., 1977). Relative electrophoretic mobilities of SDS monomers and measured by the distance of SDS micelles were which had penetrated into the gels when SDS was added only to the upper buffer compartment. Fig.1 shows typical examples of the results obtained. In Fig.1A, the SDS concentration was 0.35 mM, which was below the critical micelle concentration (1.1 mM in the buffer used). The boundary, therefore, is the front of SDS monomers electrophoresed in the 5 % gel. When the SDS concentration was increased to 3.5 mM above the critical micelle

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Fig.1. Electrophoresis of SDS from the upper buffer compartment into polyacrylamide gels. Gel concentration, 5 % for (A) and (B) and 12.5 % for (C). Electrophoresis was carried out at 6 mA/tube for 130 min for (A) and (B) and 225 min for (C). SDS concentration in the upper buffer was 0.35 mM for (A) and 3.5 mM for (B) and (C).



Fig.2. Ferguson plot of the relative mobilities of SDS micelles against gel concentration. The mobility of SDS monomers which are free from the molecular-sieving effect was used as a referenc (----). Experiments were carried out in the same manner as in Fig.1.

concentration (Fig.1B), the front of SDS with a clear boundary was found to migrate in the 5 % gel with a mobility higher than that in Fig.1A. The difference in the mobility can only be explaned assuming that SDS micelles with higher mobility than SDS monomers form the front in this case. When the gel concentration was increased up to 12.5 %, the situation was reversed, and the electrophoresis of SDS monomers could be clearly observed in front of the boundary assigned to SDS micelles as shown in Fig.1C.

Figure 2 shows the relative electrophoretic mobilities of SDS micelles as a function of gel concentration. Because the mobilities of SDS monomers were independent of gel concentration, they were used as the standard to calculate relative mobilities. Electrophoresis of SDS micelles in polyacrylamide gels was markedly influenced by the molecular-sieving effet of the gel. At 10 % gel concentration, SDS micelles and SDS monomers had the same mobility.

Figure 3 shows the results of electrophoresis of SDS when it was added only to the tops of the gels in small volumes as samples. SDS was present only in the sample solution. Figure 4 shows densitometric traces of the gels shown in Fig.3. The observed electrophoretic behavior of SDS can be clearly understood when compared with the results shown in Fig.2. When the gel concentration is 5 % (Fig.3A and Fig.4A), SDS micelles migrate ahead of SDS monomers and, therefore, are dissociated in the back to cause tailing. At 10 % gel concentration, SDS migrated as a sharp band (Fig.3B and Fig.4B). Under these conditions, SDS micelles are electrophoresed together with SDS

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Fig.3. Electrophoresis of SDS in polyacrylamide gels of various concentrations. Gel concentrations : (A) 5 %, (B) 10 %, and (C) 15 %. Twenty microliters of the sample solutions were applied on the top of each gel and contained 1 % SDS and 10 % glycerine in 0.01 M sodium phosphate buffer, pH 7.0. Electrophoresis : 2 hr, 6 mA/tube.



Fig.4. Densitometric scan of the gels shown in Fig.3. Designations are the same as in Fig.3.
monomers, which they are in equilibrium with as seen in Fig.2. Net dissociation of SDS micelles is, therefore, not expected. In 15 % gel, SDS monomers have higher mobility than SDS micelles (Fig.2), and, therefore, dissociation of micelles is observed in the frontal boundary (Fig.3C and Fig.4C). Under the conditions of Fig.3A and C, the dense SDS bands mainly composed of micelles gradually faded out during migration, since they were continuously deprived of SDS.

In Fig.5, reduced and carboxamidomethylated lysozyme was subjected to electrophoresis with SDS. SDS was again added only to the sample solutions. Figures 5A, B and C show distribution of RCAM-lysozyme visible by staining after electrophoresis in 10 % gels. Figure 5A', B' and C' show the distribution of SDS visible because of the insoluble complex formation. In Fig.5C', the SDS-protein complex is also visible as a white band at the top, as well as the micelle band electrophoresing in the front. As seen in Fig.5C and C', the SDS-protein complex migrates just behind the free SDS.

SDS monomers in equilibrium with the SDS-RCAM-lysozyme complex have higher mobility than the complex. The protein band is, therefore, continuously deprived of SDS. The SDS which lies between the protein band and the SDS micelle band in Fig. 5C' is the product of such dissociation, although it looks like a tail of the micelle band. However, the micelle band should be free from tailing, since SDS micelles and SDS monomers have the same mobility in 10 % gel as is clear from Figs.2 and 3. As SDS is depleted from the SDS-protein complex, RCAM-lysozyme precipitates

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Complex Dissociated SDS Micelle

Fig.5. Electrophoresis of RCAM-lysozyme in 10 % gel. Twenty microliters of the sample solutions were applied on the top of each gel and contained 1 % SDS and 10 % glycerine in 0.01 M sodium phosphate buffer, pH 7.0. RCAM-lysozyme added : (A) and (A') 5 μ g, (B) and (B') 10 μ g, and (C) and (C') 20 μ g. Electrophoresis: 4 hr, 6 mA/tube. (A), (B) and (C) RCAM-lysozyme was stained by Coomassie brilliant blue R; (A'), (B') and (C') SDS was visible due to the complex formation with the cationic surfactant.

as is shown in the gels of Fig.5A, B and C, which were stained for protein. The protein band disappears completely when the amount of RCAM-lysozyme is low, as in Fig.5A and B.

V. DISCUSSION

The results presented have shown that the electrophoretic behavior of SDS micelles and monomers in a polyacrylamide gel vary with the concentration of SDS and the concentration of the gels. The presence and absence of the molecular-sieving effect for SDS micelles and monomers, respectively, are the most important factors in the determination of the electrophoretic behavior of these two species in polyacrylamide gels, and should be taken into consideration when one intends to modify the standard conditions of SDS-polyacrylamide gel electrophoresis (Weber and Osborn, 1969).

The present study was prompted by the modified procedure of SDS-polyacrylamide gel electrophoresis proposed by Stoklosa and Latz (1974). The proposed procedure was based on the assumption that the complexes formed between SDS and proteins are stable even in the absence of SDS in equilibrium. As is clear from Fig. 5, this assumption is incorrect. Five percent gels were used in their study, and this is probably the reason for their apparent success. Under the condition, SDS-protein complexes are electrophoresed in the tailing region shown in Fig.4A, where the SDS concentration is maintained at the level of the critical micelle

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concentration which prevents the dissociation of the complexes. In a later study it was pointed out that enough SDS must be added to sample solutions (Stoklosa and Latz, 1975). This is necessary to maintain SDS above the critical micelle concentration in the tailing region during electrophoresis. Since SDS micelles are electrophoresed at their characteristic mobilities, the range of SDS monomers at the level of the critical micelle concentration in the tailing region is limited. Complexes formed between SDS and proteins with relatively high molecular weights would be expected to dissociate gradually to cause precipitation of the proteins in the same manner as shown in Fig.5 (left).

To perform SDS-polyacrylamide gel electrophoresis without anomalous behavior, it is strongly recommended to add SDS to the upper tray buffer up to at least the critical micelle concentration and maintain the SDS concentration in gels by SDS supplied from the upper buffer compartment (Kubo et al., 1975). In opinion, the amount of SDS saved by eliminating it from the upper buffer compartment is not worth the difficulties that might be encountered.

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3. Retarding Effect of Dodecyl Alcohol on Polyacrylamide Gel Electrophoresis of SDS Micelles and SDS-protein Polypeptide Complexes

I. SUMMARY

Micelles of sodium dodecyl sulfate (SDS) are significantly retarded by the addition of a small amount of dodecyl alcohol to a sample solution in SDS-polyacrylamide gel electrophoresis. The phenomenon can be ascribed to the decrease in charge density due to the incorporation of dodecyl alcohol into SDS micelles. The effect is extended to SDS-protein polypeptide complexes when the amount of SDS micelles is insufficient to accommodate the dodecyl alcohol. A similar effect is likely to occur when SDS-polyacrylamide gel electrophoresis is applied to a sample containing lipophilic materials.

II. INTRODUCTION

In SDS-polyacrylamide gel electrophoresis (Weber & Osborn, 1969), micelles of SDS as well as complexes formed between SDS and protein polypeptide are the main electrophoresing entities (Tsujii & Takagi, 1975a; Kubo et al., 1975). It has been suggested that SDS binds to protein polypeptide to form micelle-like clusters (Shirahama et al., 1974; Tsujii & Takagi,

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1975b; Takagi et al., 1975a). Therefore, the bound SDS is expected to have properties similar to those of SDS micelles. Steinhardt et al. (1974) showed that the bound SDS could solubilize oil-soluble dye to an extent comparable to that of micellar SDS. Since SDS-polyacrylamide gel electrophoresis is often applied to samples containing lipophilic materials, it is necessary to know the effect of solubilization of such materials on the electrophoretic behavior of SDS micelles and SDS-protein polypeptide complexes. This chapter describes the effect of the addition of dodecyl alcohol, which is known to be incoporated into SDS micelles (Sinoda et al., 1963), to sample solutions in SDS-polyacrylamide gel'electrophoresis.

III. EXPERIMENTAL PROCEDURE

Sodium dodecyl sulfate (SDS) and dodecyl alcohol were obtained from Nakarai Chemicals and Kao Soap Co., respectively. They were 96.9 99.8 and 8 pure as determined by gas chromatography. SDS-polyacrylamide gel electrophoresis was carried out essentially according to the procedure of Weber and Osborn (1969) using a temperature-controlled gel electrophoresis apparatus at 30°C. In the preparation of gels of various acrylamide concentrations, the weight ratio of acrylamide to N,N'-methylenebis(acrylamide) was kept constant (100:2.7). In most experiments, protein bands as well as the SDS band were visualized as white bands by storage of the polyacrylamide gel tube in a refrigerator for several hours

after electrophoresis (Kubo et al., 1975; Wallace et al., 1974).

IV. RESULTS AND DISCUSSION

Dodecyl alcohol, virtually insoluble in aqueous solutions, could be dissolved in 0.01 M sodium phosphate buffer in the presence of 1 % SDS. As shown in Fig.1, the electrophoretic mobility of the band chiefly composed of SDS micelles (Tsujii & Takagi, 1975a; Kubo et al., 1975) (hereafter called the SDS band) is significantly reduced by the addition of a small amount of dodecyl alcohol to sample solutions (to 88 % in the presence of 5 % dodecyl alcohol; w/w SDS). The experiments shown in Fig.1 were carried out in the absence of SDS in the gels to ensure that the SDS bands could be clearly seen. Tailing of the SDS band became more significant as the amount of dodecyl alcohol added was increased, probably due to dissociation of the retarded SDS micelles, which are left in a medium devoid of monomeric SDS ions.

With proteins it is common practice to carry out electrophoresis at various gel concentrations to estimate molecular size (Rodbard & Chrambach, 1970). We have applied the technique to SDS micelles to estimate roughly the effect of solubilization of dodecyl alcohol on the size of SDS micelles. Figure 2 shows a Ferguson plot (Ferguson, 1964) of the results obtained. The mobilities were calculated from the distance covered by the front of the plateau region formed by SDS micelles which intruded into

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Fig.1. Effect of the addition of dodecyl alcohol on the electrophoresis of the SDS band. Sample solutions (20 μ l) of SDS (1 %) in 0.01 M sodium phosphate buffer, pH 7.2, containg 10 % glycerine were layered on 7.5 % acrylamide gel. The gel contained 0.1 M sodium phosphate buffer, pH 7.2, which was also used to fill the upper and lower trays. The amounts of dodecyl alcohol added to the sample solutions were as follows (w/w SDS); controls (a, b), 1 % (c), 2 % (d), 3 % (e), 4 % (f), and 5 % (g): Malachite green (0.0025 %) was added to the sample solution only for (a). Electrophoresis; 8 mA/tube, 2.7 hr.



Fig.2. Ferguson plots of the electrophoretic mobilities of SDS micelles. The gels and trays contained 0.1 M sodium phosphate buffer, pH 7.2. SDS was added up to 0.1 % to the buffer of the upper (cathode) tray. Ten µl of 0.1 M sodium phosphate buffer containing 0.01 % bromophenol blue, 0.1 % SDS, and 10 % glycerine layered gels of various acrylamide concentrations. was on Dodecyl alcohol was added to both the buffer and the marker dye solutions up to 0.003 % for the plots designed by filled points. Electrophoresis was carried out for 3.5-7 hr, depending on the gel concentration, at 30°C at a constant current of 8 mA/tube. The tubes were stored for 6 hr in a refrigerator, and the mobilities were measured in terms of the distance of the front of the white region from the end of the gel (cathode side) using the bromophenol blue band as a reference.

the gel column from the tray buffer and which could be visualized by chilling the gel after electrophoresis. It should be noted that the line obtained with the experiments in the presence of dodecyl alcohol is parallel to that for SDS micelles in the absence of dodecyl alcohol. The slope of the line in such plots is expected to be determined solely by the size of the micelles just as in the case of spherical globular proteins (Rodbard & Chrambach, 1970). The size of SDS micelles is therefore, suggested to be almost constant despite the solubilization of dodecyl alcohol. The change of electrophoretic mobility,therefore, suggests that the effective charge density of SDS micelles is decreased as a result of the solubilization. The solubilized dodecyl alcohol probably displaces SDS, decreasing the charge density, while the size of the micelles is kept constant.

If SDS ions bound to a protein polypeptide really behave like SDS micelles regards solubilization, as discussed as by Steinhardt et al. (1974), SDS-protein polypeptide complexes are expected to solubilize dodecyl alcohol, and thus their electrophoretic behavior should change depending on the presence or absence of dodecyl alcohol. Addition of dodecyl alcohol up to 5 % (w/w SDS) to sample solutions, however, showed no appreciable effect on the mobilities of the complexes. Clearly SDS micelles are superior to SDS clusters on a SDS-protein polypeptide complex in the solubilization of dodecyl alcohol. If this preferential solubilization by SDS micelles is general, this phenomenon might well be valuable in connection with SDS-polyacrylamide gel electrophoresis.

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Fig.3. Normalization of the electrophoresis of protein bands retarded by the addition of dodecyl alcohol when SDS present in the sample solution in an amount. The gels and trays contained 0.1 M sodium phosphate buffer, pH 7.2. SDS (0.04 %) was added only to the cathode tray. The concentration of SDS was reduced so that the protein bands could be visualized as distinct bands. The sample solutions (20 μ l) contained various amounts of SDS (a, 64 μ g; b, 64 μ g; c, 613 μ g; d, 170 μ g) and dodecyl alcohol (a, none; b, 4.6 μ g; c, 4.6 μ g; d, none). Proteins added to each sample solution were 16 μ g of immunoglobulin G, 8 μ g of myoglobin, and 8 μ g of cytochrome <u>c</u>. Acrylamide concentration, 10 %; 8 mA/tube, 4.5 hr.

Subsequent experiments showed, however, that SDS-protein polypeptide complexes do have solubilizing power. Figure 3d shows an electrophoretic run comparable to an ordinary SDS-polyacrylamide gel electorophoresis (Weber & Osborn, 1969). Decrease in the amount of SDS added to the sample solution down to one-third had no appreciable effect on the electrophoretic patterns of SDS micelles and the complexes, as shown in Fig.3a. Under these conditions, however, the addition of dodecyl alcohol has a significant retarding effect on the electrophoretic behavior of the complexes as shown in Fig.3b. Further addition of SDS normalized the electrophoretic behavior of both SDS micelles and the complexes, as shown in Fig.3c. In this case, the SDS band is broad, and the retardation is not appreciable due to the presence of a large excess of SDS.

It is well-known that SDS can solubilize lipids of biomembrane origin (Helenius & Simons, 1975). When SDS-polyacrylamide gel electrophoresis was applied to biomembranes dissolved in SDS, the resulting electrophoretic patterns suggested that the lipids were solubilized in SDS micelles and electrophoresed en blok (Gahmberg, 1971; Lopez & Siekevitz, 1973). Carraway et al. (1972) reported the electrophoretic mobility of phosphatidylcholine that is dependent on the amount of SDS added to the sample solution. Their results suggest that lipids contained in biomembranes can affect the electrophoretic behavior of SDS micelles and SDS-protein polypeptide complexes. The present results indicate, however, that the effect on the complexes can be eliminated by the addition of sufficient SDS to the sample solution.

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In conclusion, it should be stressed that both SDS micelles and SDS-protein polypeptide complexes can solubilize lipophilic materials and their behavior in polyacrylamide gel electrophore-Such phenomena consequently affected. should, may be sis therefore, be taken into cosideration in the design of SDS-polyacrylamide gel electrophoresis experiments and interpretation of the results obtained, especially when the sample being investigated contains materials which can be solubilized in SDS micelles.

4. Simple Visualization of Protein Bands in SDS-Polyacrylamide Gel Electrophoresis by the Insoluble Complex Formation between SDS and a Cationic Surfactant

I. SUMMARY

Protein bands in SDS-polyacrylamide gel electrophoresis can be visualized as white bands by the mere immersion of a gel, after electrophoresis, into a dilute aqueous solution of cationic surfactant with which SDS forms an insoluble complex. Because the time-consuming and laborious step of staining is eliminated, this procedure is particularly suited for quick detection of protein bands after electrophoresis.

II. INTRODUCTION

In SDS-polyacrylamide gel electrophoresis, protein bands are generally visualized by staining. Since the procedure is laborious and takes a whole day, several alternatives have been proposed to simplify the detection of the bands. These include fluorescent labeling of proteins (Shelton, 1971), uv-scanning of a gel (Tsujii & Takagi, 1975a), and chilling of a gel to crystallize SDS as white bands (Wallace et al., 1974; Takagi et al., 1975b). We have developed a unique procedure to visualize protein bands, which is very simple and particularly suited to

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locating protein bands quickly. It depends on the insoluble complex formation between SDS and a cationic surfactant.

III. EXPERIMENTAL PROCEDURE

Alkylbenzyldimethylammonium chloride (alkyl, C₈H₁₇-C₁₈H₃₇) was found to be most satisfactory for present purpose out of several cationic surfactants tested. It was obtained from Kao-Atlas Co. Ltd., (Tokyo, Japan) as a 50 % aqueous solution named Sanisol C, and was used without further purification. This is one of the most popular germicidal cationic surfactants. The critical micelle concentration in water was found to be 0.23 % as measured by an electric conductance method (Goddard & Benson, 1957).

Other reagents were those routinely used in SDS-polyacrylamide gel electrophoresis (Weber & Osborn, 1969). Proteins used had been reduced and carboxamidomethylated (RCAM) by the method described Shirahama et al., (1974). The present procedure can, however, be applied to ordinary SDS-polyacrylamide gel electrophoresis in which proteins are reduced with 2-mercaptoethanol in a sample solution.

SDS-polyacrylamide gel electrophoresis was performed according to the procedure described by Weber and Osborn (1969). The only modification necessary was a reduction of the SDS concentration from 0.1 to 0.035-0.05 %, which caused no effect on molecular weight estimation. This modification was required to reduced the whitening all over the gel due to the complex formation between the SDS prevalent in the gel and the cationic surfactant.

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After electrophoresis, gels were withdrawn from the tubes and immersed in a dilute solution of alkylbenzyldimethylammonium chloride prepared by the 50-fold dilution of Sanisol C with water (final concentration, ca. 1 %) at room temperature. Protein bands as well as SDS-micelle band (Kubo et al., 1975) were visualized as white bands which could be located easily by the naked eye with illumination from the rear. After 10-20 min, gels were transferred into a further diluted surfactant solution prepared by a 1000-fold dilution of Sanisol C with water and were incubated in it for at least 1.5 hr to complete the development of white bands. It is preferable to maintain the temperature of the solutions around 20°C for the development of white bands. Otherwise, the appearance of the white bands was incomplete. Once the white bands have been developed, the gels may be stored indefinitely at room temperature.

Gels in test tubes, filled with the second surfactant solution used to develop white bands, were put before a black screen, and a photograph was taken by the use of illumination from the upper rear. Electrophoretic patterns were obtained using a densitometer (Toyo, Model DMU-33C).

The amount of SDS bound to a protein was measured by the equilibrium dialysis technique (Takagi et al., 1975a).

IV. RESULTS AND DISCUSSION

It is well-known that an anionic surfactant forms a

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water-insoluble complex (1:1 molar ratio) with a cationic surfactant. However, the complex is stable only when the equilibrium concentration of one of the constituent surfactants, present in excess, is below its critical micelle concentration. In the present method, a gel containing protein bands to be developed is first immersed in the cationic surfactant (alkylbenzyldimethylammonium chloride) solution at a concentration above its critical micelle concentration, to make the surfactant rapidly diffuse into the gel. To avoid solubilization of the white complexes once formed, the gel is transferred into the cationic surfactant solution with a concentration below its critical micelle concentration.

Figure 1a shows a typical example of a gel in which protein bands, containing 5 μ g each of RCAM-bovine serum albumin and RCAM-lysozyme (top and middle), as well as the micelle band are visualized by the present method. The micelle band (Kubo et al., 1975) may be used as a substitute for a band of marker dye. Figure 1 also includes two blank runs. Figure 1b shows that only the micelle band could have been observed when two protein samples were eliminated from the sample solution. Figure 1c shows a gel to which no sample solution was added; a boundary can be observed. This is the result of the retardation of the SDS micelles supplied by the upper electrode trough (Kubo et al., 1975).

Figure 2 shows that the protein bands could have been visualized even when the amount of each sample protein was decreased to more than one-tenth of that in Figure 1a.

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Fig.1. Photograph of the protein and SDS-micelle bands in 10 polyacrylamide gels, visualized by the water-insoluble complex formation between SDS and alkylbenzyldimethylammonium chloride. (a) The sample solution contained 5 µg each of RCAM-bovine serum albumin and RCAM-hen's egg white lysozyme in 20 µl of 0.01 M sodium phosphate buffer, pH 7.0, containing 1 SDS. (b) Same as (a) except that the sample solution was devoid of protein and contained only 1 SDS in the buffer. (c) Same as (a) except that no sample solution was applied to the gel. The gels and the trays contained 0.1 M sodium phosphate buffer, pH 7.0, to which SDS was added up to 0.035 . Electrophoresis was carried out at 8 mA/tube for 2.5 hr at 25°C from top to bottom. White bands are SDS-micelle (i); RCAM-lysozyme (ii); and RCAM-bovine serum albumin (iii). Only a concentration boundary (iv) with respect to SDS is observed with the right gel (c).



Fig.2. Photograph of the white protein and micelle bands visualized by the present method when the amounts of the sample proteins were varied. From left to right, 5, 2, 1, and 0.4 μ g each of RCAM-lysozyme and RCAM-bovine serum albumin were applied to the gel. All other conditions were as described in the legend to Fig.1.

As shown in Figure 3a, the white protein bands as well as the micelle band can also be located by photometric scanning using a area under each peak was found to be The densitometer. proportional to the amount of protein polypeptide in a range between 2 and 13 μg of the sample applied, as measured with the two protein polypeptides shown in Figure 3a. The slope of the plot of the area versus the amount of sample applied was, however, markedly dependent on the kind of protein polypeptide. for RCAM-lysozyme was twice that for slope the Namelv. Such a difference would not be RCAM-bovine serum albumin. expected, if the whitening were only due to the complex formation between SDS and the cationic surfactant, because no significant difference was observed in the amount of SDS bound to these in the buffer solution used the for protein polypeptides SDS-polyacrylamide gel electrophoresis (1.5 and 1.6 for a/a RCAM-bovine serum albumin and RCAM-lysozyme, respectively). The difference in the slopes might be due to the difference in the state of a protein polypeptide which has been deprived of SDS by the protein The more insoluble formation. the complex polypeptide is, the more significantly it will contribute additionally to the development of the corresponding white band.

During the first immersion in the cationic surfactant solution, the gel seems to be supplied with enough of the cationic surfactant to complex with the SDS contained in it. Use of a more concentrated solution of the cationic surfactant caused solubilization of the white bands once formed, and use of a more dilute solution caused no development of the white band,

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Fig.3. Electrophoretic patterns obtained with a gel in which protein bands and the SDS-micelle band were visualized by the present method (a) as well as another gel stained with Coomassie brilliant blue R (b). Electrophoretic conditions were the same as in Fig.1a except that the electrophoresis was continued for 3.8 hr. Gels were scanned either at 440 (a) or 565 nm (b) using an aperture of 0.2 X2.0 mm. Bars on the left side indicate the absorbance scale. Electrophoresis was from right to left. due to the diffusion of SDS into the solution without forming the complex in the gel. The visualization was virtually completed during the first immersion. If only the measurements of mobilities of major protein bands are necessary, one may finish all experiments within 20 min after terminating electrophoresis. The rate of development of the protein bands was somewhat dependent on the kind of protein polypeptide. The first immersion should not be prolonged beyond 20 min, because the complex is slowly solubilized by the micelles of the cationic surfactant.

The white bands thus developed became further clarified during the second immersion in the more dilute solution of the cationic surfactant with a concentration below its critical micelle concentration. The bands were so stable that no significant change was observed up to 7 months after the visualization. Gels may be kept in test tubes filled with the second surfactant solution or water.

The present method has the following advantages. (i) The protein bands as well as the micelle band can be promptly and easily visualized after electrophoresis. (ii) The gel is not deformed by the immersion into surfactant solutions, contrary to the marked shrinkage in the aqueous methanol- or isopropanol-acetic acid mixture in the ordinary staining procedures. (iii) The protein polypeptide in a visualized band can be easily extracted from a sliced section of the gel by the addition of excess SDS.

On the other hand, the present method has the following disadvantages. (i) The visualization as white bands is not as

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distinct as in the ordinary staining procedures. (ii) The present method actually measures the distribution of SDS in the gel. Sometimes, a noisy or inclined baseline was observed due to uneven distribution of SDS in the background as a result of, as yet, unknown reasons.

The present method has been applied successfully to the visualization of various protein polypeptides. It is also particularly suited to gain knowledge about the distribution of SDS in a gel which is pertinent to understanding the basis of SDS-polyacrylamide gel electrophoresis, as has been pointed out by the author (Kubo et al., 1975) and Takagi et al., (1975b).

5. Solubilization of Oil-Soluble Dyes by Sodium Dodecyl Sulfate-Protein Polypeptide Complexes with Reference to SDS-Polyacrylamide Gel Electrophoresis

I. SUMMARY

Sodium dodecyl sulfate binds to the linear polypeptide derived from a protein to form micelle-like clusters (Takagi et al., 1975a) which are expected to solubilize lipophilic materials. afford the complexes strong found to Such clusters were solubilizing power against oil-soluble dyes, comparable or slightly superior to SDS micelles, by conventional solubilizing technique (equilibration with solid dye) and by gel chromatography technique first applied to measure solubilization by such complexes. Solubilization behavior of the bound SDS was insensitive to the kind of protein polypeptide, in contrast to the variety found in the pioneering study on the complexes between SDS and initially native proteins (Steinhardt et al., 1977). The knowledge of the solubilizing power of SDS-protein polypeptide complexes seems to be valuable in design of experiments and interpretation of the results obtained in application of SDSpolyacrylamide gel electrophoresis to samples containing lipophilic materials.

II. INTRODUCTION

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Weber and Osborn, 1969) is now extensively used to analyze protein and to estimate their molecular weights. We have been studying the complexes formed between sodium dodecyl sulfate (SDS) and linear polypeptides derived from proteins (SDS-protein polypeptide complexes) which are the major electrophoresing entities in the technique. In a recent study, Takagi et al. (1975a) have proposed a model of such complexes in which SDS binds to a polypeptide chain to form clusters similar to SDS micelles along its length. If SDS is really bound in such a manner, the bound SDS is expected to make lipophilic materials apparently soluble in an aqueous solution by the incorporation of materials in the micellar cluster with an efficiency the comparable to that of SDS micelle.

SDS-polyacrylamide gel electrophoresis is frequently applied to membrane proteins which are often contaminated by lipophilic materials. Dodecyl alcohol, which is easily incorporated into a SDS micelle, has been found to have a profound effect on the electrophoretic behavior of SDS-protein polypeptide complexes in a polyacrylamide gel (Takagi et al., 1975b). It is therefore necessary to understand the solubilizing power of SDS bound to a protein polypeptide.

The solubilization of oil-soluble dyes by SDS protein complexes was first reported by Blei (1960), and has been extensively studied by Steinhardt and his colleagues (1974; 1977;

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Birdi & Steinhardt, 1978). They found that the bound SDS has strong solubilizing power comparable to that of SDS micelles. Their studies are, however, mostly concerned with complexes formed between SDS and initially native proteins. Most of the proteins they studied necessarily retain disulfide cross-linkage, even after denaturation by SDS. No detailed study has been made on complexes formed between SDS and linear polypeptides lacking the cross-linkages. It is such complexes, however, that are actually electrophoresed in SDS-polyacrylamide gel electrophoresis. The study described in this chapter was planned specifically to make clear the solubilizing power of such complexes with references SDS-polyacrylamide gel to electrophoresis. The results obtained clearly show that SDS bound to protein polypeptide has a strong solubilizing power to oil-soluble dyes, such as Yellow OB, Orange OT, and Butter Yellow, comparable to that of SDS micelle. Yellow OB, however, behaved somewhat exceptionally.

III. EXPERIMENTAL PROCEDURE

Sodium dodecyl sulfate designated as SPS-4 was obtained from Nakarai Chemicals as specially prepared reagent. It was 97 % pure as determined by gas chromatography, and contained decyl and tetradecyl sulfates as the major contaminants. The critical micelle concentration of the SDS preparation in 50 mM sodium phosphate buffer, pH 7.0, was determined to be 1.85 mM at 25.0 \pm 0.2°C by the drop number method. Proteins were the best

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available products and have been reduced and caboxymethylated or carboxamidomethylated, as has been described (Shirahama et al., 1974). Protein concentrations were determind spectrophotometrically using values of $E_{1cm}^{1\%}$ at 280 nm (6.7 and 26.9 for bovine serum albumin and egg white lysozyme, respectively). No correction was made for the effect of chemical modification and the presence of SDS. A 50 mM sodium phosphate buffer, pH 7.0, prepared by mixing 50 mM Na₂HPO₄ and 50 mM NaH₂PO₄ and adding sodium azide to a final concentration of 0.02 %, was routinely The dyes, Butter Yellow, Yellow OB, and Orange OT, were used. obtained from Daiwa Chemicals (lot No. 28-46-2), Daiwa Chemicals (lot No. 75-5), and Tokyo Chemicals Industry (lot No. CI 12100), respectively. They were recrystallized from 80 8 aqueous ethanol, and futher purified by partition chromatography using a mixture of petroleum ether and ethyl ether (9 : 1, v/v) as an eluent, and a silica gel column (5 X 20 cm; Waco Gel c-100). Melting points of the final products were 114-116°C (Merck Index, 114-117°C), 124-125°C (Merck Index, 125-126°C) and 130°C (no reference data available) for Butter Yellow, Yellow OB, and Orange OT, respectively. The molar extinction coefficients of the dyes in 50 mM sodium phosphate buffer, pH 7.0, containing 1 % SDS, was determined with solutions prepared by the addition of a small amount of an acetone solution of known concentration of each of the dyes to the above SDS solution from a micrometer The molar extiction coefficients of Butter Yellow, syringe. Yellow OB, and Orange OT were determined to be 2.35 X 10^4 at 415 nm, 1.3 \times 10⁴ at 445 nm, and 1.9 \times 10⁴ at 496 nm, respectively.

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The maximum solubilization of a dye by SDS-protein polypeptide complexes or SDS micelles were determined as follows: 5 ml of a 50 mM sodium phosphate buffer, pH 7.0, containing an SDS-protein polypeptide complex and/or SDS micelles were added to a brown ampoule, together with an excess of a solid dye to be solubilized. The SDS concentration was various, while the protein concentration was kept at about 0.5 mg/ml. The ampoules were sealed and shaken in a water bath, kept at 25 ± 0.2°C with the aid of a thermostat for 40 hr, which had been shown to be long enough for the system to attain equilibrium. The supernatants were filtered through a glass filter (No. 4) which was kept at 25 ± 0.2°C by a thermostat. The amount of solubilized dye was estimated by the measurement of absorbancy after appropriate dilution with 50 mM sodium phosphate buffer, pH 7.0, containing 1 % SDS, using the values of molar extinction coefficients given above.

The partition of a dye between an SDS-protein polypeptide complex and the pure SDS micelle in conditions before the attainment of saturation was estimated as follows: 5 mg of a protein polypeptide and 7.5 mg of SDS were dissolved in 10 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 % SDS and 1 % glycerine. The solution was dialyzed against 1000 ml of the buffer solution. The dialyzate in an amount of 1.5 ml was applied to a Sephadex G-100 column (2 x 60 cm) which had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 % SDS and the desired amount of a solubilized dye. Elution was carried out with the same buffer used for the

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equilibration, at a flow rate of 16 ml/hr. Aliquots of 1.8 ml were collected. Absorbance for each fraction was measured at 280 nm and at the wavelength of maximum absorption of the dye being used. The protein concentration was estimated after correction for the contribution of the solubilized dye at 280 nm. The concentration of a dye and the equilibrium concentration of SDS in the baseline region in a chromatogram allowed the estimation of solubilization of a particular dye by SDS micelles. Solubilization of a dye by SDS-protein polypeptide complex was estimated from the concentration of the dye and that of SDS in the peak region. The concentration of SDS bound to protein polypeptide was estimated from the protein concentration determined from the absorbance curve shown in Fig.5b and from the corresponding binding isotherm (Takagi et al., 1975a).

IV. RESULTS AND DISCUSSION

Solubilization in the presence of solid dye

In the present experiments, crystalline oil-soluble dyes were suspended in 50 mM sodium phosphate buffer, pH 7.0, containing SDS alone or an SDS-protein polypeptide complex in equilibrium with various concentration of SDS. The absorbance of the solutions, measured at the maximum absorption wavelength of the dye, plotted as a function of the total concentration of SDS, is the most direct presentation of the experimental data obtained.

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Fig.1. Solubilization of Butter Yellow by SDS in the presence (o-----o) and in the absence (-----) of reduced carboxymethylated bovine serum albumin (0.52 mg/ml).

Fig.1 shows a typical example of such plots. In the absence of protein polypeptide, the solubilization of Butter Yellow began to be significant only above the critical micelle concentration of SDS (1.85 mM), as shown by a dotted line. In the presence of protein polypeptide the solubilization of Butter Yellow proceeded quite differenly from that shown by the dotted line. One gram of reduced and carboxymethylated bovine serum albumin binds maximally 1.25 g of SDS before attainment of the critical micelle concentration (Takagi et al., 1975a). SDS micelles, therefore, begin to appear only at the total concentration of 4.1 mM. Clearly, the complex formed between SDS and reduced and carboxymethylated bovine serum albumin can solubilize the dye. The two curves in Fig.1 eventually cross, and finally become parallel to each other at higher concentration of SDS. This means that the formation of SDS-bovine serum albumin (reduced and carboxymethylated) complex is completed, and futher solubilization is due to newly formed SDS micelles. Now slightly less of the dye was solubilized in the presence of reduced and carboxymethylated bovine serum albumin.

The same solubilization experiments were carried out three modified protein polypeptides derived from bovine serum albumin and egg white lysozyme; the data obtained are shown collectively in Fig.2. The eight curves in Figs.1 and 2 representing solubilization data in the presence of protein polypeptides, shown by the solid lines, can be classified according to their correlation with the corresponding curves for SDS micelles shown by the dotted lines in the figure. In Fig.2e, f, and g, the

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Fig.2. Data similar to those of Fig.1 in the presence -) and in the absence (----) of protein polypeptides. Protein polypeptides and dyes are (a) reduced and carboxamidomethylated bovine serum albumin and Butter Yellow, (b) reduced and carboxamidomethylated bovine serum albumin and Orange OT, (C) reduced and carboxymethylated bovine serum albumin and Orange OT, (d) reduced and carboxamidomethylated lysozyme and Orange OT, (e) carboxamidomethylated bovine reduced and serum albumin and Yellow OB, (f) reduced and carboxymethylated bovine serum albumin and Yellow OB, and (g) reduced and carboxamidomethylated lysozyme and Yellow OB.

curves in the presence of polypeptide are always significantly above the solubilization curves for the SDS micelles. It should be noted that all of these cases were observed when the dye used was Yellow OB. The curve in Fig.2a for reduced and carboxamidomethylated bovine serum albumin and Butter Yellow virtually overlaps the "SDS curve". Other curves in Fig.2b, c, and d have a cross-over with the corresponding SDS curve like those in Fig.1.

Solubilization isotherms

We wanted to correlate the solubilization curves with the binding isotherms of SDS to protein polypeptides previously reported (Takagi et al., 1975a). To make comparison easy, we have replotted the ordinates of the solubilization curves in Figs.1 and 2 against the equilibrium concentrations of SDS (concentration of SDS not engaged in the formation of complexes) to give "solubilization isotherms". The equilibrium concentration was obtained by subtraction of the concentration of SDS bound to a protein polypeptide from the total concentration of The concentration of SDS bound was obtained from the SDS. binding isotherms (Takagi et al., 1975a). A typical example of the isotherms obtained is shown in Fig.3. The lysozyme derivatives gave insoluble complexes below the equilibrium concentration of SDS of 1 mM. The presentation of data is, therefore, limited to the derivatives of bovine serum albumin. Corresponding binding isotherms are also included in Fig.4. Each of the

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Fig.3. Solubilization of Butter Yellow. Replot of the data of Fig.1 vs. equilibrium concentration of SDS.



Fig.4. Replotted data similar to those of Fig.3 in the region below the critical micelle concentration of SDS. Corresponding binding isotherm (----) was cited from the data of Takagi et al., Points (•) indicate solubilization data in the presence (1975a). of protein polypeptide and broken lines the near ordinates ----) those in the absence of protein polypeptide. (a) reduced and carboxamidomethylated bovine serum albumin and Butter Yellow, (b) reduced and carboxymethylated bovine serum albumin and Butter Yellow, (c) reduced and carboxamidomethylated bovine serum albumin and Orange OT, (d) reduced and carboxymethylated bovine serum albumin and Orange OT, (e) reduced and carboxamidomethy1ated bovine serum albumin and Yellow OB, and (f) reduced and carboxymethylated bovine serum albumin and Yellow OB.

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abscissa scale of the two curves was adjusted so that their spans coincide at the points where binding of SDS and the solubilization by SDS-protein polypeptide complex are completed, respectively.

It is to be noted that the data points measured in solubilization experiments fall on, or very close to, the corresponding isotherms. This indicates that SDS bound to the protein polypeptides has solubilizing power comparable to that after saturation of the binding throughout the region where the binding isotherms were measured.

Direct measurements of dye partition between complex and micelle

Solubilization has been studied under the condition where a solubilized dye is in equilibrium with its crystal. Such a saturation condition is rarely met in actual operation of SDS-polyacrylamide gel electrophoresis. We wanted to compare SDS-protein polypeptide complexes and SDS micelles with respect to their solubilizing power in conditions where their solubilizing capacity is not yet filled. For this purpose, the partition coefficient of a dye between SDS-protein polypeptide complex and SDS micelle should be directly estimated. It was tried to estimate the coefficients by making SDS-protein polypeptide complexes in equilibrium with SDS micelles solubilize an oil-soluble dye by gel chromatography. Amounts of the dye solubilized could be varied. Fig.5 shows a typical example of elution data obtained by monitoring at 445 and 280 nm. The presence of


Fig.5. Gel chromatography of reduced and carboxymethylated bovine serum albumin through a Sephadex column (2 x 60 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 0.1 % SDS in which Yellow OB had been solubilized to 36 % of the saturation level. Elution was monitored at 445 nm (a) and at 280 nm (b). For details, see Experimental Procedure.

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TABLE I

DISTRIBUTION OF SOLUBILIZED OIL-SOLUBLE DYES BETWEEN SDS BOUND TO PROTEIN POLYPEPTIDE AND MICELLAR SDS

The amounts of dye solubilized shown in the last line for each dye was obtained with micellar and bound SDS by separate experiments of equilibrium with solid dye. Distribution coefficients in these cases were evaluated assuming that the two groups of solubilized dyes are in equilibrium through solid dye. Other data were obtained by gel chromatography experiments. RCAM, reduced and carboxamidomethylated; RCM, reduced and carboxymethylated; BSA, bovine serum albumin.

Dye .	Dye solubilized (mol/100 mol SDS)				Distribution coefficients between SDS bound to protein		
	Micellar	Bound to RCAM BSA	Bound to RCM BSA	Bound to RCAM lysozyme	RCAM BSA	RCM BSA	RCAM lysozyme
0.59	0.76			1.29			
0.64	0.68		•	1.06			
1.21	1.22	1.17		1.01	0.97		
Yellow OB	0.28		0.48			1.71	
	0.28		0.47			1.68	
	0.28	0.55	•		1.96	-	
	0.43	0.89			2.07		
	0.43		0.79			1.84	
	0.57		1.23			2.16	
	0.57	0.94			1.65		
	0.57		1.17			2.05	
	1.12	2.27	2.22	1.67	2.03	1.98	1.49
Orange OT	0.24	0.22			0.92	• .	
	0.24	0.20			0.83		
	0.46	0.42			0.91		<i>,</i> '
	0.46	0.46			1.00		
	0.49	0.52			1.06		
	0.55	0.64			1.16		
	0.83	1.00			1.20		
	0.94	1.06			1.13		
	0.97	0.96	0.95	0.84	0.99	0.98	0.87

ו 0

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baseline region between the peak and the trough indicates that the partition equilibrium of the dye between SDS-reduced carboxymethylated bovine serum albumin complex and SDS micelle has been established. Details have been described in Experimental procedure.

Table I shows amounts of dye solubilized in SDS micelles and SDS-protein polypeptide complexes which are in equilibrium with each other. The amounts are expressed in moles of each of the dye solubilized per 100 moles of SDS. Distribution coefficients for the dyes partitioned between the complex and the micelle are also included in Table I. In the last line for each of the dye, the data obtained in the experiments of equilibrium with solid dye are included. The coefficients observed for Butter Yellow and Orange OT were nearly unity, and indicate that these dyes are almost equally partitioned between SDS bound to protein polypeptide and micellar SDS. The above data support the previous view that SDS bound to protein polypeptides forms clusters similar to micellar SDS, and that the clusters began to be formed below the critical micelle concentration (Takagi et al., 1975a). Twice as much Yellow OB was apparently solubilized by SDS bound to protein polypeptides than by micellar SDS. This can be explained if the dye has affinity not only to micellar clusters of SDS but also to the protein polypeptide. Ιt is interesting that the only difference between Yellow OB and Orange OT is that the former has an amino group, whilst the latter has a hydroxyl group. It is not clear how such a minor difference leads to the significant difference in the solubilization. The interpretation of this

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phenomenon must await futher study.

The solubilization of oil-soluble dyes by complexes formed between SDS and initially native proteins has been extensively studied by Steinhardt and his colleagues (1974; 1977; Birdi & Steinhardt, 1978). The majority of such complexes were shown to be quite different in their solubilization behavior from the complexes which were the objects of the present study. As shown in Figs.3 and 4, the solubilization of oil-soluble dye proceeded essentially in parallel with the binding. Although the solubilization curve went somewhat above the corresponding binding isotherm when Yellow OB was the solubilizate, the solubilization efficient of the bound SDS was not sensitive to the kind of modification of bovine serum albumin used here. On the other hand, the experiments with the initially native proteins (Steinhardt et al., 1974; Birdi & Steinhardt, 1978) showed that "the proteins differed greatly in the extent to which they form solubilizing complexes with SDS, and the effectiveness of such complexes could also vary" (Steinhardt et al., 1977). The above difference in solubilizing behavior is easily understood if it is taken into consideration that the native proteins vary widely in their stability against denaturation by SDS and that the protein polypeptides are devoid of rigid structure from the beginning.

The implication of the results of the present study on the complexes formed between SDS and protein polypeptides, which are major electrophoresing entities in SDS-polyacrylamide gel electrophoresis, is as follows: (1) lipophilic materials present in a sample solution in SDS-polyacrylamide gel electrophoresis, as

well as gel chromatography, in the presence of SDS may be almost equally partitioned between SDS clusters on the complexes and the SDS micelles; (2) the degree of contamination of the complexes may decrease with the increase of the amount of SDS added to the sample solution, since this leads to the increase of miceller SDS concentration; (3) in either SDS-polyacrylamide gel electrophoresis or SDS-gel chromatography, SDS-protein polypeptide complexes differ in their velocity from miceller SDS. The complexes always encounter new unloaded SDS micelles, which are supplied from the buffer of the upper reservoir and pass the complexes, in SDS-polyacrylamide gel electrophoresis. In SDS-gel chromatography, the complexes pass new unloaded SDS micelles which are present in a gel column. Re-establishment of the partition of solubilized materials may occur between SDS micelles and SDSprotein polypeptide complexes. Continuous re-establishment of solubilization equilibrium has been verified in model experiments of SDS-polyacrylamide gel electrophoresis using Yellow OB as the substance solubilized al., (Kubo et 1980). The partition experiments (Fig.5) actually utilized the above phenomenon in SDS-gel chromatography and the successful application is the strong supporting evidence of the reality of the phenomenon.

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6. Model Study on the Fate of Lipophilic Materials Incorporated into Sodium Dodecyl Sulfate-Protein Polypeptide Complexes in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

I. SUMMARY

Complexes formed between sodium dodecyl sulfate (SDS) and polypeptides, derived from proteins by reductive cleavage of disulfide crosslinkages and denaturation, are the electrophoresing entities in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. SDS bound to the polypeptides has something in SDS constituting micelles, and can incorporate common with lipophilic materials (Takagi et al., 1980). Lipophilic materials often contaminating samples of SDS-polyacrylamide gel electrophoresis are thus presumed to be incorporated into the complexes. Model experiments using an oil-soluble dye, 1-o-tolylazo-2-naphthylamine, were carried out to confirm the above assumption, and to follow the fate of such materials during the electrophoresis. The dye incorporated into the complexes was found to be turned over to SDS micelles migrating from the upper buffer reservoir through the gel and overtaking the complexes. The complexes are thus continuously deprived of the dye. A larger-sized complex is freed of the dye more promptly than a smaller-sized complex, is electrophoresed more slowly former and is because the overtaken by more SDS micelles than the latter. The dye is retained in the complexes only when the overtaking micelles have been also saturated with the dye.

II. INTRODUCTION

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophorefrequently applied to biomembrane proteins. Samples sis is prepared from biomembranes are often contaminated with lipophilic When such a sample is treated with SDS prior to materials. electrophoresis, the lipophilic materials, otherwise insoluble in an aqueous solution, are incorporated into the nonpolar environment afforded by clustered long alkyl hydrocarbon chains of SDS. Such a cluster can be found not only in a SDS micelle but also in a complex formed between SDS and protein polypeptide (Sirahama et Steinhardt and his colleagues have shown that SDS al., 1974). involved in such a complex has a strong ability to incorporate micelle lipophilic materials comparable to SDS forming а (1980) have further (Steinhardt et al., 1977). Takagi et al. studied the ability of SDS-protein polypeptide complexes bearing SDS-polyacrylamide gel electrophoresis in mind, and arrived at the same conclusion. Takagi et al. (1975a) have shown that the incorporation of a lipophilic material perturbs electrophoretic properties of both SDS micelles and SDS-protein polypeptide model of lipophilic dodecyl alcohol complexes using aś а Lopez and Siekevitz (1973) suggested that natural material. lipids also have an effect similar to that of dodecyl alcohol.

In the present chapter, the fate of such lipophilic materials

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in SDS-polyacrylamide gel electrophoresis was followed using an oil-soluble dye as a model substance. The terms, "incorporation" and "incorporate" are often used. They are equivalent to the terms, "solubilization" and "solubilize", respectively, which are used in the field of physical chemistry of surfactants.

III. EXPERIMENTAL PROCEDURE

Sodium dodecyl sulfate (SDS) was obtained from Nakarai Chemicals. It was 97 % pure by the criteria of gas chromatography, and contained decyl and tetradecyl sulfate as the major contaminants. Bovine serum albumin and hen's egg white lysozyme were of the best commercially available quality, and were reduced and carboxamidomethylated (RCAM) by the method described elsewhere (Shirahama et al., 1974). Yellow OB was obtained from Daiwa Chemicals, recrystallized three times from 80 % aqueous ethanol, and finally purified by silica gel partition chromatography (Wako Gel c-100, 5 X 20 cm) using a mixture of petroleum ether and ethyl ether (9:1 (v/v)) as an eluant (mp. 124-125°C, Merck Index 125-126°C).

SDS-polyacrylamide gel electrophoresis was carried out essentially according to the procedure of Weber and Osborn (1969), except that the concentration of SDS in the upper tray buffer was reduced to half and that SDS was added neither to the lower tray buffer nor the gels. Elimination of SDS from the gels and the lower tray buffer causes no trouble, since protein

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samples are electrophoresed surrounded by SDS supplied from upper tray buffers (Kubo et al., 1979). Gel tubes were immersed in a bath maintained at 25 \pm 0.2°C by circulation of water in the jacket during electrophoresis.

Electrophoretic patterns were obtained by scanning gels using a Toyo densitometer Model DMU-33C (Fig.1a) and a Shimadzu dual-wavelength scanner Model CS-900 (Figs.1b and c).

IV. RESULTS AND DISCUSSION

In chapter 3, it had been appeared that the addition of dodecyl alcohol to a sample solution of SDS-polyacrylamide gel variously retarded electrophoresis of SDS micelles and SDSprotein polypeptide complexes. The phenomenon was ascribed to the effect of incorporation of dodecyl alcohol by SDS micelles and the complexes. SDS micelles were retarded most markedly. The retardation of a complex became less significant as the molecular weight of its protein moiety became higher. The observation apparently conflicts with the result described by Takagi et al. (1980) that the SDS involved in the complex formation effective is as as the SDS forming micelle in incorporation of lipophilic materials and that the efficiency is independent of the size of the polypeptide moiety. it is presumed that the observed perplexing phenomenon must be related to transport of micelles and complexes during electrophoresis, and the following working hypothesis is assumed.

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SDS micelles have mobility higher than that of any SDS-protein polypeptide complex in SDS-polyacrylamide gel electrophoresis (Kubo et al., 1975). SDS micelles electrophorese into a gel column from the upper tray buffer, and overtake and go ahead of the SDS-protein polypeptide complexes. SDS micelles surrounding a complex are, therefore, always replaced by the "newcomers". Tn such a situation, the lipophilic materials incorporated into the complex are rapidly partitioned between complexes and micelles. The complexes are thus deprived of the lipophilic materials. This phenomenon may be called a "washing effect by micelles". The larger the difference in velocity between micelles and SDS-protein polypeptide complexes, the more promptly the materials will be removed from the complexes. The phenomenon described in the beginning of this section can be clearly interpreted by the above hypothesis.

Experiments using a "visible lipophilic compound" were carried out to testify the above hypothesis. Protein polypeptides, derived from bovine serum albumin and lysozyme by reductive cleavage of disulfide bonds and subsequent carboxamidomethylation, were used as samples and will be called RCAM-bovine serum albumin and RCAM-lysozyme, respectively. These polypeptides were treated with excess SDS. The resultant sample solution contained the two kinds of SDS-protein polypeptide complexes and SDS micelles. The solution was contacted with finely dispersed crystals of an oil-soluble dye, Yellow OB, under stirring, and was colored orange due to incorporation of the dye into the complexes and SDS micelles.

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Fig.1. Patterns of SDS-polyacrylamide gel electrophoresis revealed by staining with Coomassie brilliant blue and subsequent scanning at 565 nm (a) and by direct scanning at 445 nm (b, c) to localize proteins and Yellow OB, respectively. Gel concentration: T = 10 % and C = 2.7%; upper tray buffer: 0.1 M sodium phosphate buffer, pH 7.0, containing 0.05 % SDS ; sample buffer: 0.01 M sodium phosphate buffer, pH 7.0, containing 1 % SDS and 5 % glycerine; samples, 2.0 µg of RCAM-bovine serum albumin and 2.0 µg of RCAM-lysozyme dissolved in 20 µl of the sample buffer (a), 30 µg each of the above two protein derivatives in 30 µl of the sample buffer (b, c); temperature: 25 0.2°C. ± The sample

solutions for each run and the upper tray buffer for (c) had been equilibrated with solid Yellow OB by shaking with it 40 hr at 25°C. Arrows in (a) and (b) indicate 1.0 and 0.1 absorbance scales, respectively. The latter is common to (c). The three gel columns were prepared at the same time using the same acrylamide solution.

The colored solution was used as a sample in SDS-polyacrylbands located qel electrophoresis. Protein were by amide staining with Coomassie brilliant blue as shown in Fig.1a. The fast-moving band (left) and the slow-moving band (right) were assigned to RCAM-lysozyme and RCAM-bovine serum albumin, respectively, with reference to the patterns obtained in the control runs for each of them. In the electrophoretic run shown in Fig. 1a, the concentrations of the protein polypeptides were too low to be revealed by visual or photometric inspection of Yellow OB incorporated into the complexes between SDS and either of the polypeptides.

Fifteen times the amount of the two protein polypeptides was then applied to SDS-polyacrylamide gel electrophoresis otherwise under the same condition. Now two orange-colored bands could be observed in the frontal region and the scanning at 445 nm for Yellow OB of the gel column in the glass tube just after the electrophoretic run gave the pattern shown in Fig.1b. The second peak (from left) was identical in position to the band of RCAM-lysozyme in Fig.1a. Staining of the gel with Coomassie brilliant blue revealed two bands.

They appeared at positions identical to those of RCAM-lysozyme and RCAM-bovine serum albumin in Fig.1a, and could be assigned to each of them, respectively. The first peak (from left) in Fig.1b electrophoresed in the top of the frontal region, and failed to be stained by Coomassie brilliant blue. These properties are just those expected for the micelle band formed by the excess SDS added to the sample solution (for details of the

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behavior of the band, see chapter 2). According to the results obtained for the solubilization of oil-soluble dye by SDS-protein polypeptide complexes (Takagi et al., 1980), the Yellow OB molecules must have been equally distributed among the three species, namely, the two kinds of complexes and the SDS micelle. Seemingly the Yellow OB molecules incorporated into the complex between SDS and RCAM-bovine serum albumin with the lowest mobility among the three are "washed away" by the overtaking micelles most effectively and thus failed to be visualized.

The "washing effect by micelles" was no more expected if the SDS micelles overtaking the SDS-protein complex with lower mobility had been saturated with Yellow OB. Figure 1c shows the electrophoretic pattern obtained by scanning a gel column at 445 nm after an electrophoretic run in which the upper reservoir buffer had been saturated with Yellow OB but other conditions were the same as in Fig.1b. The rise of the baseline region indicated that micelles electrophoresing into the gel from the reservoir were actually loaded with the dye as expected. Now a band was observed near the starting point at a position identical to that of RCAM-bovine serum albumin (Fig.1a) other than the two bands appeared at the positions identical to those in Fig.1b. Clearly the dye incorporated into the complex between SDS and RCAM-bovine serum albumin was not "washed away" in this case.

The above results are those expected from the hypothesis of "washing of SDS-protein polypeptide complex by SDS micelles", and strongly support the reality of the above hypothesis. Knowledge of the above phenomenon seems helpful in designing

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experiments and in interpreting the results obtained in the application of SDS-polyacrylamide gel electrophoresis to samples containing lipophilic materials.

It is to be noted that acrylamide gel concentration (T %) must be equal to or higher than 10 % to reproduce the results described in this chapter. This is because SDS micelles are electrophoresed in advance of SDS monomers in equilibrium with them in a gel of concentration below 10 % as described in chapter 2, and are dissociated gradually to "dump" the dye molecules to cause anomalies in the frontal region. Presence of a shoulder in front of the micelle peak in both Figs. 1b and c seems to suggest that some less serious anomalies are inevitable even in a 10 % gel. 7. Sodium Dodecyl Sulfate-Protein Polypeptide Complexes in 8 M Urea with Special Reference to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

I. SUMMARY

The effect of 8 M urea on the complexes formed between sodium dodecyl sulfate and protein polypeptide were found to be as follows: (1) The maximum amount of SDS bound is reduced by almost half, and the minimum equilibrium concentration of SDS necessary to reach saturation of the binding was nearly doubled; (2) The apparent content of α -helical structure deduced from CD measurement is only reduced to the level of 50-70 % of that in the presence of sodium dodecyl sulfate alone; (3) The effective size of the sodium dodecyl sulfate-protein polypeptide complex deduced from viscosity measurements is increased, but is still smaller than the effective size of the protein in 8 M urea alone.

II. INTRODUCTION

Both sodium dodecyl sulfate (SDS) and urea have been extensively used as strong protein denaturants. Polacrylamide gel electrophoresis of proteins is often carried out in the presence of both of the two denaturants. Swank and Munkres (1971) recommended the addition of 8 M urea to the buffer used in

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SDS-polyacrylamide gel electrophoresis, since the presence of both 8 M urea and SDS improves the resolution of the system for proteins of molecular weight less than 10,000 and facilitates dissociation of protein aggregates (Swank & Munkres, 1971; Bachrach & Hess, 1973; Abraham & Cooper, 1976; Downer et al., 1976).

To evaluate data obtained from SDS-polyacrylamide qel electrophoresis in the presence of 8 M urea, it is necessary to understand the effect of urea on the complexes formed between SDS and a protein polypeptide. The complexes formed in the absence of urea have been extensively studied (Reynolds & Tanford, 1970; Shirahama et al., 1974; Takagi et al., 1975a; Wright et al., 1975; Mattice et al., 1976; Rowe & Steinhardt, 1976; Dunker & Kenyon, 1976). No study has been made, however, on the complexes in the This presence of urea. chapter describes the result of comparative experiments on the states of protein polypeptides in aqueous solutions containing either SDS or urea or both.

III. EXPERIMENTAL PROCEDURE

Sodium dodecyl sulfate (SDS) designated as SPS-4 was obtained from Nakarai Chemicals as a specially prepared reagent. It was 97 % pure by gas chromatography, the major contaminants being the decyl and tetradecyl sulfates. Dodecyltrimethylammonium chloride (special grade) was obtained from Tokyo Kasei Chemicals. Urea (special grade) was obtained from Wako Chemicals, and used

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without further purification. Other reagents including proteins were special grade or those routinely used in polyacrylamide gel electrophoresis. Proteins with disulfide groups were reduced and carboxamidomethylated (RCAM) according to the method described elsewhere (Shirahama et al., 1974).

Protein concentration was determined spectrophotometrically using authentic values of $E_{1cm}^{1_{\%}}$ at 280 nm (6.7, 7.4, 9.1, and 26.9 for bovine serum albumin, ovalbumin, β -lactoglobulin, and lysozyme, respectively) or at 410 nm (90.7 for cytochrome <u>c</u>). The concentration of hemoglobin was determined by the method of Van Kampen-Zijlstra (1961) as ferrihemoglobin cyanide, assuming the molar extinction coefficient for the heme group to be 7290 at 503 nm; the absorption was not affected by the presence of SDS at this wavelength.

Sodium phosphate buffer of pH 7.0 was prepared by mixing 0.05 M NaH_2PO_4 and 0.05 M Na_2HPO_4 and adding sodium azide to a final concentration of 0.02 %; hereafter, this buffer will be referred to as 0.05 M sodium phosphate buffer, pH 7.0. When urea was added to this buffer to a final concentration of 8 M, the pH value increased to 7.6.

The critical micelle concentration of SDS in the presence or absence of 8 M urea was determined by a method utilizing the marked change of electrophoretic mobility of SDS on micelle formation in a polyacrylamide gel. SDS was added only to the upper reservoir of the electrophoretic apparatus and several runs were carried out at various SDS concentration around that expected to be the critical mucelle concentration. The mobility

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of SDS was estimated by measurement of the position of a white band formed when the zonal front of the dodecyl sulfate ion ran counter to that of the dodecyltrimethylammonium ion which had been added to the lower reservoir. The concentration of the cationic surfactant was kept constant just below its critical micelle concentrations throughout the runs.

The binding of SDS to a protein polypeptide was measured by the equilibrium dialysis technique, essentially according to the method described by Takagi et al., (1975a). Dialysis was continued for 14 days at 25.0 \pm 0.2°C. When the final equilibrium concentration of SDS was above the critical micelle concentration, the starting condition of equilibrium dialysis was brought close to the expected final condition. SDS was analyzed by colorimetry of the Methylene Blue-SDS complex extracted with chloroform (Takagi et al., 1975a).

CD spectra were measured with a JASCO J-20 CD spectrophotometer. The temperature of the sample solution was controlled at 25 ± 0.1°C using a brass muffle obtained from JASCO. CD data are reported as mean residue ellipticity. Mean residue weight was assumed to be 112 for all proteins used. Sample solutions containing SDS were prepared in the same manner as in viscosity measurements.

Viscosity was measured with an Ubbelohde type viscometer with flow time for water of 200 sec. Protein samples were dissolved to a final concentration of between 2 and 4 mg/ml in 0.05 M sodium phosphate buffer, pH 7.0 containing 3.5 mM SDS and/or 8 M urea. Enough SDS was further added to the solutions to supply

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sufficient SDS for binding to the proteins; the required amounts of SDS were calculated from corresponding binding isothrms. A sample solution thus prepared was dialysed against a hundred volumes of an appropriate buffer. Dialysis was carried out for 14 days for the buffer containing SDS and several days for that lacking it. For dilution of a sample solution in the viscometer, the outer solution was used as the solvent. Both the inner and the outer solutions were filtered through a Millipore Filter before pouring into the viscometer. Cytochrome <u>c</u> and hemoglobin were dissolved in the same manner as described above, and heated at 100° C in boiling water for 3 min before starting dialysis. Other proteins had been reduced and carboxamidomethylated to make the above denaturation procedure unnecessary.

IV. RESULTS

Protein polypeptides

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis is usually carried out with proteins in which the intra- and inter-chain disulfide groups have been reductively cleaved. In the present study, sample proteins having disulfide groups were reduced and carboxamidomethylated to prevent reformation of disulfide groups during preparation and measurements. Cytochrome \underline{c} and hemoglobin, which lack disulfide group, were simply heated at 100°C in the presence of necessary amounts of SDS and/or 8 M

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urea to complete denaturation.

Binding isotherms

Fig.1 shows binding isotherms of SDS to RCAM-lysozyme and RCAM-bovine serum albumin in the presence and the absence of 8 M The presence of 8 M urea had the following major effects urea. on the binding isotherms: (1) The maximum amount of SDS bound was reduced by almost half; (2) The maximum equilibrium concentration of SDS necessary to attain maximum binding was nearly doubled. These effects have also been observed with two kinds of plant virus coat proteins by Sano et al., (1978). The above effects of urea, therefore, may be taken to be common to binding isotherms of SDS to various kinds of protein polypeptides. RCAM-lysozyme was precipitated when the equilibrium concentration was below half of the critical micelle concentration either in the presence (curve 2) or in the absence (curve 1) of 8 M urea. Presumably the complex formed between SDS and the highly basic RCAM-lysozyme at low binding ratio is highly aggregated, making it insoluble even in 8 M urea.

As has been described in the paper on the binding isotherms (Takagi et al., 1975a), the binding of SDS to a protein polypeptide proceeds in two or three steps, as shown by curve 3 in Fig.1. As shown by curve 4 in Fig.1, in the presence of 8 M urea the first step disappeared and the amount of bound SDS increased monotonously over a wide concentration range of SDS. The same effect has been observed with the virus coat proteins



Binding isotherms of SDS to protein polypeptides in the Fig.1. presence (curve 2 and 4) and in the absence (curve 1 and 3) of 8 M urea in 0.05 M sodium phosphate buffer, pH 7.0, at 25.0°C. a, RCAM-lysozyme; b, RCAM-bovine serum albumin. Arrows indicate the critical micelle concentrations of SDS in the presence (right) and in the absence (left) of 8 M urea. Curve 3 is cited from the report by Takagi et al. (1975a). Due to the formation of insoluble material in the region indicated as "ppt" in Fig.1a, measurement at lower concentrations was impossible.

(Sano et al., 1978). The present study was initiated with special reference to SDS-polyacrylamide gel electrophoresis, which is carried out in the presence of SDS in a concentration above its critical micelle concentration. The binding process before attainment of the saturation value was, therefore, not further investigated in the present study.

CD spectra

spectra Fiq.2 shows CD of RCAM-bovine serum albumin, RCAM-lysozyme, and RCAM-ovalbumin in the presence of 3.5 mM SDS and/or 8 M urea. In 3.5 mM SDS (curves labeled 1), CD spectra for the three protein polypeptides showed patterns anticipated for a mixture of α -helix and random coil with the amount of α-helix present ca. 50 %, according to Greenfield and Fasman (1969). In 8 M urea (curves labeled 3), CD spectra for the three polypeptides showed patterns anticipated for a random coil (Greenfield & Fasman, 1969). In the presence of both 3.5 mM SDS and 8 M urea, CD spectra showed patterns intermediate between the above two cases. The presence of 8 M urea made measurements below 205 nm impossible. It is to be noted that the CD patterns observed for the three polypeptides derived from different proteins are similar in each of the three different conditions. Such a convergence to a common CD pattern has been reported for many SDS-protein polypeptide complexes by Mattice et al. (1976).



Fig.2. CD spectra of protein polypeptides in 3.5 mM SDS (curve 1), in 3.5 mM SDS plus 8 M urea (curve 2), and in 8 M urea (curve 3) at 25.0°C. a, RCAM-bovine serum albumin; b, RCAM-lysozyme; c, RCAM-ovalbumin. The buffer solution was the same as in Fig.1.

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Fig.3. Plots of reduced viscosity versus concentration for RCAM-lysozyme (a) and RCAM-bovine serum albumin (b) at 25.00°C in the presence of 3.5 mM SDS (o—o), 3.5 mM SDS plus 8 M urea (x - x), and 8 M urea (•—•), respectively. The buffer was the same as in Fig.1.

Viscosity

Fig.3 shows plots of reduced viscosity of SDS-protein polypeptide complex versus concentration for RCAM-lysozyme and RCAM-bovine serum albumin. Such viscosity measurements were carried out with six other kinds of protein polypeptides. The reduced viscosity was almost independent of the concentration of each protein polypeptide. Intrinsic viscosities obtained are plotted in Fig.4 versus molecular weights of the protein polypeptides.

V. DISCUSSION

The proposal by Swank and Munkres (1971) of the addition of 8 M urea to the medium of SDS-polyacrylamide gel electrophoresis was followed by the widespread use of this modified technique. The improvement gained by the modification may be ascribed to two major factors: the first is the change of the nature of the aqueous SDS solution by the addition of 8 M urea. The second is that the presence of 8 M urea makes it possible to prepare а transparent and highly cross-linked polyacrylamide gel with an improved performance for small-sized protein polypeptides. The present study concerns the first factor. SDS-polyacrylamide gel electrophoresis in the presence of 8 M urea has been carried out in various buffer solutions. In the present study, 0.05 M sodium phosphate buffer, pH 7.0, was the sole buffer solution used.

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Similar conclusions will be obtained with other buffers as far as the comparision with behavior of a protein polypeptide in the three denaturing media (3.5 mM SDS, 3.5 mM SDS plus 8 M urea, and 8 M urea) is concerned.

SDS binding

The major premises of SDS-polyacrylamide gel electrophoresis concerning SDS binding are as follows (Reynolds & Tanford, 1970; Takagi et al., 1975a): (1) SDS binding is saturated; (2) The intrinsic charge of a protein polypeptide is smeared out to give a constant surface charge density, as the result of the high and nonspecific affinity of SDS, to give a nearly constant binding ratio of near 1.5 (weight to weight basis). SDS-polyacrylamide gel electrophoresis in the presence of 8 M urea is generally carried out in an SDS concentration of 3.5 mM (0.1 %) or above. Though a significant lateral shift of the binding isotherm is observed for RCAM-lysozyme and RCAM-bovine serum albumin (Fig.1), the SDS binding is saturated in this concentration range. The first premise is still satisfied even in the presence of 8 M urea.

Reduction of the maximum amount of SDS bound almost by half was observed with both RCAM-lysozyme and RCAM-bovine serum albumin in the present study, and with two virus coat proteins by Sano et al. (1978), and clearly jeopardizes the second premise. Tung and Knight (1971) have demonstrated using a model system (maleylated and unmaleylated virus coat proteins) that SDS-polyacrylamide gel electrophoresis gives incorrect molecular weight

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for proteins with relatively high net charge. The presence of 8 M urea evidently promotes such a tendency to give an incorrect estimation, due to insufficient smearing out of the intrinsic charge.

Conformation

To understand the effect of urea on the conformation of an SDS-protein polypeptide complex, it is prerequisite to have a knowledge about its conformation in the absence of 8 M urea. Several models have been proposed for this kind of complex (Reynolds & Tanford, 1970; Shirahama et al., 1974; Takagi et al., 1975a; Wright et al., 1975; Mattice et al., 1976; Rowe & Steinhardt, 1976; Dunker & Kenyon, 1976). The popular "rod-like" model of Reynolds and Tanford (1970) was introduced to interpret the deviation of intrinsic viscosities of the complexes from those expected for compact globules assuming them to be rigid ellipsoids from the beginning, and, therefore, is not realistic. Models proposed thereafter (Shirahama et al, 1974; Takagi et al., 1975; Wright et al, 1975; Mattice et al, 1976) laid emphasis on the flexible nature of the complexes. In this chapter, the results obtained will be discussed in the light of the "necklace model" proposed by Shirahama et al. (1974) and Takagi et al. (1975a). In summary, the necklace model is characterized as follows: (1) The polypeptide chain of a complex is essentially flexible; (2) dodecyl sulfate ions bind to a protein polypeptide to form a micelle-like cluster; (3) the polypeptide chain locally

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assumes α -helical structure. According to the result of CD measurements by Mattice et al. (1976), α -helical content ranges from 30 to 50 % for many kinds of protein polypeptides.

spectra in Fig.2 indicate that most (50-70 %) of the CD α -helical structure present in 3.5 mM SDS still persists in solution containing both 8 M urea and 3.5 mΜ SDS. This was judged from the CD intensity at 220 nm which is generally regarded as a measure of α -helical content, assuming that no α-helical structure was present in 8 M urea (Sano et al., 1978). It is surprising that the α -helical structure still persists to such an extent in 8 M urea which forces most protein polypeptides to expand to a randomly coiled state (Tanford, 1968). Presumably the hydrocarbon moieties of SDS molecules associate around a protein polypeptide to afford an environment sequestered from the concentrated urea solution and favor formation of hydrogen bonds to maintain the α -helical structure. Such an effect is expected only if SDS is bound to a protein polypeptide in groups to afford an extended hydrophobic environment which allows formation of α -helical structure. This is precisely the situation assumed in the necklace model (Shirahama et al., 1974).

The fact that urea and SDS act in opposite way on the <u>b</u>₀ value of ovalbumin even in each other's presence was pointed out by Meyer and Kauzmann (1962). The ORD parameter, <u>b</u>₀, suggested the same situation concerning the α -helical content for ovalbumin as that suggested from the CD data of Fig.2 for the three protein polypeptides. At that time, urea as well as SDS was assumed to rupture hydrophobic interactions (Meyer & Kauzmann, 1962).

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Fig.4. Logarithmic plots of intrinsic viscosity, $[\eta]$, versus molecular weight for various protein polypeptides in the presence of 3.5 mM SDS (o—o), 3.5 mM SDS plus 8 M urea (x—x), and 8 M urea (•—•), respectively. Proteins are, from left to right, bovine heart cytochrome <u>c</u>, bovine pancreatic ribonuclease A, hen's egg white lysozyme, human hemoglobin, bovine β -lactoglobulin, rabbit muscle lactic dehydrogenase, ovalbumin, <u>Bacillus subtillis</u> α -amylase, and bovine serum albumin.

The interpretation of the observed phenomena was, therefore, quite confusing (Meyer & Kauzmann, 1962). Similar experimental results were subsequently obtained by Jirgensons (1963).

The intrinsic viscosity, a measure of hydrodynamically effective size of a complex, decreased with the change of medium in the following order: 8 M urea, 3.5 mM SDS plus 8 M urea, and 3.5 mM SDS (Fig.4). This suggests that the increase in the effective size of a protein polypeptide by the binding of SDS is superseded by the decrease due to formation of α-helical The α -helical structure is presumed to be local, structure. short-range, and intermitted to allow flexibility of the complex. A similar decrease of intrinsic viscosity has been observed when amylose in an alkaline aqueous solution assumes a helical structure on the formation of a complex with SDS (Takagi & Isemura, 1960; Rao & Foster, 1963).

All three logarithmic plots of intrinsic viscosity versus molecular weight of the polypeptide moiety of the complexes. (Fig.4) are almost parallel, having inclination around 0.7. The value indicates that the polypeptide chains of the complexes behave as flexible coils from macroscopic point of view (Tanford, 1961) in each of the three denaturing media. Intrinsic viscosities of the complexes in the absence of urea have been measured, and plots equivalent to the bottom one in Fig.4 have been reported by Reynolds and Tanford (1970) and Hamauzu et al. (1975). The two reported plots and ours, however, differ from each other. The inclination obtained by the other two groups Tanford, 1970; Hamauzu et al, 1975) were (Reynolds & in

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agreement, and were around 1.2, apparently indicating that the complexes are asymmetric in support of the rod-like model of Reynolds and Tanford (1970). The present author can offer no explanation for the difference between the previously reported results and the present results, except to point out that the previous measurements were made in media of salt concentrations significantly lower than that of the buffer solution used in the present study. The viscosity of an SDS-protein polypeptide complex is quite sensitive to salt concentration (Takagi et al., 1981).

It is to be noted in Fig.4 that the intrinsic viscosity of the complexes in 3.5 mM SDS alone levels off as the molecular weight becomes less than 15,000. This phenomenon must be closely related to the lowering of the resolution of SDS-polyacrylamide gel electrophoresis for proteins in the regions of molecular weight less than 15,000 (Williams & Gratzer, 1971). In the presence of 8 M urea, no leveling off was observed, and the value of intrinsic viscosity was still variable in this region. This might be a major factor improving the resolution of SDS-polyacrylamide gel electrophoresis in the presence of 8 M urea. The plot was, however, less monotonous and points were scattered more than either of other two plots in Fig.4. This will lead to erroneous estimates of molecular weight. The auther wanted to investigate the correlation between the effective sizes of complexes in 8 M urea and their molecular weights for small-sized protein polypeptides in more detail. Preparation of a sample solution of SDS-protein polypeptide complex for a protein with

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molecular weight less than 10,000 is quite difficult due to the lack of appropriate dialysis membrane. The present auther, therefore, used gel chromatography to investigate the problem, but found that all conventional gels for molecular sieving deteriorated in aqueous solutions containing both of 3.5 mM SDS and 8 M urea, making gel chromatography impracticable. The solution of this problem requires further work. 8. Interaction Between the α1 Chain of Rat Tail Collagen and Sodium Dodecyl Sulfate with Reference to Its Behavior in SDS-polyacrylamide Gel Electrophoresis

I. SUMMARY

The interaction between the α 1.chain of rat tail collagen and sodium dodecyl sulfate (SDS) was studied to provide knowledge necessary to understand the behavior of the complex between them in a molecular sieving technique such as SDS-polyacrylamide gel electrophoresis. Measured properties include binding isotherm, CD spectrum, viscosity and behavior in the free boundary electrophoresis, the gel electrophoresis and the high performance silica gel chromatography. The complex differed in most respects from the complexes between SDS and polypeptides derived from watersoluble globular proteins, reflecting its peculiar amino acid composition. The hydrodynamically effective volume of the SDS- α 1 chain complex was the most significantly deviated among the properties studied. The complex of the $\alpha 1$ chain consisted of 1052 amino acid residues was found to have the same effective volume as that of a standard polypeptide consisting of about 800 residues. The abnormal behavior of the SDS-al chain complex was interpreted on the basis of these result.

II. INTRODUCTION

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Shapiro et al., 1967; Weber & Osborn, 1969) is based on the assumption that sample proteins as well as proteins used as molecular weight standards (standard proteins) form complexes with SDS which are homologous with one another (Reynolds & Tanford, 1970; Shirahama et al., 1974). It is, however, being applied to proteins, such as those of the collagen family, which are hardly expected to fulfill the premise.

acid Collagens are unique with respect to their amino composition and sequence. Glycine residues appear every three residues, and proline and hydroxyproline residues frequently appear at the positions of X and Y, respectively, in the repeating sequence of Gly-X-Y (Piez, 1976). Collagen polypeptides thus lack the ability to form the α -helical structures predominant in the complexes formed between SDS and polypeptides derived from standard proteins (standerd complexes) (Mattice et al., 1976). Due to the unusual amino acid composition, the mean residue weight of the al chain, 91.8 (Piez et al., 1963), is significantly lower than those of the standard proteins, which are around 115 (Freytag et al., 1979). It is quite natural to expect that the nature of the complexes formed between SDS and the polypeptides derived from collagen is significantly different from that shared by the standard complexes.

Furthmayr and Timpl (1971) found that the α 1 chain of collagen shows electrophoretic mobility expected for a protein with

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molecular weight 40 % higher than the true value (96000) in SDS-polyacrylamide gel electrophoresis. Several studies have since been carried out on the applicability of the SDS-polyacrylamide gel electrophoretic technique to estimate molecular weights of polypeptides derived from collagen (Freytag et al., 1979; Furthmayr & Timpl, 1971; Hayashi & Nagai, 1980). However, no definite conclution has been reached concerning the point. It thus seemed desirable to make clear how collagen polypeptide chains differ from those derived from standard proteins in the mode of interaction with SDS.

The present study specifically on the al chain of rat tail tendon collagen was therefore initiated. The study followed the pattern of the previous studies on the interaction of SDS with polypeptides derived from standard proteins (Shirahama et al., 1974; Takagi et al., 1975) and from a membrane protein (Miyake et al., 1978). The binding isotherm of SDS to the al chain was measured in parallel with measurement of CD spectra. In the condition of SDS-polyacrylamide gel electrophoresis where the binding had been saturated, measurements of free boundary electrophoresis and viscosity of the complex between SDS and the α1 chain were carried out to estimate its intrinsic electrophoretic and hydrodynamic properties in the absence of the polyacrylamide gel matrix.

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III. EXPERIMENTAL PROCEDURE

Acid-soluble collagen was prepared from tail tendons of Wistar rats. The α 1 chain was isolated essentially according to the procedure of Chung and Miller (1974). The preparation exhibited a major and a minor band in SDS-polyacrylamide gel electrophoresis. The latter accounted for only 0.5 % and was assigned to the β 11 chain. Concentration of the α 1 chain was determined spectrophotometrically. The value of $A_{1cm}^{1\%}$ at 230 nm was determined to be 22.5 by the dry weight method.

SDS was prepared by sulfation of <u>n</u>-dodecyl alcohol (99.8 % with respect to the chain length distribution) (Dreger et al., 1944). Critical micelle concentration of the SDS preparation was found to be 1.66 mM at 25°C in 50 mM sodium phosphate buffer, pH 7.0, by measurement of the solubilization of Yellow OB (Takagi et al., 1980). In SDS-polyacrylamide gel electrophoresis and high performance silica gel chromatography, SDS obtained from Nakarai Chemicals (SPS-4, 97 %) was used. Sodium phosphate buffer of pH 7.0 was prepared by mixing 50 mM NaH₂PO₄ and 50 mM Na₂HPO₄ and adding sodium azide to a final concentration of 0.02%.

Binding of SDS was measured by the equilibration of a solution of the α 1 chain and the buffer solution with respect to SDS by dialysis (Takagi et al., 1975) or by gel chromatography. In the chromatography technique, 1 mg of the α 1 chain was dissolved in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM SDS and 1.5 % glycerol. The solution was applied to a Sephadex G-100 column (1.6 X 65 cm; thermostatically maintained at 25°C)
equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing SDS at the desired concentration, and eluted with the same buffer at a flow rate of 20 ml/h. Aliquots of 2 ml each were collected. SDS was analyzed colorimetrically (Takagi et al., 1975a).

CD spectra were measured with a JASCO J-500 CD spectropolari-The data obtained were expressed as mean residue molar meter. ellipticity. The mean residue weight of the α 1 chain was assumed to be 91.8 (Piez et al., 1963). Sample solutions for the CD measurements were prepared as follows. One mg of the α 1 chain was dissolved in 2 ml of 50 mM sodium phosphate buffer, pH 7.0, containing the desired amount of SDS. The solution was heated for 2 min in boiling water. Measurements of CD spectra as well as absorbance at 230 nm were made after standing for 20-30hr at room temperature near 25^oC. The concentration of free SDS was calculated from the amount of added using the binding SDS isotherm.

Free boundary electrophoresis was carried out at $25.0 \pm 0.1^{\circ}$ C using a Tiselius-type apparatus, Hitachi model HTB-2A. The sample solutions were prepared in the same manner as in the viscosity measurements.

SDS-polyacrylamide gel electrophoresis was carried out according to the standard procedure (Weber & Osborn, 1969). After pre-electrophoresis (1 hr, 8 mA/tube), the samples were electrophoresed at various gel concentrations at 6 mA per tube (thermostatically maintained at 25°C). Reduced-carboxamidomethylated (RCAM) bovine serum albumin used as a marker was prepared

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as described elsewhere (Shirahama et al., 1974).

Viscosity was measured with an Ubbelohde type viscometer (flow time of 200 sec for water). The α 1 chain was dissolved in 50 mM sodium phosphate buffer, pH 7.0, containing 2.0 mM SDS. SDS was added further to the solution to supply SDS to be bound to the α 1 chain. The amount of SDS required was calculated using the binding isotherm. A sample solution thus prepared was heated in a boiling water bath for 3 min, and dialyzed against 100 vol. of the solvent for 14 days. The outer solution was used as the solvent for the dilution of a sample solution. The solution and the solvent were filtered through a membrane filter (pore size, 2.0 µm).

Analytical high performance silica gel chromatography was carried out using two TSK-GEL G-4000SW columns (Toyo Soda Co., each 60 cm X 7.5 mm i.d.). A TSK RI-8 differential refractometer (Toyo Soda Co.) was used to monitor elution.

IV. RESULTS

Binding isotherm

Fig.1 shows the progress of binding of SDS to the α 1 chain as a binding isotherm. The isotherm for RCAM-bovine serum albumin is included as a reference. Below 1.3 mM free SDS, the α 1 chain formed precipitates.



Fig.1. Binding isotherm of SDS to the α_1 chain of rat tail collagen (-----) in 50 mM sodium phosphate buffer, pH 7.0, at 25.0 ± 0.2°C. Data designated by filled and open squares were obtained by the equilibrium dialysis (14 days) and the gel chromatography techniques, respectively. Height and width of each square indicate mean deviation. The arrow on the abscissa indicates the critical micelle concentration of SDS. The isotherm of RCAM-bovine serum albumin (-----) is cited from a report by Takagi et al.(1975a).

Circular dichroism

Fig.2 shows typical examples of CD spectra of the α^1 chain. In the absence of SDS, the CD spectrum of the α1 chain was similar to curve 3 at the beginning, and changed with time finally to have a pattern like curve 1 after 20 h at 25°C. Ά maximum at 222 nm is characteristic of native collagen. The collagen preparation used in this study showed molar residue ellipticity of 4900 deg·cm²· dmol⁻¹ in 0.05 M acetic acid, pH 3, at the wavelength. Curve 2 shows the CD spectrum of the α 1 chain in the presence of 0.5 mM free SDS. Further addition of SDS destroyed the maximum to give curve 3 in the presence of 2.3 mΜ free SDS. Curve 3 coincided with the spectrum of the α 1 chain at 50°C in the absence of SDS (not shown).

The CD intensity of the α 1 chain at 222 nm is plotted against the concentration of free SDS in Fig.3. The intensity first increased progressively to become flat above 1.7 mM free SDS, which is critical micelle concentration of SDS in the buffer used.

SDS-polyacrylamide gel electrophoresis is usually carried out in the presence of 0.1 % (3.5 mM) SDS using a sodium phosphate buffer with concentration between 0.05 and 0.1 M. The binding of SDS, as well as the consequent conformational change, has been completed in such conditions. Since the present study was planned with reference to the technique, further experiments were carried out with the α 1 chain to which SDS had been maximally bound.

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Fig.2. CD spectra of the α 1 chain in the absence (curve 1) and the presence (curve 2, 0.5 mM; curve 3, 2.3 mM) of free SDS at 25.0 ± 0.2°C. The three curves coincided at 212 nm as shown on the left side and continued so at least down to 208 nm (not shown).



Fig 3. Variation in the mean residue molar ellipticity of the α 1 chain with concentration of free SDS.



Fig.4. CD spectra of the complex between the α 1 chain and SDS in equilibrium with 2.3 mM SDS in 50 mM sodium phosphate buffer, pH 7.0 (curve 1) and the α 1 chain dissolved in 6 M guanidine hydrochloride, pH 4.6 (curve 2) in comparison with that between RCAM-bovine serum albumin and SDS in the same buffer containing 3.5 mM SDS. Curve 2 coincides with curve 1 above 235 nm, and could not be extended below 210 nm due to the high absorbance of the medium. Temperature, 25.0 \pm 0.2°C. Fig.4 shows the CD spectrum of the α1 chain in 50 mM sodium phosphate buffer, pH 7.0, containing 2.3 mM free SDS at 25°C (curve 1) in comparison with that in 6 M guanidine hydrochloride (curve 2). These spectra are significantly different from those of the complexes between SDS and polypeptides derived from standard proteins (Mattice et al., 1976; Takagi et al., 1975a). A corresponding CD spectrum of RCAM-bovine serum albumin is cited as a reference (curve 3).

Free boundary electrophoresis

Free boundary electrophoretic mobilities of the complex between the α 1 chain and SDS are plotted against concentration of the chain in Fig.5. The ascending and the descending boundaries showed positive and negative dependence on the concentration, respectively, and converged on the ordinate. Such a concentration dependence of electrophoretic mobility has been observed with the standard complexes (Shirahama et al., 1974).

Gel electrophoresis

The complex between the $\alpha 1$ chain and SDS and several standard coplexes were electrophoresed in polyacrylamide gels of various concentrations. The results obtained are plotted in Fig.6. The values of the relative mobilities called "free electrophoretic mobilities" (Banker & Cotman, 1972) obtained by the extrapolation to zero gel concentration showed no systematic relation.



Fig.5. Free boundary electrophoresis of the complex between the α 1 chain and SDS in 50 mM sodium phosphate buffer, pH 7.0, containing 2.0 mM SDS, at 25.0 ± 0.1°C. Variation in the electrophoretic mobility with concentration of the chain calculated for the descending (o---o) and the ascending (----o) boundaries, respectively.

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Fig.6. Change in the electrophoretic mobilities of the complex between the α 1 chain and SDS and several standard complexes with concentration of polyacrylamide gel having a constant weight ratio of acrylamide to N,N'-methylenebisacrylamide, 100:2.7. Protein polypeptides are, from the top to the bottom; carbonic anhydrase, ovalbumin, immunoglobulin G heavy chain, RCAM-bovine serum albumin (marker), phosphorylase <u>b</u>, and the α 1 chain. To /*/avoid confusion, data points are omitted for the two proteins.



The slope of each of the plots in Fig.6 gives the retardation coefficient, $K_{\rm R}$, of the respective protein polypeptide, which is a measure of its hydrodynamic effective size (Banker & Cotman, 1972). The coefficients thus obtained are plotted in Fig.7 against residue numbers of the proteins. The point for the α 1 chain deviated from the straight line for the standard complexes.

Viscosity

Fig.8 shows the plot of reduced viscosity of the complex between the α 1 chain and SDS against concentration for the polypeptide moiety. The intrinsic viscosity was not affected by the change of shear rate brought about by alteration of the holding angle of the viscometer.

Gel chromatography

Fig.9 shows the elution profiles in the high performance silica gel chromatography of the α 1 chain and the five standard protein polypeptides. When the α 1 chain was applied to the same column, it was eluted at a retention time just ahead of the peak of phosphorylase <u>b</u> with molecular weight of 97000, as is shown in Fig.9.



Fig.8. Plot of the reduced viscosity at $25.00 \pm 0.02^{\circ}$ C against concentration of the α 1 chain for the complex between the chain and SDS in 50 mM sodium phosphate buffer, pH 7.0, containing 2.0 mM SDS.



Elution patterns in the high performance silica gel Fig.9. chromatography of the α 1 chain (-----) and five standard protein polypeptides (----), phosphorylase <u>b</u>, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor, from the left to the right). The $\alpha 1$ chain preparation in an amount of 213 μ g was dissolved in 500 μ l of the buffer solution containing 10 mg of SDS and heated at 100°C for 5 min. One hundred μl of the sample solution were applied to the column equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 3.5 mM SDS. The elution was made with the same buffer solution with a flow rate of 2.8 ml/min. The sample solution of the standard proteins was prepared in the same manner as above, except for the addition of dithiothreitol to the reaction mixture (for details, see Takagi, 1981). The standing up of the elution curve of standard proteins is the elution of SDS micelles.

V. DISCUSSION

Binding of SDS and consequent conformational changes

The mode of binding of SDS to various kinds of protein polypeptide is not unique but diverse within a certain range (Takagi et al., 1975a). The diversity can be taken to reflect the composition and the sequence of amino acid residues of the protein polypeptides.

The al chain of collagen with a well-known unique composition and sequence of amino acid residues was revealed to show a characteristic binding isotherm as shown in Fig.1. The corresponding isotherm of RCAM-bovine serum albumin may be taken as a standard one for polypeptides derived from water-soluble globular The α 1 chain binds a small amount of SDS below 1.0 mM proteins. of free SDS. The isotherm thus lacks the "first plateau region" observed with most polypeptides derived 'from standard proteins. In this region, the addition of SDS induced precipitation of the al chain during the dialysis. CD spectra which could be measured at lower concentration without formation of precipitates revealed the appearance of a maximum at 222 nm which seemed to be characteristic of the collagen fold, as shown in Fig.2. Hayashi and Nagai (1972) have shown that the addition of a small amount of SDS promotes the fibril formation of collagen in vitro. The phenomenon seems to have its origin in the level of the collagen molecule.

Above 1.0 mM of free SDS, the binding proceeded progressively

and finally attained the maximum of 1.4 g/g at 2.0 mM free SDS, which is above the critical micelle concentration of 1.7 mM. The behavior is in contrast with that observed with most polypeptides derived from water-soluble globular proteins. They show binding isotherms with a steep increase in the amount of binding above 1.0 mM and the levelling-off at a free SDS concentration below the critical micelle concentration. Concomitant with the beginning of appreciable binding, a drastic change was observed in the CD spectrum, as shown in Fig.3. Above 1.7 mM, the spectrum attained a final form which agreed well with that seen in 6 M guanidine hydrochloride, except for a slight difference at around 220 nm, as shown in Fig.4. The CD spectrum of the $\alpha 1$ chain finally attained is naturally quite different from corresponding ones observed with standard protein polypeptides for which binding of SDS induced the formation of α -helix.

Electrophoretic behavior

Limiting free boundary electrophoretic mobility (Shirahama et al., 1974) of α 1 chain with maximally bound SDS was estimated by the extrapolation of the observed electrophoretic mobilities to zero concentration to be -2.48 X 10⁻⁴ cm²·s⁻¹·V⁻¹, as shown in Fig.5. The value is only slightly smaller than the value, -2.8 X 10^{-4} cm²·s⁻¹·V⁻¹, expected for a standard complex with an equivalent molecular size (see Fig.7 in Takagi et al., 1975a).

Extrapolation to zero concentration in the Ferguson plots shown in Fig.6 was expected to give the mobility of the α 1 chain

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in the absence of gel matrix relative to those of the standard complexes. The plots, however, showed a fuzzy focal point at the concentration around 1 %. It has been shown that that the free boundary electrophoretic mobilities of the complexes between SDS and standard protein polypeptides are virtually independent of molecular weight of the latter (Takagi et al., 1975a). The electrophoretic mobility extrapolated to zero gel concentration, thus, showed no exact correlation with the corresponding free boundary electrophoretic mobility.

Estimation of molecular weight in the conventional way (Weber Osborn, 1969) at each of the five gel concentrations gave & values of molecular weight ranging between 110000 and 120000 for the α 1 chain with molecular weight of 96000. This is at variance with the overestimation which was reported to be as high as 40 % (Freytag et al., 1979; Furthmayr & Timpl, 1971). In the experiments of Furthmayr and Timpl (1971), the α 1 chain was electrophoresed less than 2 cm in distance. Electrophoresis in the top region of a gel is liable to be affected by various factors. In the present experiments, the α 1 chain was electrophoresed over a long distance using RCAM-bovine serum albumin as a marker. We have no immediate explanation concerning the failure to reproduce the well-known overestimation. Hayashi and Nagai (1980) carried out a careful run of SDS-polyacrylamide gel electrophoresis in a Tris-glycine buffer, and concluded that the free electrophoretic mobility of the al chain was 10 % lower than the monochromatic value observed with standard protein polypeptides.

Behavior related to hydrodynamically effective size

As shown in Fig.7, the retardation coefficients estimated from the Ferguson plots for the complexes between SDS and the standard protein polypeptides are linearly correlated with their residue numbers. The point for the α 1 chain with residue number of 1052 is not on the line for the complexes, and its retardation coefficient is equivalent to that of a standard complex with residue number of 840.

Intrinsic viscosity of the α 1 chain complexed with SDS was 41.9 ml/g, as shown in Fig.8. When the plots of intrinsic viscosities against molecular weights (see Fig.4 in Takagi & Kubo, 1979) are used as a calibration curve, the intrinsic viscosity of the α 1 chain complexed with SDS is that expected for a complex between SDS and a standard protein polypeptide with residue number of 780.

Elution of the $\alpha 1$ chain at the retention time almost the same as that of phosphorylase <u>b</u> in the high performance silica gel chromatography (Fig.9) has the same implication as above.

VI. CONCLUTION

The mode of interaction of the collagen $\alpha 1$ chain with SDS has been examined. The process of the binding of SDS to the chain was unique reflecting the characteristic amino acid composition and sequence. The final product with maximally bound SDS differed

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from standard complexes, namely those between SDS and polypeptides derived from typical water-soluble globular proteins in the following points: (1) the amount of SDS bound is similar on a weight basis, but 25 % less on a residue molar basis than that monochromatically observed with standard protein polypeptides; (2) the hydrodynamically effective size of the complex of the $\alpha 1$ chain with residue number of 1052 is equivalent to that of a standard complex with a chain length 75 % of the former. Such a standard protein polypeptide has a molecular weight near 91000, which is close to that of the $\alpha 1$ chain (96000). An empirical technique of molecular weight estimation based on the molecular sieving effect usually relies on the use of standard proteins. Such a technique, therefore, may give a fairly correct estimate It must be noted, however, that this is a for the $\alpha 1$ chain. The compactness of the complex between the fortuitous situation. al chain and SDS may be ascribed to the abundance of glycine, proline and hydroxyproline, all of which have been shown to have effect to minimize the expansion of a polypeptide chain an (Flory, 1969).

A complex between SDS and protein polypeptide in the condition of SDS-polyacrylamide gel electrophoresis behaves like a heavily charged polyelectrolyte through which solvent molecules as well as ions are freely permeable (Shirahama et al., 1974; Takagi et al., 1975a). The electrophoretic behavior of such a polyelectrolytic chain can be interpreted based on a model in which many identical segments with equal charge are tied in a row with a frictionless string (Shirahama et al., 1974).

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The electrophoretic mobility of the chain is expected to be proportional to the charge on the individual segment and is inversely proportional to the frictional coefficient of the The electrophoretic mobility of the complex between SDS segment. snd the $\alpha 1$ chain was not significantly different from that expected for a standard complex with a size comparable to the The low charge density as the result of the low capacity former. of binding to SDS (on residue molar basis) and the possible small effetive size of the segment have a counterbalancing effect on the electrophoretic mobility of the complex. The latter may be ascribed to either of the small number of bound SDS per segment and the abundance of amino acid residues of small size (the closed loop of proline and hydroxyproline may not be bulky) or both of these factors.

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9. The α1 and α2 Chains of Collagen Separate in Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Due to Differences in Sodium Dodecyl Sulfate Binding Capacities

I. SUMMARY

The $\alpha 1(I)$ and $\alpha 2(I)$ chains of rat tail collagen, indistiguishable with respect to their chain lengths, are well separated in polyacrylamide gel electrophoresis in the presence of sodium the present chapter the results dodecyl sulfate (SDS). In demonstrated: 1) the $\alpha 2$ chain maximally binds SDS in an amount of 1.6 g/g which is significantly larger than 1.4 g/g found for the α 1 chain; 2) the α 2 chain acquires electrophoretic mobility in a free solution 14 % higher than that of the α1 chain as the result of the larger amount of SDS bound; 3) although both of the chains have virtually the same hydrodynamically effective size, the above difference in mobility persists in a gel, and brings about the separation in a high resolution. It was also found that the $\alpha 2$ chain binds appreciable amounts of SDS in the initial phase of the binding where the α 1 chain hardly binds SDS.

II. INTRODUCTION

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) (Shapiro et al., 1967; Weber & Osborn,

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1969) is now indispensable in the field of collagen research. This is mostly attributable to the high performance of the technique in the separation of the $\alpha 1$ and $\alpha 2$ chains to make the type analysis of collagen possible (Hayashi & Nagai, 1979). The two chains have been reported to be virtually identical with respect to their chain lengths according to the sedimentation equilibrium analysis (Lewis & Piez, 1964). This seems to be quite natural as judged from the mechanism of the formation of the collagen molecule by the post-translational scission of the triple-stranded precursor (Bornstein & Traub, 1979). It is quite interesting that the two chains can be separated by SDS-PAGE generally taken to separate protein polypeptides according to their chain lengths, regardless of the above situation. In this chapter, The interaction of SDS with the $\alpha 2$ chain of rat tail tendon collagen has been studied, and their results were compared with that of the $\alpha 1$ chain described in chapter 8 to make clear the reason for the favorable separation.

III. EXPERIMENTAL PROCEDURE

The $\alpha 1$, $\alpha 2$, $\beta 11$ and $\beta 12$ chains were isolated from tail tendon type I acid-soluble collagen of Wistar rat by CM-cellulose (Whatman CM 52) chromatography (Chung & Miller, 1974) and subsequent Sepharose 4B gel chromatography (Piez, 1968). Each of the chains thus prepared was confirmed to be free from any contaminant polypeptide by SDS-PAGE. Concentration of the

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collagen polypeptides was determined spectrophotometrically assuming A ${}^{1\%}_{1cm}$ at 230 nm to be 22.5 (Kubo et al., 1982). Reducedcarboxamidomethylated (RCAM) bovine serum albumin (BSA) and reduced-carboxymethylated (RCM) BSA were prepared as described by Shirahama et al.(1974).

Sodium dodecyl sulfate (SDS) was prepared by sulfation of n-dodecyl alcohol (99.8 8 with respest to chain length distribution). A commercially available SDS preparation (Nakarai Chemicals, SPS-4: nominally 97 % pure) was used only for SDS-PAGE. All measurements except SDS-PAGE were carried out using sodium phosphate buffer of pH 7.0 prepared by mixing 50 mM NaH_2PO_4 and 50 mM Na_2HPO_4 . Sodium azide was added to the solution to a final concentration of 0.02 % (w/v).

Binding of SDS to the collagen polypeptides was measured by the equilibrium dialysis technique (Takagi et al., 1975a) or by the gel chromatography technique (Takagi et al., 1980; Kubo et al., 1982). SDS was analyzed colorimetrically (Takagi et al., 1975a).

CD spectra were measured with a JASCO J-500 Spectropolarimeter. Sample solutions for the CD measurements were prepared by the equilibrium dialysis against the buffer solution containing required amount of SDS in the same manner as described (Kubo et al., 1982). The data obtained are expressed as residue molar ellipticities. The mean residue weights were assumed to be 91.8, 91.3, 91.8 and 91.6 (Piez et al., 1963) for the $\alpha 1$, $\alpha 2$, $\beta 11$ and $\beta 12$ chains, respectively.

Free-boundary electrophoresis was carried out using a

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Tiselius-type apparatus (Hitachi model HTB-2A) with a schlieren optics.

Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the standard procedure (Weber & Osborn, 1969) with a slight modification (Kubo et al., 1979).

Viscosity was measured in an Ubbelohde type viscometer with a flow-time of 200 sec for water. Sample solutions for viscosity measurements were prepared in the same manner as for the CD measurements.

IV. RESULTS AND DISCUSSION

Figure 1a illustrates the separation of the $\alpha 1$ and $\alpha 2$ chains as well as that of the β 11 and β 12 chains in SDS-PAGE. Each of these two pairs of the collagen polypeptides has virtually identical chain length (Lewis & Piez, 1964). The present study was planned to make clear why such separation is realized. The separation has been observed in the wide varieties of buffer salt concentrations and solutions in pH's. The following experiments were carried out using 50 mM sodium phosphate buffer solution. This is only because the critical micelle concentration of SDS increases with the decrease in buffer concentration so that measurements can be carried out over a wide range of SDS concentration. Figure 1b shows another example of separation of two polypeptides with identical chain length, namely polypeptides derived from BSA by reductive cleavage of

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disulfide bonds and subsequent alkylation of resultant sulfhydryl groups.

SDS-PAGE is generally considered to separate protein polypeptides according to their chain lengths. This is, however, not always the case (Swank & Munkers, 1971; Tung & Knight, 1972). According to Rodbard and Chrambach (1970), the electrophoretic mobility of a protein in a gel matrix can be expressed as follows,

$$U_q = U_0 \exp(-K_R T)$$
 (1)

where U_g is mobility in the gel matrix; U_0 , mobility in a solvent in the absence of a gel matrix; K_R , retardation coefficient roughly proportional to the square of hydrodynamically effective radius; and T, concentration of the gel matrix. The difference in the electrophoretic mobilities observed in an electrophoretic run can be ascribed to both or either of the two factors: 1) difference in intrinsic electrophoretic mobility, U_0 ; and 2) difference in hydrodynamically effective size reflected in K_R .

We first examined the second factor, the size. Intrinsic viscosity is a reliable measure of the hydrodynamically effective volume. Figure 2 shows the plots of reduced viscosities of the $\alpha 1$, $\alpha 2$, $\beta 11$ and $\beta 12$ chains against concentration for the polypeptide concentrations. No significant difference in intrinsic viscosity obtained by extrapolation to zero concentration was observed either between the α chains or between the β chains. The difference in the observed mobilities in SDS-PAGE, therefore,



Fig.1. SDS-PAGE of the collagen chains in comparison with that of derivatives of BSA. Electrophoresis was performed in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1 % SDS on 5 % gel at 25.0 \pm 0.2°C (negative electrode in the right side). Bands are assignable, from right to left, to the β 11, β 12, α 1 and α 2 chains of the collagen for a) and RCM-BSA and RCAM-BSA for b), respectively. Samples: a) 7.1 µg of the collagen, and b) 0.65 µg of each of the derivatives of BSA.



Fig.2. Plots of reduced viscosities against concentration of the collagen chains. x, $\beta 12$; o, $\beta 11$; \Box , $\alpha 2$. Solution of the chains had been equilibrated with 50 mM sodium phosphate buffer, pH 7.0, containing 2 mM SDS. The buffer in the outer solution was used as the solvent. Viscosities were measured at 25.00 \pm 0.02°C. Plots for the $\alpha 1$ chain (-----) is cited from the authors, paper (Kubo et al., 1982).

cannot be ascribed to the difference in size.

CD spectra were measured to detect any possible difference in coformation (not shown). No significant difference was observed between the spectra observed for the collagen chains, except in the amplitude of the depression at 222 nm. The amplitudes at the wavelength are included in Table 1. It is to be noted that the amplitudes observed for the β 11 and β 12 chains coincide with the averages of the amplitudes observed for the respective constitu- α 2 chains presumed that the $\alpha 1$ and assume ents. It is conformations slightly different with each other in the presence The difference is, however, too small to be reflected in of SDS. the viscosity behavior.

Now, the $\alpha 1$ and $\alpha 2$ chains must differ in U₀ value. The Un value is electrophoretic mobility in the absence of gel matrices. The value of U_0 can be evaluated in principle by extrapolation of mobilities obtained in PAGE to zero gel concentration. The extrapolation is hardly reliable to obtain the values suitable for quantitative discussion. For the purpose, one must resort to mobilities measured by the free-boundary electrophoretic technique using a Tiselius type apparatus. In Figure 3, the electrophoretic mobilities are plotted against chain concentration for each of the complexes between SDS and the collagen chains. The ascending and the descending boundaries differed in the inclination in the concentration dependence, and the plots for the boundaries coverged on the ordinate. The plots for the β chains were somewhat irregular, and the lines were drawn by the force. above concentration dependence is not confined The to the

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the collagen chains in free-boundary Fig.3. Mobilities of electrophoresis at 25.0 \pm 0.1°C in the presence of 2.1 mM free SDS as function of the concentrations of the chains. The filled and open marks represent the data for the ascending and descending boundaries, respectively. \Box , \blacksquare ; α 2: o, \bullet ; α 1: \diamondsuit , \blacklozenge ; β 12: Δ , \blacktriangle ; β 11. Data of the α 1 chain is cited from the authors, paper (Kubo et al., 1982). The buffer solution used is the same as in Fig.2.

Table 1. Summary of several properties of the collagen chains in the presence of SDS. All measurements were performed in 50 mM sodium phosphate buffer, pH 7.0, containing 0.02 % sodium azide, at 25°C. Data of the α 1 chain was cited from the authors, paper (Kubo et al., 1982).

Collagen	Intrinsic	Residue molar	Electrophoretic	Bound
chain	viscosity	ellipticity at 222 nm	mobility X 104	SDS
<u> </u>	(ml/g)	(deg·cm ² ·dmol ⁻¹)	(cm ² ·s ⁻¹ ·v ⁻¹)	(g/g)
α1	41.9	-900	-2.48 1.36	± 0.08
α2	41.2	-1250	-2.88 1.57	± 0.09
β11	60.0	-900	-2.71 1.36	± 0.05
β12	60.1	-1100	-2.90 1.44	± 0.04

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Fig.4. Binding isotherm of SDS to the $\alpha 2$ chain (-----) in 50 mM sodium phosphate buffer containing 0.02 % sodium azide, pH 7.0, at 25.0 ± 0.2°C. Data designated by filled and open squares were obtained by the equilibrium dialysis (14 days) and the gel chromatography techniques, respectively. Height and width of each square indicate mean deviations. The arrow on the abscissa indicates the critical micelle concentration of SDS. The isotherm of the $\alpha 1$ chain (-----) is cited from the authors, paper (Kubo et al., 1982).

collagen chains that has been observed for the complexes between SDS and protein polypeptide derived from various globular proteins (Shirahama et al., 1974). A significant difference was observed in the electrophoretic mobility between $\alpha 1$ and $\alpha 2$ chains. For the $\beta 11$ and $\beta 12$ chains, a smaller difference was observed and was consistent with that expected from their compositions.

To interpret the above observation, the complex between SDS and α 2 chains must have a higher negative charge than that betweem SDS and α 1 chains. Since the effective charge of such a complex is dominated by the bound dodecyl sulfate ions, the amount of bound SDS was compared between the α 1 and α 2 chains by measurement of binding isotherms of SDS.

As shown in Figure 4, a significant difference was observed between the α 1 and α 2 chains. The latter maximally bound 1.57 g SDS per g which is significantly higher than the corresponding value of 1.36 g SDS per g reported for the former (Kubo et al., 1982). It is interesting to note that the complex between SDS and RCAM-BSA shows significantly higher mobility in the gel than that between SDS and RCM-BSA (Fig.1). The former has been reported to be more abundant in SDS bound than the latter (Takagi et al., 1975a).

When the results obtained are taken into consideration, the reason of separation of the $\alpha 1$ and $\alpha 2$ chains can be interpreted as follows: 1) the $\alpha 2$ chain differs from the $\alpha 1$ chain in the mode of the interaction with SDS and maximally binds SDS in an amount 15 % larger than the $\alpha 1$ chain; 2) this higher capacity of SDS has



Fig.5. Change in the electrophoretic mobilities of complexes between protein polypeptide chains and SDS with concentration of polyacrylamide gel having a constant weight ratio of acrylamide to N,N'-methylene-bis-(acrylamide), 100 : 2.7. Protein polypeptide chains are from the top to the bottom; RCAM-BSA used as the reference, RCM-BSA, α 2 chain, α 1 chain, β 12 chain and β 11 chain, respectively.

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no significant effect on the hydrodynamically effective size of the α 2 chain-SDS complex but increase the charge density of the complex to give the complex with an electrophoretic mobility higher than that of the α 1 chain-SDS complex. The higher affinity of the α 2 chain to SDS may be ascribed to the abundance of hydrophobic amino acid residues in the chain compared with the α 1 chain (Piez et al., 1963; Hayashi & Nagai, 1980). To obtain supplementary data, effort was also made to evaluate the U₀ and K_R values directly by polyacrylamide gel electrophoresis carried out at various gel concentrations.

Figure 5 shows the results thus obtained, in the form of Ferguson plot. For the $\alpha 1$ and $\alpha 2$ chains, difference was observed not in K_R but in U_0 in agreement with the above discussion. However when the plots for the $\beta 11$ and $\beta 12$ chains are taken into consideration, the validity of extrapolation to zero gel concentration to obtain U_0 becomes questionable. The same question has been raised by Frank and Rodbard (1975). They showed that Ferguson plots of their results gave a focal point at 1.3 % gel concentration.

Hayashi and Nagai (1980) has reached conclution similar to the above conclusion concerning the difference between the $\alpha 1$ and $\alpha 2$ chains in their behavior in SDS-PAGE using the Ferguson plots. Due to the above mentioned ambiguity inherent in the approach using Ferguson plot, the above conclusion based on more sound experimental data has been here reported.

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10. Separation of Cross-linked Products of Rat Tail Tendon Collagen from Monomeric Components by Gel Chromatography in the Presence of Tris Dodecyl Sulfate at 4^oC

I. SUMMARY

Rat tail tendon collagen can be solubilized by a neutral aqueous solution containing 2 % Tris dodecyl sulfate at 4°C without being unfolded to its constituent polypeptides. The solvent at this temperature is particularly suited for qel chromatographic separation of native collagen. Thus Sepharose CL-2B gel chromatography was found to separate the monomeric collagen molecule from the products of intermolecular cross-linking. The procedure can be effectively used to follow of intermolecular cross-linking with aging. progress The monomeric collagen can be subsequently applied to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to evaluate the mode of intramolecular cross-linking.

II. INTRODUCTION

Collagen fibrils are strengthened by the formation of covalent cross-linkages between constituent polypeptides. Some of the linkages are intra- and others are intermolecular. Formation of cross-linkages between collagen polypeptides can be detected efficiently by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). To study formation of intermolecular cross-links, collagen molecules must be separated depending on the degree of oligomerization without being unfolded. Subsequent analysis of each component will give insight into the mode of intramolecular cross-linking. Previously, the separation has been attempted using techniques such as fractional salt precipitation (Chandrakasam et al., 1976), membrane filtration None of (Silver and Trelstad, 1980), and gel chromatography. these techniques, however, brought about satisfactory results. Although gel chromatography was most promising, the monomeric collagen molecule could be only partially separated from the oligomers or gelatinous by-products because of the lack of solvent suitable for making collagen molecules soluble without causing denaturation. Use of an ionic surfactant can eliminate unfavorable intermolecular noncovalent interaction by the introduction of charges just as in polyacrylamide gel electrophoresis in the presence of SDS. SDS cannot be used because of its denaturing action against the collagen molecule at normal However, use of SDS at low temperature might make temperatures. the collagen molecule soluble without causing denaturation. This idea is unrealistic, since the surfactant reduces its solubility in water remarkably at temperature below 10°C.

In contrast, lithium dodecyl sulfate (LDS) is highly soluble in water even at 0°C (Noll and Stutz, 1968). It has been reported that this analog of sodium dodecyl sulfate can be effectively used for solubilization of some functional protein
units of photosynthesis without destruction of their oligomeric structures (Delepelaire and Chua, 1979). LDS was found, however, to have denaturing action against the collagen molecule even at 4°C. It was assessed, then, to use Tris dodecyl sulfate which is highly soluble even at 0°C like LDS. As will be described below, gel chromatography in the presence of Tris dodecyl sulfate was found highly promising as a way to analyze a collagen preparation according to their sizes which reflect difference in the degree of formation of intermolecular cross-linking.

III. EXPERIMENTAL PROCEDURE

Tris dodecyl sulfate

Sodium dodecyl sulfate (SDS) was obtained from Nakarai Chemicals (SPS-4), and converted to Tris (tris(hydroxymethyl) methylammonium) dodecyl sulfate by passage through a column (2.5 X 23 cm) of Dowex 50W-X8 (Tris form) essentially according to the method of Noll and Stutz (1968)originally designed for preparation of lithium dodecyl sulfate (LDS). Conversion to Tris form was confirmed to be quantitative by analysis of sodium using UHF Plasma Spectra Scan-300 (Hitachi, Ltd.). а Only a trace amount of sodium was detected in the preparation of Tris dodecyl sulfate. LDS was obtained as "lauryl sulfate lithium salt" from Sigma Chemical Co..

Lathyritic rats

Four-week-old Wister rats were purchased and bred on foods supplemented with β -aminopropionitrile fumarate for 2-3 weeks before slaughter.

Collagen

Samples for solubility and CD measurements were obtained by extraction of Wister rat tail tendon with 0.5 M acetic acid. The extracted collagen was lyophilized and stored in a refrigerator. The lyophilized collagen was solubilized as follows: 1) the collagen preparation was soaked at 4°C in 0-100 mM Tris-HCl, pH 7.2, containing 1-4 % Tris dodecyl sulfate in an amount of 2 mg/ml; 2) after storage for 24 h small amounts of insoluble materials were removed by centrifugation (130,000 X g) for 60 min.

Collagen samples for gel chromatography were prepared as follows. Tail tendon of a 30-week-old rat was extracted with 10 mM Tris-HCl, pH 7.2, containing 0.4 % Tris dodecyl sulfate, and the insoluble materials were collected by centrifugation (60,000 X g) for 30 min. Proteins other than collagen are left in the supernatant. The pellet was solubilized in the same buffer as above containing 6 % Tris dodecyl sulfate. After three days, a insoluble materials slight amount of was removed by centrifugation (130,000 X g) for 60 min. A collagen stock solution of a final concentration of 1-2 mg/ml was obtained as the supernatant. Tendons from 5-week-old rats were extracted with

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0.5 M acetic acid. Tendons from lathyritic rats were extracted with 50 mM Tris-HCl, pH 7.2, containing 1 M NaCl. The extracted lathyritic collagen was dialyzed against 0.1 M acetic acid. The collagen preparation in aqueous acetic acid were lyophilized. For solubilization, they were treated with buffer solutions containing 4 % and 2 % Tris dodecyl'sulfate for 5-week-old and lathyritic rats, respectively, for 2 days. Collagen stock solutions were prepared in the same manner as described above. All the procedures described above were carried out at 4°C.

Concentration of collagen was determined spectrophotometrically assuming absorbance at 230 nm (1 % solution, 1 cm light path) to be 23.5. The value was determind based on the dry weight method. The absorbance was measured with the collagen polypeptides denatured by dodecyl sulfate at room temperature.

CD spectra

A JASCO J-500 CD spectrophotometer was used. Temperature of the sample solution was controlled by circulation of thermostated ethanol through a brass muffle obtained from JASCO. CD data are reported as mean residue ellipticity. Mean residue weight of collagen was assumed to be 91.6.

<u>Gel</u> chromatography

Sepharose CL-4B or Sepharose CL-2B was used. A Pharmacia column, K 16/100, was used, and temperature was controlled by

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passing water at 4° C through the envelope. Bed size was 1.6 x 95 cm in both cases. Elution was monitored by an LDC Spectromonitor model III at 230 nm using a cell of 1 cm light path.

Gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1 % SDS was carried out essentially according to the method of Weber and Osborn (1969) using 0.1 M sodium phosphate buffer, pH 7.0, at 20°C. Gels were stained with 0.02 % Coomassie Brilliant Blue R in a mixture of isopropanol-acetic acid-water (volume ratio, 25:10:65).

III. RESULTS

Solubility

A lyophilized preparation of the collagen from rat tail tendon (15-week-old) was suspended at a concentration of 2 mg/ml in Tris-HCl buffer solution, pH 7.2, of various concentrations between 0 and 100 mM each containing 2 % Tris dodecyl sulfate for 24 hr at 4°C. Concentration range of Tris-HCl between 10 and 50 mM was optimum, and about 90 % of the collagen could be solubilized as shown in Fig.1a. The maximum solubilization could be attained when the concentration of Tris dodecyl sulfate was about 2 % as shown in Fig.1b.

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Fig.1. Solubility of rat tail tendon collagen extracted with acetic acid as a function of concentration of Tris-HCl buffer, pH 7.2 (a) and of Tris dodecyl sulfate (b). Concentrations of Tris dodecyl sulfate and Tris-HCl buffer were kept at 2 % and 50 mM at 4° C.

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CD spectra

Fig.2 shows CD spectra of the collagen in the presence of 2 % Tris dodecyl sulfate at various temperatures. At lower temperatures, the collagen showed a positive CD band with an intensity of 6200 deg $cm^2 \cdot dmol^{-1}$ at 222 nm. The CD band is characteristic The above intensity is collagen in the native state. of comparable to that of the collagen in 5 mM acetic acid at pH 3.6 and 4°C as also included in Fig.2. Clearly collagen retains its native structure in 2 % Tris dodecyl sulfate at lower temperatures like in 5 mM acetic acid which is known to preserve of collagen. CD spectrum at the lower the native state tempera-ture range and in the presence of LDS is included in Amplitude of the CD band is half of that in the presence Fig.2. of Tris dodecyl sulfate indicating that the lithium salt is not as effective as the Tris salt to maintain the native structure of collagen. With increase of temperature, the CD peak of the collagen in 2 % Tris dodecyl sulfate became attenuated, and disappeared at 22°C finally to give a negative CD spectrum monotonously increasing its nagative amplitude with decrease in wavelength. The change reflects denaturation of the collagen in the presence of Tris dodecyl sulfate with increase of temperature.

Gel chromatography

The finding of the stability of the collagen molecule in the presence of Tris dodecyl sulfate at 4°C prompted one to examine

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Fig.2. CD spectra of the collagen at various temperatures. Solvent, 50 mM Tris-HCl buffer, pH 7.2, containing 2 % Tris dodecyl sulfate. Full lines from top to bottom, 4-10°, 15°, 17°, 19°, 20°, 22° and 25°C, respectively. Dotted line: upper, 5 mM acetic acid, pH 3.6 at 4°C; lower, 50 mM lithium chloride-5 mM lithium citrate buffer, pH 6.4, containing 2 % LDS at 4-7°C.

the possibility of fractionation of the collagen by qel chromatography in the above condition. Fig.3 shows typical examples of elution patterns of the collagen from a Sepharose CL-4B column in the above condition. The collagen eluted as a peak near the void volume as shown by the curve a in Fig.3. Presence of at least two components is suggested from double-headed feature of the peak. Heat-treatment for 3 min at 50°C drastically changed elution pattern obtained in the identical condition as shown by curve b in Fig.3. The collagen molecule is dissociated into constituent polypeptides with The main peak and the shoulder in front of it can be heating. assigned to the α and β chains, respectively. Comparison of the two elution curves in Fig.3 indicates that the collagen molecule in the presence of 2 % Tris dodecyl sulfate retains its native structure at 4°C and is denatured irreversibly by the heat-treatment.

Further to fractionate the components which eluted near the void volume (curve a in Fig.3), the collagen was chromatographed by Sepharose CL-2B instead of CL-4B under the same condition. The collagen from a 30-week old rat showed an elution curve with two major peaks as shown in Fig.4a. The first peak eluted between 50 and 80 ml had a notched appearance suggesting heterogeneity. The other peak eluted between 80 and 120 ml showed a symmetrical shape. The collagen from a younger rat (5-week-old) contained less amount of the higher molecular weight components when compared to the former as shown in Fig.4b. The collagen from a lathyritic rat contained only a single major



Fig.3. Elution patterns from a Sepharose CL-4B column (1.6 X 95 cm) of the collagen before (a) and after (b) heat-treatment (50°C, 3 min). Sample, 0.4 ml of the collagen (1.3 mg/ml) extracted from rat tail tendon. Solvent, 50 mM Tris-HCl, pH 7.2, containing 2% Tris dodecyl sulfate. Flow rate, 17.4 ml/hr. Temperature, 4°C. Double-headed arrow, 0.05 absorbance scale at 230 nm.



Fig.4. Elution patterns from a Sepharose CL-2B column (1.6 X 95 cm) of the collagen. Each 0.4 ml of sample solutions contained following amounts of the collagen obtained from rats of age indicated: a) 1.2 mg, 30 weeks; b) 1.3 mg, 5 weeks and c) 0.7 mg, 6-7 weeks (lathyritic). Elution conditions, the same as in Fig.3 except flow rate (10 ml/h).

component as shown by curve c in Fig.4. As far as the separation of α -chains and β -chains of the collagen is concerned, the Sepharose CL-2B column is inferior to the Sepharose CL-4B column. Both components were eluted at elution volume of 140 ml in Fig.4 without being separated. Therefore Sepharose CL-2B gel chromatography in the presence of 2 % Tris' dodecyl sulfate at 4°C can separate a collagen preparation into cross-linked products and into collagen molecules without intermolecular cross-links.

Polyacrylamide gel electrophoresis

The collagen from 5-week-old the rat eluted from the Sepharose CL-2B column was separated into five fractions as shown by curve b in Fig.4. The fractions were analyzed by polyacrylamide gel electrophoresis in the presence of SDS to evaluate the degree of separation of collagen by the Sepharose CL-2B gel chromatography. Electrophoretic patterns obtained are shown in Fig.5.

It is to be noted that the fraction V revealed only two α , two β and one γ component. This indicates that the particular fraction contained only the monomeric collagen molecule without intermolecular cross-linking. In comparison, the fraction IV showed an electrophoretic pattern similar to that for the fraction V except the presence of a very small amount of polymeric cross-linked products consisted of four or more collagen polypeptides. Fractions II and III showed almost the same electrophoretic patterns and were found to contain a larger



Fig.5. Polyacrylamide gel electrophoresis in the presence of SDS of fractions of the collagen from a 5-week-old rat (curve b in Fig.4) at 20^oC. The pattern for Fraction III is omitted, because it is virtually identical to that for Fraction II. Gel, 5 % (w/v). Electrophoresis, left to right. Double-headed arrow, 0.5 absorbance scale at 565 nm. Major peaks are 1) $\alpha 2$, 2) $\alpha 1$, 3) $\beta 22$, 4) $\beta 12$, 5) $\beta 11$, 6) γ and 7) polymeric component, respectively.

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amount of γ and higher polymeric components. In the β region, a band assignable to β 22 chain (Bornstein et al., 1964) appeared in addition to the β 11 and β 12 chains. The γ component increased not only its content but also the number of peaks called collectively under this grouping. The above results indicate that the fractions II and III contain intermolecularly cross-linked polypeptides. The fraction I was found to contain larger amount of polymeric components than the fractions II and III.

V. DISCUSSION

Fractionation of collagen by gel chromatography has been attempted using various non-denaturing solvents indicated in Dancewicz (1975) and co-worker (Majeska parentheses. and Dancewicz, 1977) applied rat skin tropocollagen to a Sepharose 6B column (solvent, 0.05 M acetic acid) after irradiation of various doses of X-ray and found that cross-linked components eluted in advance of intact molecules giving two distinct peaks. Meredith and Kezdy (1981) applied calf and rat skin collagens to a Sepharose CL-6B column (solvent, 0.16 M sodium phosphate, pH 7.4, containing 0.3 M sucrose) and found that the collagens eluted as a single peak being preceded by unfolded collagen polypeptides. It is to be noted that collagen was eluted near the total column volume in the above experiments. Fujimori and Shambaugh (1983) applied rat tendon collagen to a Sephacryl S-300 column (solvent, 0.1 M acetate buffer, pH 4). The collagen eluted earlier with

the increase of the age of rat used as the source. Noelken and Bettin (1983) found that calf-skin collagen eluted from a Fractogel TSK HW-65F colmn (solvent, 0.1 M Tris-HCl, pH 7.3, containg 0.5 M urea) as a single peak. They found that the collagen eluted in advance of the unfolded collagen polypeptides if present.

From the results of the studies cited above, it can be concluded that: 1) the gels used as supporting materials were not porous enough to separate collagen molecules according to the degree of intermolecular cross-linking; 2) the collagen molecule is retarded due to protein-gel interaction when Sepharose 6B, Sepharose CL-6B or Fractogel HW-65F was used; and 3) gel chromatographic separation of collagen purely on the basis of molecular sieving effect has not yet been successful.

A breakthrough was made in the gel chromatographic separation of collagen taking the advantage of the following situations: 1) presence of 2 % Tris dodecyl sulfate made the collagen molecule soluble in a neutral buffer solution; and 2) charges introduced by the bound surfactants into both the collagen molecule and its cross-linked products excluded the protein-protein interaction as well as protein-gel interaction which led to decrease of resolution.

It is proposed to apply to gel chromatography of collagen in the presence of 2 % Tris dodecyl sulfate at 4°C for the following purposes: 1) separation of collagen from unfolded collagen polypeptides and other contaminating protein polypeptides using Sepharose CL-4B; and 2) separation of monomeric collagen molecule

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from intermolecularly cross-linked collagen molecules using Sepharose CL-2B. The latter is particularly suited for following the formation of intermolecular cross-linking with aging. It seems possible further to fractionate the cross-linked products according to the degree of oligomerization, if a gel chromatographic supporting material with higher resolution for the cross-linked products become available.

It is not yet clear why the concentration of Tris dodecyl sulfate as high as 2 % is required for the solubilization of the collagen and its cross-linked products. Critical micelle concentration of Tris dodecyl sulfate in the buffer solution used in this study is 0.09 %. It is suggested that there is a mode of interaction between Tris dodecyl sulfate and collagen which becomes effective at a concentration far exceeding the critical micelle concentration and has not yet been made clear. Binding of Lithium Dodecyl Sulfate to Polyacrylamide Gel at 4°C Perturbs Electrophoresis of Proteins

I. SUMMARY

Although polyacrylamide gel has no affinity to lithium dodecyl sulfate (LDS) at 25°C, the gel maximally binds 17 mg of LDS per gram dry weight at 4°C. When polyacrylamide gel electrophoresis is carried out at 4°C in the presence of LDS instead of sodium dodecyl sulfate (SDS) using a continuous buffer system, migration of proteins with lower molecular weight is accelerated as a result of the deficiency of LDS in the frontal region of the gel. When the gel is saturated with LDS, electrophoresis in the presence of LDS and at 4°C shows a resolution higher than that of SDS-polyacrylamide gel electrophoresis at 25°C.

II. INTRODUCTION

dodecyl sulfate-polyacrylamide gel electrophoresis Sodium (SDS-PAGE) is now widely used in the field of biochemistry. Electrophoretic runs of SDS-PAGE at temperatures near 0°C are impeded by crystal formation of SDS. The difficulty was overcome by the use of lithium dodecyl sulfate (LDS) instead of SDS. Polyacrylamide gel electrophoresis in the presence of LDS (LDS-PAGE) was first applied to the analysis of thylakoid

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membrane proteins of Chlamydomonas reinhardtii by Delepelaire and Chua (1979). Visualization of the gel after the electrophoresis showed the presence of several colored bands assignable to chlorophyll-containing proteins. In SDS-PAGE at room temperature, chlorophyll proteins were mostly denatured to release chlorophylls and give a major colored band assignable to SDS micelles in which chlorophylls were incorporated. Only one or two chlorophyll-containing protein bands were observed. Since then, LDS-PAGE at temperatures near 0°C has been utilized in the research field of photosynthesis as an analytical method of molecular assembly of chlorophyll-containing proteins without cleavage of intermolecular interactions. In the original recipe of LDS-PAGE that has been followed since (Delepelaire & Chua, 1979), LDS is added only to the sample solution and the upper electrode trough. We noticed that proteins complexed with dodecyl sulfate ions become free from lithium ions as soon as the electrophoresis is started. It turned out that the stabilization of the molecular assemblies could be ascribed to the extremely slow denaturation of the chlorophyll-containing proteins at 4°C in the presence of Tris dodecyl sulfate (TrisDS). This procedure, called "LDS-PAGE", is actually "TrisDS-PAGE". We have found that polyacrylamide gel electrophoresis in the presence of TrisDS or an analogous alkanolammonium dodecyl sulfate is very efficient in the analysis of the molecular assemblies of spinach thylakoid membrane proteins and possibly other such assemblies as will be reported in an another paper (Kubo & Takagi, 1986).

We have modified the conventional procedure for LDS-PAGE so

that it is true to its name. Buffer components were designed so that lithium ion exists as the sole cation. LDS was found to be comparable to or stronger than SDS in its denaturing power even at temperatures near 0°C. As is shown by an example in this chapter (Fig.6), LDS-PAGE at 0-4°C is superior to SDS-PAGE at This real "LDS-PAGE" will be welcomed as an room temperature. improved version of SDS-PAGE. Special care is required, however, to carry out LDS-PAGE at 0-4°C, because LDS interacts with the polyacrylamide gel matrices in this temperature range. This chapter is written first to point out this phenomenon, and second to show what kind of care is required to be free from anomalies We have reviewed the result of caused by the interaction. the assessment of LDS-PAGE elsewhere (Kubo & Takagi, 1985).

III. EXPERIMENTAL PROCEDURE

Lithium dodecyl sulfate (LDS) was prepared from SDS obtained from Nakarai Chemicals (SPS-4), essentially according to the procedure of Noll and Stutz (1968). An ion-exchange column of Dowex 50W-X8 (2.5 X 23 cm, 100-200 mesh) was converted from H to Li form by passage of 500 ml of 2 M lithium chloride, and washed with distilled water. SDS was converted to LDS by passage of 500 ml of 10 % (w/v) aqueous solution of the former through the column. The column was washed with 60 ml of distilled water. Eluates were combined and lyophilized to obtain solid LDS. The yield of the conversion was nearly 100 %. LDS is also available

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commercially. We preferred conversion from SDS, since identical purity can be expected with respect to the alkyl chain for both preparations of the dodecyl sulfates thus obtained. A stock solution of lithium azide was prepared by passage of 2 % sodium azide through the Li-form ion-exchange column. Lithium citrate was obtained from Wako Pure Chemicals (grade for amino acid analysis).

A buffer solution with the following composition was used throughout the present study: 0.1 M lithium citrate, pН 6.0, containing 0.02 % lithium azide. Hereafter it is called "lithium citrate buffer, pH 6.0". The critical micelle concentration of LDS in this buffer solution was determined to be 1.1 mM at $4^{\circ}C$ and 0.9 mM at 25°C by measurement of the solubilization of Yellow OB as described previously (Takagi et al., 1980). Polvacrylamide gel electrophoresis in the presence of LDS was carried out essentially according to the method described by Weber and Osborn (1969), with the following modifications: (i) SDS was replaced by LDS; (ii) lithium citrate buffer, pH 6.0, was used instead of 0.1 M sodium phosphate buffer, pH 7.0. Distribution of LDS in the gel was visualized immediately after electrophoresis by formation of the insoluble complex with a cationic surfactant according to the procedure described previously (Takagi et al., 1977). White zones thus revealed were scanned at 440 nm by a densitometer.

Binding of LDS to Bio-Gel P-60 (100-200 mesh; control No. 163572; polyacrylamide beads) was measured as follows. Bio-Gel P-60 beads were immersed in water, washed repeatedly by

decantation, immersed in ethanol, and finally dried under vacuum over phosphorous pentaoxide. To each 4 ml of lithium citrate buffer, pH 6.0, in a vial containing a known amount of LDS in the range between 0.14 and 50 mM, 200 mg of the dried Bio-Gel P-60 added. The vials were kept overnight beads was at room temperature and shaken in a water bath at 4, 15, or 25°C for 2 to hr to attain equilibrium. The beads were separated by 3 centrifugation at the same temperature as in the equilibration, and the supernatants were analyzed for LDS by colorimetry of the methylene blue-dodecyl sulfate complex extracted with chloroform according to the method described by Takagi et al. (1975).The amount of LDS bound was determined by subtraction of the amount of free LDS from the total amount of LDS added.

A mixture of proteins designated as "low molecular weight calibration kit" (Pharmacia Fine Chemicals) and β -lactoglobulin were fully reduced and carboxamidomethylated according to the procedure described by Shirahama et al. (1974) and used as molecular weight standards. Broken spinach chloroplasts were prepared according to the method described previously (Kubo & Takagi, 1986).

IV. RESULTS AND DISCUSSION

Electrophoresis of lithium dodecyl sulfate

Anomalous behavior of LDS in polyacrylamide gel electrophoresis



Fig.1. Polyacrylamide gel electrophoresis of LDS is markedly dependent on temperature. Twenty μ l of 40 mM LDS in 0.01 M lithium citrate containing 5 % glycerine was layered on each of the gels prepared using 0.1 M lithium citrate, pH 6.0. The upper and lower troughs were filled with 0.1 M lithium citrate, pH 6.0. Experimental conditions, namely, gel concentration, temperature, electric current per tube (applied voltage), and duration of run were as follows: a) 5 %, 25°C, 6 mA (32 V), 2.5 hr; b) 10 %, 25°C, 6mA (38 V), 3 hr; c) 5 %, 4°C, 6 mA (50 V), 2.5 hr; d) 10 %, 4°C, 6 mA (60 V), 3 hr. The white arrows indicate the top of the gels. Electrophoresis from top to bottom. is exemplified when LDS alone is analyzed. Here, LDS had been added neither to the electrode buffer nor to the gel. When the corresponding electrophoretic patterns observed for SDS (Kubo et 1979) are taken into account, the patterns in Fig.1 can be al.. explained as follows. At 25°C and in 5 % gel, LDS micelles were electrophoresed ahead of monomeric LDS (Fig.1a). At 25°C and in 10 % gel, LDS micelles were retarded and had the same mobility as monomeric LDS (Fig.1b). Thus LDS, upon electrophoresis formed a narrow band in which micelles and monomers were in equilibrium. When temperature was lowered to 4°C, LDS showed quite different electrophoretic behavior (Figs.1c and d). LDS was markedly retarded, and mostly concentrated at the rear edge. At 4°C, gel concentration had no marked effect on the electrophoretic behavior.

We compared the electrophoresis of LDS in agarose gel at 4 and 25° C, and observed no marked difference (data not shown). Since the agarose gel is devoid of molecular sieving effect on LDS micelles, this indicates that there is no significant change with temperature in the electrophoretic behavior of LDS in free solution whether it is micellar or monomeric. The observed difference can be interpreted, if it is assumed that LDS acquired high affinity to polyacrylamide gel at 4° C.

Binding of lithium dodecyl sulfate

Binding of LDS to polyacrylamide gel was, therefore, measured at 25, 15, and 4°C as shown in Fig.2. Commercially available

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Fig.2. Binding of LDS to polyacrylamide gel (Bio-Gel P-60) with increase in concentration of free LDS. Temperature, $4^{\circ}C$ (o, o), $15^{\circ}C$ (Δ), and $25^{\circ}C$ (x). For the filled circles, refer to the upper and right scales. Solvent, 0.1 M lithium citrate containing 0.02 % LiN₃, pH 6.0.

polyacrylamide gel beads, Bio-Gel P-60, were used since homemade gel is inconvenient for such a purpose. Although binding was not appreciable at 25°C, the polyacrylamide gel bound a small amount of LDS at 15°C, and much more LDS at 4°C. Above 20 mM free LDS, about 17 mg of LDS was bound per gram dry weight of polyacrylamide gel. This amount of binding might seem trivial. It is to be noted, however, that 10 % polyacrylamide gel binds about 0.7 mg LDS per ml of gel in the presence of 0.1 % (3.7 mM) free LDS. The concentration of LDS in the buffer solution in the upper electrode trough is usually 0.1 %.

Seemingly the anomaly in the electrophoresis of LDS at 4°C is due to the binding of LDS to polyacrylamide gel matrices. LDS in the sample solution is mostly bound to the gel matrices and consequently markedly retarded. Presumably unbound LDS present in equilibrium was electrophoresed in advance of the "densely stained" band, and formed the less dense region in front of the band (Figs.1c and d).

<u>Behavior of lithium dodecyl sulfate in an actual electrophoretic</u> <u>run</u>

In a practical LDS-PAGE run, LDS is added not only to the sample solution but also to the buffer of the upper electrode trough. If the conditions were similar to those for SDS-PAGE, addition of LDS to the gel buffer would not be required. Figure 3 shows the electrophoretic behavior of LDS in LDS-PAGE carried out in such a situation without application of a sample solution.

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Electrophoresis

Fig.3. Profiles of the distribution of LDS in electrophoresis from the upper electrode trough into 7.5 % polyacrylamide gel at 25°C (a) and 4°C (b). The lithium citrate buffer used to fill the upper trough was 0.1 % LDS, pH 6.0. Electrophoresis was carried out at 5 mA for 3.5 hr (a) and 5.0 hr (b). The arrows in indicate the tops of the gels. Vertical the right ends double-headed arrow, 1.0 absorbance scale at 440 nm. Horizontal double-headed arrow, 2 cm.

At 25°C, LDS was electrophoresed, giving a single plateau region. The behavior is the same as that observed with SDS at 25°C (Kubo et al,. 1979). At 4°C, the distribution of LDS was depressed at the frontal region of electrophoresing LDS. This can be interpreted as a result of the binding of LDS to polyacrylamide gel. The plateau seems to appear only after LDS is bound to the level where bound LDS is in equilibrium with free LDS at a concentration of 0.1 %.

Figure 4 shows electrophoretic patterns similar to those in Fig.3 except that the sample solution contained two proteins. The major peaks appeared in the front and the middle in Figs,4a and b respectively and can be assigned to LDS. The minor two peaks appearing in both can be assigned to soybean trypsin inhibitor and bovine serum albumin (left to right). It is to be noted that LDS added in excess to the sample solution was electrophoresed at 4°C between the two protein bands (Fig.4b). This is not expected for SDS-PAGE or LDS-PAGE at 25°C (Fig.4a) where excess SDS or LDS in a sample solution is always electrophoresed in advance of all protein bands.

Comparison of Fig.3b and Fig.4b indicates that the concentration of LDS is variable in most of the frontal region of the polyacrylamide gel. The expected profile of the distribution of LDS not bound to protein is illustrated in Fig.4b by shading. We have measured electrophoretic mobilities of six standard proteins (available from Pharmacia as "low molecular weight calibration kit") and of β -lactoglobulin. The conditions were the same as those in Fig.3 except for the addition of proteins to



Fig.4. Profiles of the distribution of LDS in 7.5 % polyacrylamide gel at 25° C (a) and 4° C (b). Each twenty µl of sample solution containing 37 mM LDS, 4 µg bovine serum albumin, and 4 µg soybean trypsin inhibitor was layered on top of polyacrylamide gel. The concentration of LDS in the upper electrode buffer was decreased to 1.2 mM (0.03 %) just above the critical micelle concentration so that the protein bands could be easily localized. Both proteins had been S-carboxamidomethylated according to the procedure described by Shirahama et al. (1974). Electrophoresis was carried out at 6 mA for 4 hr (a) and 4.5 hr (b). Arrows at both ends indicate bottoms (left) and tops (right) of the gels. Double-headed arrow, 1.0 absorbance scale at 440 nm.



Fig.5. Semilogarithmic plot of electrophoretic mobility versus molecular weight in different concentrations of LDS at 4° C. Mobilities are expressed relative to that of bovine serum albumin. Concentration of LDS in polyacrylamide gel (7.5 %) and in the upper electrode buffer were as follows: x, 0.1 and 0.1 %; o, 1.0 and 0.4 %, respectively. The solvent was 0.1 M lithium citrate buffer, pH 6.0. Proteins had been S-carboxamidomethylated, and are, from the right to left, phosphorylase <u>b</u>, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, β -lactoglobulin and α -lactalbumin.

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the sample solution and of 0.1 % LDS to the gel. At 25°C, plots of the logarithms of the observed relative mobility versus molecular weight were linear for all of the proteins. At 4°C, the plot was linear in most of the region, but curved upward for proteins with molecular weights less than 30,000 as shown in Fig.5. The anomaly seems to be related to the deficiency of LDS at 4°C in the frontal region of the electrophoresing LDS (see Fig.3b). On the other hand, the presence of retarded LDS derived from the excess amount in the sample solution seems to have no appreciable influence on the electrophoretic behavior of the When Malachite Green was used as a marker dye it proteins. stained the "excess LDS band". It is likely, therefore, that this band will incorporate lipophilic materials if present, and they will be stained mistaken for a protein band.

<u>High resolution in LDS-PAGE at 4°C</u>

The abnormal distribution of LDS in polyacrylamide gel must be eliminated to make the LDS-PAGE technique reliable. This was expected to be attained if binding sites for LDS had been saturated. Addition of 0.1 % LDS not only to the upper electrode buffer but also to the buffer for gel preparation was found to be insufficient as shown in Fig.5. Addition of 0.4 and 1.0 % LDS to the buffer solution in the upper electrode trough and to the buffer for the gel preparation, respectively, was found to totally eliminate the anomaly. Thus, in an electrophoretic run carried out under the above conditions, the "excess LDS band" was



Fig.6. Resolution of spinach thylakoid membrane proteins in polyacrylamide gel electrophoresis was markedly improved when SDS was replaced by LDS and temperature was lowered from 25 to 4°C.

Thylakoid membrane proteins (1.5 µg per tube as chlorophylls) solubilized using LDS (a) or SDS (b) at the same temperature used in the elec-

trophoresis were layered on the tops of polyacrylamide gels (7.5 %). (a) LDS-PAGE (1.0-0.4 %) at 4°C, 4 mA/tube, 6 hr; (b) SDS-PAGE (0.1-0.4 %) at 25°C, 5mA/tube, 4 hr (the concentration (%) of LDS or SDS in the polyacrylamide gel (before dash) and in the upper electrode buffer (after dash) is shown in parentheses). The solvents were 0.1 M lithium citrate buffer, pH 6.0 (a), and 0.1 M sodium phosphate buffer, pH 7.0 (b). Vertical arrows to the right indicate the position of the free pigment band. Double-headed arrow, 0.4 absorbance scale at 565 nm. electrophoresed in advance of all proteins, and the semilogarithmic plot of relative mobility versus the molecular weight was linear throughout the entire region as also shown in Fig.5. When care (conditions described above) is taken to avoid the anomaly, the performance of LDS-PAGE is far superior to that of conventional SDS-PAGE especially with respect to resolution. As an example, Fig.6 shows the electrophoretic pattern of LDS-PAGE at 4° C of spinach thylakoid membrane proteins in comparison with that of SDS-PAGE at 25° C.

In this study, we paid attention only to the affinity of LDS to polyacrylamide and related phenomena. The mechanism of the binding of LDS to polyacrylamide is not clear and is under investigation. 12. Marked Effect of Cations on the Separation of Spinach Thylakoid Chlorophyll-proteins on Polyacrylamide Gel Electrophoresis at 0°C in the Presence of Alkanolammonium Dodecyl Sulfates.

I. SUMMARY

The separation of spinach thylakoid chlorophyll-proteins on polyacrylamide gel electrophoresis at 0°C in the presence of dodecyl sulfate is markedly influenced by the kind of surfactant cation in the media used for solubilization and electrophoresis. The mode of separation can thus be modulated through the cation. Three kinds of alkanolammonium dodecyl sulfates were selection. and their abilities as to the dissociation tested of the molecular assemblies of chlorophyll-proteins were found to decrease in the following order: tris(hydroxymethyl)methylammonium, triethanolammonium and triisopropanolammonium. Comparison of the electrophoretic patterns obtained with different kinds of cation may help clarify the hierarchy of the molecular assemblies of chlorophyll-proteins.

II. INTRODUCTION

The chlorophyll-protein complexes of chloroplast thylakoid membranes of higher plants and green algae can be resolved by

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polyacrylamide gel electrophoresis carried out at around 0°C in the presence of dodecyl sulfate into three to several chlorophyllcontaining bands (see, Anderson et al., 1978). The retention of non-covalently bound chlorophylls in the protein bands has been ascribed to prevention of denaturation of the chlorophyll-containing proteins due to the low temperature during solubilization and electrophoresis. On surveying the reported procedures, we noticed that the cation in the media for solubilization and electrophoresis plays a major role in determination of the mode of separation, as well as the temperature. The present chapter was written to show that the use of one of certain ions, tris(hydroxymethyl)methylammonium (Tris) or one of its analogues, namely alkanolammonium ions, as the sole cation in the media opens the way to new variations of polyacrylamide gel electrophoresis in the presence of dodecyl sulfate for studies on protein molecular assemblies related to photosynthesis. We chose the above family of cations, because Tris was found to be the major cation component in the recipes for the buffer solutions used for electrophoreses in which chlorophyll-proteins were reported to be at least partly free from denaturation.

III. EXPERIMENTAL PROCEDURE

Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate was carried out according to the method of Weber and Osborn (1969). The acrylamide concentration was 5 (T, w/v) and

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Tube	_{DS} 1	Chl ² /DS	Buffer	Concentration		Current	Duration
		(g/g)	(0.1 M)	of DS Gel	(%) Upper	(mA/tube) (hr) ³
			electrode				
a	Lithium	1:40	Lithium	1.04	0.4	5	4
	(LDS)		citrate,				
			рН 6.0	ı	·		
		·		0	0.1	2	2 (1)
b	Tris	1:40	Tris-HCI	0	0.1	3	3(1)
	(TrisDS)		рН /.2				
_	mad a the sec	1 1.40	Maiothone	0 1	0 1	3 5	3(1)
С	Trietnanoi- 1:40		amino UCI)1- 0	0.1	2.5	5(1)
	(Tri-ebs	>)	pn 7.0				
đ	Triisoprooanol-		Triisopropanol-				
	ammonium	1 : 60	amine-HC]	. 0	0.1	3.5	3.5(1)
	(Tri-ipDS	5)	pH 7.2				
					·		

Table I. Conditions for Electrophoreses shown in Fig.1.

¹Dodecyl sulfate. ²Chlorophyll.

 $3_{\rm Numbers}$ in the parentheses denote duration of preruns.

⁴Poltacrylamide gel has appreciable affinity sites for LDS at 0°C but not at room temperature. Addition of LDS to the gel buffer is required for freedom from the anomaly caused by the affinity. bis-acrylamide added accounted for 2.7 % (w/v) of the total The buffers used are shown in Table I. Sodium acrylamide. dodecyl sulfate (SDS) was replaced by lithium dodecyl sulfate (LDS), tris(hydroxymethyl)methylammonium dodecyl sulfate (TrisDS), triethanolammonium dodecyl sulfate (Tri-eDS) or triisopropanoldodecyl sulfate (Tri-ipDS). Each buffer solution ammonium contained only a single cation, which was the same as that of the The alkanolammonium ions in the buffer dodecyl sulfate used. solutions accelerated the polymerization of acrylamide. Compared to the procedure of Weber and Osborn (1969), the amount of N,N,N',N'-tetramethylethylenediamine was reduced by half in the cases of Tris and Tri-ip and by one-fourth in the case of Tri-e, and that of ammonium persulfate was reduced by half in the case of Tri-e. Both reagents were added just before initiation of the polymerization. Tubes (5 X 70 mm) were immersed in the buffer solution in a positive electrode trough maintained at 0°C.

SDS was obtained from Nakarai Chemicals (SPS-4). Triethanolamine and triisopropanolamine were obtained from Wako Pure Chemicals and Kanto Chemicals, respectively. Dodecyl sulfates, each with a cation other than sodium, were prepared from SDS by means of ion-exchange essentially according to Noll and Stutz (1968). (LDS and TrisDS are available commercially.) Dowex 50W-X8 cation exchanger of 100-200 mesh in particle size was pretreated to obtain the H⁺ form and then packed into a glass column to obtain a 2.5 X 25 cm bed size. Then the lithium, Tris, triethanolammonium and triisopropanolammonium forms were obtained by passage of the following aqueous solutions, respectively: 2 M

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lithium chloride, 500 ml; 2 M tris(hydroxymethyl)aminomethane adjusted to pH 3.7 with hydrochloric acid, 500 ml; 1.5 M triethanolamine hydrochloride adjusted to pH 3.9 in the same way as above, 670 ml; and 1.5 M triisopropanolamine hydrochloride (pH 3.9), 670 ml. The triisopropanolamine hydrochloride had been prepared by the addition of concentrated hydrochloric acid to 3 M triisopropanolamine to pH 3.5. The solution was dried in a rotary evaporator, and the residue was dissolved in ethanol at 60°C for crystallization. The column was washed with 350 ml of distilled water. SDS was converted to the respective derivative by the passage of 500 ml of a 10 % solution through the column. The residual product was washed out with 60 ml of distilled The eluted solutions were combined and used as the stock water. The exchange was so quantitative that estimation of solution. the concentration of dodecyl sulfate after correction for dilution was possible.

Broken spinach chloroplasts were prepared as follows according to the method described by Strotmann et al. (1973) and Henriques and Park (1976). Forty grams of spinach leaves were homogenized in 200 ml of 50 mM sodium phosphate buffer, pH 7.8, containing 0.4 M sucrose, 10 mM sodium chloride, 0.1 mM phenylmethylsulfonyl fluoride, for 10 sec in a blender. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 3000 x q for 8 min at 4°C. The precipitate was suspended in 100 ml of the above buffer. The suspension was centrifuged at 500 x The supernatant was centrifuged at 3000 x g g for 5 min at 4^oC. 4°C to obtain the thylakoid membrane а as 8 min at for

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precipitate. The precipitate was washed by centrifugation at $3000 \times \text{g}$ for 8 min at 4°C with 50 mM sodium phosphate buffer, pH 7.8, containing 0.15 M sodium chloride, with 10 mM sodium pyrophosphate, pH 7.4, and finally with 0.3 M sucrose, twice, three-times and twice, respectively. The final product obtained as a pellet was suspended in 0.3 M sucrose containing 0.1 mM phenylmethylsulfonyl fluoride, and then frozen at -30°C until use.

Spinach thylakoid was solubilized at 0°C with one of the following dodecyl sulfates: lithium, tris(hydroxymethyl)methylammonium (Tris), triethanolammonium (Tri-e) and triisopropanolammonium (Tri-ip) dodecyl sulfates, with the respective dodecyl sulfate at the ratio and in the buffer solution given in Table I. Each solution obtained was analyzed by polyacrylamide gel electrophoresis in the presence of respective dodecyl sulfate under the conditions given in Table I. Special care was taken that there was only a single kind of cation, identical to the counter-ion of the particular dodecyl sulfate, in the medium for solubilization and electrophoresis.

Samples for spectral measurements were prepared as follows. Chloroplast preparation, equivalent in amount to 300 μ g of chlorophylls, was solubilized with Tri-ipDS by means of the same procedure as that described in Table I (d). The solubilized sample was layered on the top of each of two polyacrylamide slab gels without sample slots (same composition as in Fig.1d; 80 X 80 X 2.7 mm). Electrophoresis was carried out at 40 mA/slab for 3.5 hr using a Pharmacia gel electrophoresis apparatus (GE-2/4). Each of the major bands corresponding to those in Fig.1d was cut

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out, and homogenized after addition of a small amount of 0.1 % Tri-ipDS-0.1 M triisopropanolamine-HCl buffer, pH 7.2, in a Teflon homogenizer. The homogenized sample was centrifuged at 3000 rpm for 5 min, and the supernatant was filtered through a Pasteur pipet outlet of which was filled with glass wool. All the above procedures were carried out in a cold room at 4°C. Absorption spectra were measured promptly at room temperature (20°C) using a Hitachi model 557 dual wavelength double beam spectrophotometer.

IV. RESULTS AND DISCUSSION

Fig.1 shows photographs of the gels taken immediately after the end of the electrophoretic runs. For each of the sets of electrophoretic conditions, three parallel runs were made in which the weight ratios of chlorophyll to dodecyl sulfate were 1:80, 1:40 and 1:20, respectively, except in the case of Tri-ipDS where the ratios were 1:120 and 1:60. No appreciable difference was observed among the groups. This indicates that such electrophoretic runs are not significantly affected by differences in the ratio of sample to dodecyl sulfate.

Comparison of the electrophoretic patterns in Fig.1 clearly indicates that the mode of separation is heavily dependent on the kinds of cation used. In the experiments with LDS, all chlorophylls migrated to the frontal region as a single band, usually termed the "free pigment band (FP)". This band is

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Fig.1. Distribution of pigments in 5 % polyacrylamide gels just after electrophoreses at 0°C in the presence of the following dodecyl sulfates: a, LDS; b, TrisDS; c, Tri-eDS; d, Tri-ipDS. Sample, 5 μ g per tube as chlorophylls. The visible bands in gel b are numbered i,i', ii, iii, iv and v. The corresponding bands in different gels are linked by dotted lines. Bands i' and ii seem to be overlapped in gels c and d. Minor bands in gel "d" were too faint to be seen in the photograph. For the experimental conditions, see Table I. ascribed to micelles of dodecyl sulfate in which all liberated pigments from chlorophyll-proteins are incorporated. This result indicates that polyacrylamide gel electrophoresis in the presence of LDS cannot be used to separate the proteins retaining chlorophylls. Inspection of the procedures for so-called "LDSpolyacrylamide gel electrophoresis" showed that the chlorophyllcontaining protein bands were actually electrophoresed with media rich in the Tris cation. For example, Delepelaire and Chua (1979) added LDS only to the upper-trough buffer and the sample This indicates that protein bands are electrophoresed solution. with a medium totally lacking the lithium ion. In the present experiments, the number of heavily or moderately chlorophyll-containing protein bands decreased in the following order without intensification of the FP band: TrisDS, Tri-eDS and Tri-ipDS. We investigated by means of re-electrophoresis as to whether the chlorophyll-containing proteins are stable entities or not, under the electrophoretic conditions. For TrisDS (Fig.1b) and Tri-eDS (Fig.1c), electrophoresis of samples recovered from each section of the chlorophyll-containing protein bands, using the same medium as used for the electrophoresis, showed that parts of particular components had been transformed to others: $i \rightarrow i'$ and iv, iii \rightarrow v, iv \rightarrow v and FP, and v \rightarrow FP (TrisDS); and i \rightarrow ii and iv, iii \rightarrow v, iv \rightarrow v and FP, and v \rightarrow FP (Tri-eDS); respectively. In the case of Tri-ipDS (Fig. 1d), only a minor fraction of i was transformed to ii. Tri-ipDS seems to be the mildest surfactant among the three alkanolammonium dodecyl sulfates.

Fig.2 shows the absorption spectra of the samples recovered



Fig.2. Absorption spectra of extracts from the major colored bands on polyacrylamide gel electrophoresis in the presence of Tri-ipDS under the same conditions as in Fig.1d. Each of the spectra is numbered in the same way as in Fig.1. The bars on the right indicate an absorbance scale of 0.1. from the four chlorophyll-containing protein bands shown in Fig.1d. Bands i and ii are rich in chlorophyll <u>a</u>, and bands iii and v contain both chlorophyll <u>a</u> and <u>b</u>. It is a common practice to assign each of the bands to a particular functional unit of photosynthesis according to their spectra (Anderson et al., 1978). The present results, however, show that the mode of separation of the bands is very variable, depending on the composition of the electrophoretic medium. Thus comparison of the present results with the previous ones is so difficult that we cannot make assignments at present time.

Since the pioneering work of Ogawa et al. (1966), chlorophyllcontaining protein assemblies have been frequently analyzed by polyacrylamide gel electrophoresis at temperatures around 0°C in the presence of dodecyl sulfate. The present results, however, emphasize the necessity for careful revision of the experimental conditions, especially the cation composition of the media used for solubilization and electrophoresis. This chapter is written to point out the above problem which has been overlooked by the people in the field of photosynthesis. On the other hand, electrophoreses in the presence of the alkanolammonium dodecyl sulfates are promising as a means to clarify the hierarchy of the molecular assemblies of thylakoid proteins.

ACKNOWLEDGEMENTS

The author expresses his thanks to Dr. Toshizo Isemura for his encouragement and helpful comments throughout this doctoral work. He is indebted to Prof. Nobuo Okabe of the Faculty of Pharmacy, Kinki University, for his encouragement with the experiments. He also thanks the people who were once the students of the Laboratory of Phamaceutical Physical Chemistry, Faculty of Pharmacy, Kinki University, for their skilful assistance in the graduation studies. The author expresses his heartfelt thanks to Professor Toshio Takagi of the Division of Physical Chemistry, Institute for Protein Research, Osaka University, for his constant encouragement and guidance throughout this doctoral thesis.

REFERENCES

Abraham, G. & Cooper, P. D. (1976). Anal Biochem. 73, 439-446.

Anderson, J. M., Waldron, J. C. & Thorne, S. W. (1978). FEBS Lett. 92, 227-233.

Bachrach, H. L. & Hess, W. R. (1973). Biochem. Biophys. Res. Commun. <u>55</u>, 141-149.

Banker, G. A. & Cotman, C. W. (1972). J. Biol. Chem. <u>247</u>, 5856-5861.

Birdi, K. & Steinhardt, J. (1978). Biochim. Biophys. Acta <u>534</u>, 219-227.

Blei, I. (1960). J. Colloid Sci. 15, 370-380.

Bornstein, P., Martin, G. R. & Piez, K. A. (1964). Science <u>144</u>, 1220-1221.

Bornstein, P. & Traub, W. (1979). In : <u>The Proteins, Third</u> <u>Edition, Volume IV, pp.411-632.</u> eds. by Neurath, H. & Hill, R. L., Academic Press, New York.

-174-

Carraway, K. L., Lam, A., Kobylka, D. & Huggins, J. (1972). Anal Biochem. <u>45</u>, 325-331.

Chandrakasan, G., Torchia, D. A. & Piez, K. A. (1976). J. Biol. Chem. <u>251</u>, 6062-6067.

Chung, E. & Miller, E. J. (1974). Science 183, 1200-1201.

Dancewicz, A. M. (1975). Nukleonika 20, 867-875.

Delepelaire, P. & Chua, N.-H. (1979). Proc. Natl. Acad. Sci. USA 75, 111-115.

Downer, N. W., Robinson, N. C. & Capaldi, R. A. (1976). Biochemistry 15, 2930-2936.

Dreger, E. E., Keim, G. I., Miles, G. D., Schedlovsky, L. & Ross J. (1944). Ind. Eng. Chem. 36, 610-617.

Dunker, A. K. & Kenyon, A. J. (1976). Biochem. J. 153, 191-197.

Ferguson, K. A. (1964). Metabolism 13, 985-1002.

Flanagan, M. T. & Ainsworth, S. (1968). Biochim. Biophys. Acta 168, 16-26.

Flory, P. J. (1969). <u>Statistical Mechanics of Chain Molecules</u>. pp. 248-274. Wiley, New York.

Frank, R. N. & Rodbard, D. (1975). Arch. Biochem. Biophys. <u>171</u>, 1-13.

Freytag, J. W., Noelken, M. E. & Hudson, B. G. (1979). Biochemistry <u>18</u>, 4761-4768.

Fujimori, E. & Shambaugh, N. (1983). Biochim. Biophys. Acta <u>742</u>, 155-161.

Gamberg, C. G. (1971) Biochim. Biophys. Acta 249, 81-95.

Goddard, E. D. & Benson, G. C. (1957). Canad. J. Chem. <u>35</u>, 986-991.

Greenfield, N. & Fasman, G. D. (1969). Biochemistry 8, 4108-4116.

Hamauzu, Z., Nakatani, N. & Yonezawa, D. (1975). Arg. Biol. Chem. 39, 1407-1410.

Hayashi, T. & Nagai, Y. (1972). J. Biochem. <u>72</u>, 749-758.

Hayashi, T. & Nagai, Y. (1979). J. Biochem. 86, 453-459.

Hayashi, T. & Nagai, Y. (1980). J. Biochem. 87, 803-808.

-176-

Hedrick, J. L. & Smith, A. J. (1968) Arch. Biochem. Biophys. <u>126</u>, 155-164.

Helenius, A. & Simons, K. (1975). Biochim. Biophys. Acta <u>415</u>, 29-79.

Henriques, F. & Park, R. B. (1976). Arch. Biochem. Biophys. <u>176</u>, 472-478.

Jirgensons, B. (1963). J. Biol. Chem. 238, 2716-2722.

Kubo, K., Isemura, T. & Takagi, T. (1975). J. Biochem. <u>78</u>, 349-354.

Kubo, K., Isemura, T. & Takagi, T. (1979). Anal. Biochem. <u>92</u>, 243-247.

Kubo, K., Isemura, T. & Takagi, T. (1980). Anal. Biochem. <u>105</u> 188-191.

Kubo, K., Isemura, T. & Takagi, T.(1982). Biochim. Biophys. Acta 703, 180-186.

Kubo, K. & Takagi, T. (1985). Seikagaku 57, 117-120.

Kubo, K. & Takagi, T. (1986). J. Biochem. <u>99</u>, 1545-1548.

-177-

Kubo, K. & Takagi, T. (1986). Anal. Biochem. 156, in press.

Lewis, M. S. & Piez, K. A. (1964). Biochemistry 3, 1126-1131.

Lopez, R. M. Y. & Siekevitz, P. (1973) Anal. Biochem. <u>53</u>, 594-602.

Mattice, W. L., Riser, J. M. & Clark, D. S. (1976). Biochemistry <u>15</u>, 4264-4272.

Mattice, W. L., Srinivasan, G. & Santiago, G. (1980). Macromolecules <u>13</u>, 1254-1260.

Majewska, M. R. & Dancewicz, A. M. (1977). studia biophysica, Berlin, <u>63</u>, 65-74.

Meredith, S. C. & Kezdy, F. J. (1981). Biochim. Biophys. Acta 668, 357-369.

Meyer, M. L. & Kauzmann, W. (1962). Arch. Biochem. Biophys. <u>99</u>, 348-349.

Miyake, J., Ochiai-Yanagi, S., Kasumi, T. & Takagi, T. (1978). J. Biochem. <u>83</u>, 1679-1686. Mukerjee, P. & Mysels, K. J. (1971). <u>Critical Micelle</u> <u>Concentrations of Aqueous Surfactant Systems U.S. Government</u> <u>Printing Office</u>, Washington.

Nelles, L. P. & Bamburg, J. R. (1976). Anal. Biochem. <u>73</u>, 522-531.

Noelken, M. E. & Bettin, B. D. (1983). Anal. Biochem. <u>134</u>, 347-381.

Noll, H. & Stutz, E. (1968). In : <u>Methods in Enzymology</u> (Grossman, E. & Moldave, K., eds.), Vol. 12(B) pp. 129-155. Academic Press, New York.

Ogawa, T., Obata, F. & Shibata, K. (1966). Biochim. Biophys. Acta 112, 223-234.

Piez, K. A. (1976). In : <u>Biochemistry of Collagen</u> (Ramachandran, G. N. and Reddi, A. H., eds.), pp. 1-40. Plenum Press, New York.

Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963). Biochemistry <u>2</u>, 58-66.

Piez, K. A. (1968). Anal. Biochem. <u>26</u>, 305-312.

Rao, V. S. R. & Foster, J. F. (1963). Biopolymers 1, 527-544.

Reynolds, J. A. & Tanford, C. (1970). J. Biol. Chem. <u>245</u>, 5161-5165.

Rodbard, D. & Chrambach, A. (1970). Proc. Natl. Acad. Sci. USA 65, 970-977.

Rowe, E. S. & Steinhardt, J. (1976). Biochemistry 15, 2579-2585.

Sano, Y., Nozu, Y. & Inoue, H. (1978). Arch. Biochem. Biophys. <u>186</u>, 307-316.

Shapiro, A. L., Vinuela, E. & Maizel, J. V. Jr. (1967). Biochem. Biophys. Res. Comm. <u>28</u>, 815-820.

Shapiro, A. L. & Maizel, J. V., Jr. (1969). Anal. Biochem. <u>29</u>, 505-514.

Shelton, K. R. (1971). Biochem. Biophys. Res. Commun. <u>43</u>, 367-371.

Shinoda, K., Nakagawa, T., Tamamushi, B. & Isemura, T. (1963). In : <u>Colloidal Surfactants</u>, Some Physicochemical properties, pp. 69-74, Academic Press, New York.

Shirahama, K., Tsujii, K. & Takagi, T. (1974). J. Biochem. <u>75</u>, 309-319.

Silver, F. H. & Trelstad, R. L. (1980). J. Biol. Chem. <u>225</u>, 9427-9433.

1

Steinhardt, J., Scott, J. R. & Birdi, K. S. (1977). Biochemistry <u>16</u>, 718-725.

Steinhardt, J., Stocker, N., Carroll, D. & Birdi, K. S. (1974). Biochemistry <u>13</u>, 4461-4468.

Stigter, D. & Mysels, K. J. (1955). J. Phys. Chem. 59, 45-51.

Stoklosa, J. T. & Latz, H. W. (1974). Biochem. Biophys. Res. Comm. <u>58</u>, 74-79.

Stoklosa, J. T. & Latz, H. W. (1975). Anal. Biochem. 68, 358-370.

Strotmann, H., Hesse, H. & Edelmann, K. (1973). Biochim. Biophys. Acta <u>314</u>, 202-210.

Summers, F., Maizel, J. V. & Darnell, J. E. Jr. (1965). Proc. Natl. Acad. Sci. USA 17, 505-513.

Swank, R. T. & Munkres, K. D. (1971). Anal. Biochem. <u>39</u>, 462-477.

Takagi, T. (1981). J. Chromatogr. 219, 123-127.

-181-

Takagi, T. & Isemura, T. (1960). Bull. Chem. Soc. Japan <u>33</u>, 437-441.

Takagi, T., Tsujii, K. & Shirahama, K. (1975a). J. Biochem. <u>77</u>, 939-947.

Takagi, T., Kubo, K., Asakura, J. & Isemura, T. (1975b). J. Biochem. <u>78</u>, 1297-1300.

Takagi, T., Kubo, K. & Isemura, T. (1977). Anal. Biochem. <u>79</u>, 104-109.

Takagi, T. & Kubo, K. (1979). Biochim. Biophys. Acta 578, 68-75.

Takagi, T., Kubo, K. & Isemura, T. (1980). Biochim. Biophys. Acta 623, 271-279.

Takagi, T., Takeda, K. & Okuno, T. (1981). J. Chromatogr. <u>208</u>, 201-208.

Tanford, C. (1961). <u>Physical Chemistry of Macromolecules</u>, pp. 407-411, Wiley and Sons, New York.

Tanford, C. (1968). Adv. Protein Chem. 23, 121-282.

Tanford, C. (1973). <u>Hydrophobic Effect</u>, pp. 170, Wiley and Sons, New York.

Tsujii, K. & Takagi, T. (1975a). J. Biochem. 77, 117-123.

Tsujii, K. & Takagi, T. (1975b). J. Biochem. 77, 511-519.

Tung, J.-S. & Knight, C. A. (1971). Biochem. Biophys. Res. Commun. <u>42</u>, 1117-1121.

Tung, J.-S. & Knight, C. A. (1972). Anal. Biochem. <u>48</u>, 153-163.

Van Kampen, E. J. & Zijlstra, W. G. (1961). Clin. Chim. Acta <u>6</u>, 538-544.

Wallace, R. W., Yu, P. H., Diekert, J. P. & Diekert, J. W. (1974). Anal. Biochem. <u>61</u>, 86-92.

Weber, K. & Osborn, M. (1969). J. Biol. Chem. 244, 4406-4412.

Weber, L., Meigel, W. N. & Rauterberg, J. (1977). Arch. Derm. Res. <u>258</u>, 251-257.

Williams, J. G. & Gratzer, W. B. (1971). J. Chromatogr. <u>57</u>, 121-125.

Wright, A. K., Thompson, M. R. & Miller, R. L. (1975). Biochemistry <u>14</u>, 3224-3228.

-183-