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Author(s)	Maezawa, Shigenori
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Assessment Study on the Application of the Low-angle Laser Light Scattering Technique to the Characterization of Membrane Proteins Solubilized by Surfactants

A Doctoral Thesis
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1984

Shigenori Maezawa

Division of Physical Chemistry
Institute for Protein Research
Osaka University

Approvals

This thesis is approved
as to style and content

by

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Member-in-Chief

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前澤重禮
Shigenori Maezawa

Tokushima
July, 1984

CONTENTS

1. Introduction

1-1 Introductory remarks

In this thesis, the author wishes to present and discuss results of the "assessment study on the application of the low-angle laser light scattering (LALLS) technique to the characterization of membrane proteins solubilized by surfactants". In this chapter, the author would like to point out the reasons why the theme described above was taken up.

1-1-1 Why low-angle laser light scattering technique ?

Determination of molecular weight and its distribution is undoubtedly major items in the characterization of biological macromolecules. Prominent scientists have made their full efforts to develop methods for determination of molecular weights of biological macromolecules. Namely, Svedberg and Rinde (1924) were the first who developed an analytical ultracentrifuge, and demonstrated that proteins have definite molecular weights. Adair (1928) determined molecular weights of various proteins by osmometry. They have contributed not only to figure out the nature of protein molecules but also to foster the concept of macromolecules. Since then, these physicochemical techniques have been extensively utilized in determination of molecular weights of

biological macromolecules.

Light scattering technique was demonstrated also to be effective for the above purpose by Debye (1944). The technique, however, failed to settle in the field of biochemistry. This was largely due to the "dust problem". Namely, in a conventional light scattering technique, enormous enduring effort was required to make sample solutions as well as solvents free from dust particles. If present, they introduce great errors in molecular weight determination. The problem was especially serious in the field of biochemistry where one was forced to use aqueous solvents which were hardly made free from dust particles and bubbles.

Innovations in the light scattering technique itself as well as in various peripheral techniques seem to have changed the above situation. Among them, the technique called under the name of low-angle laser light scattering technique is worthy of special attention. It started from the development by Kaye et al. (1971) of a novel type of light scattering photometer called a "low-angle laser light scattering photometer" after its unique optics. The photometer passed under an abbreviated name of "LALLS" photometer. It has several advantages over a conventional light scattering photometer as will be described later. A LALLS photometer is a particularly useful detector in gel permeation chromatography (GPC). The technique monitoring of elution from a GPC column using a LALLS photometer and

supplementary equipments will be abbreviated to the GPC/LALLS technique. Kotaka (1977) has treated the averaged molecular weights estimated by the GPC/LALLS technique theoretically for macromolecules with molecular weight distribution. It has proved its merits in the field of research on synthetic polymers where organic solvents were generally used. Use of the photometer as a detector in GPC has been outside the reach of people in the field of research on biological macromolecules. It was mostly due to the lack of GPC column suitable for use of aqueous solvents. The situation was improved very recently by the development of gel permeation columns suitable for aqueous solvents (Kato et al., 1980). Use of a LALLS photometer as a detector for aqueous GPC, however, has attracted least attention. The research field of biochemistry seems to be prevailed by an allergy against light scattering techniques due to the malfunction of the conventional light scattering technique experienced two decades ago. We planned to put the LALLS technique to practical use in the field especially for characterization of membrane proteins.

1-1-2 Why membrane proteins ?

The fundamental structure common to most of the biomembranes is phospholipid bilayer. The bilayer functions not only as a barrier dividing the interior from

the outer environment but also as a substructure to which various proteins adhere or are embedded. Such proteins are called "membrane proteins", and the general feature of their presence in the membrane has been figured out beautifully by Singer and Nicolson (1972) as the "fluid mosaic model" (Fig.1-1).

Membrane proteins are classified into two categories on the basis of the mode of interaction with the lipid bilayer (Fig.2-2). Some of the proteins are loosely bound to the surface of the bilayer and are readily soluble in aqueous solvents. Such proteins are called peripheral membrane proteins. On the other hand, a majority of membrane proteins are embedded in the bilayer and often traverse it. Such proteins are called integral membrane proteins, and bound strongly to the bilayer. Integral membrane proteins are mostly insoluble in aqueous solvents due to their hydrophobic nature. Accordingly, characterization of integral membrane proteins is extremely difficult compared to that of most of the water-soluble proteins. Solubilization of the membrane proteins by use of surfactants is a breakthrough leading to their characterization.

1-1-3 Why surfactants ?

A part of an integral membrane protein molecule is embedded in the phospholipid bilayer, and the other is

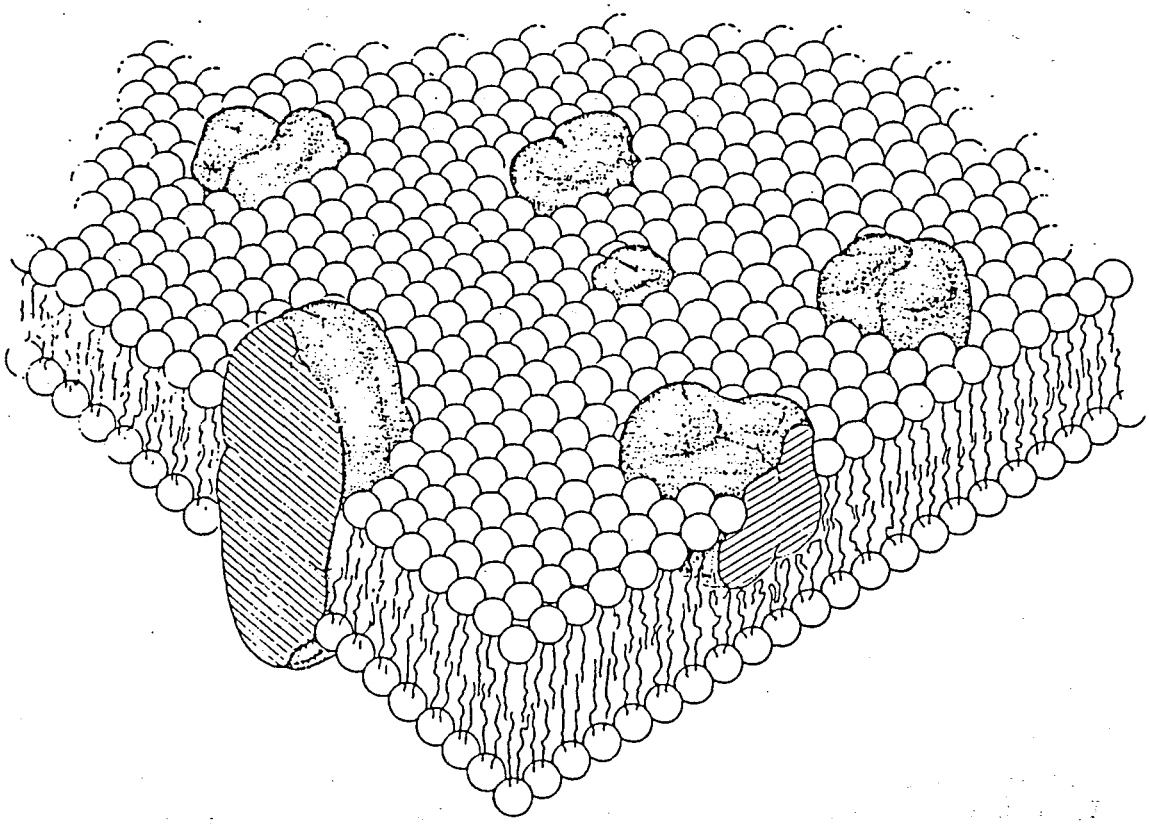


Fig.1-1 Fluid mosaic model (Singer and Nicolson, 1972)

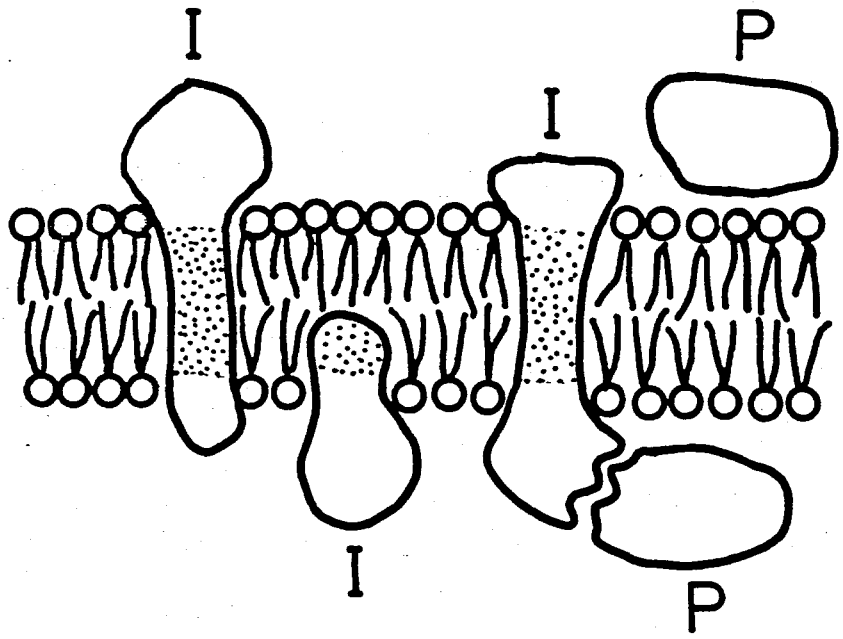


Fig.1-2 Sketch of the membrane proteins in a biomembrane. I, integral membrane proteins; P, peripheral membrane proteins.

exposed to the aqueous medium. The first step in characterization of the membrane proteins is their separation from the membrane and purification in an active form. The membrane proteins are extracted by disruption of membrane and solubilization of the membrane components by appropriate solubilizing agents. The most effective and extensively used ones are surface active agents (surfactants). Other procedures, involving the use of organic solvents or chelating agents, manipulation of ionic strength or pH, enzymatic digestion, do not lead to effective solubilization of the membrane proteins, particularly those tightly bound to the lipid matrix of membranes. The membrane proteins coexist with phospholipid in a membrane as shown in Fig.1-2. Therefore, they should preferably be characterized in a form of complex between proteins and lipids. However, since the complexes are usualy insoluble in water, they are not suitable for physicochemical characterization by conventional techniques such as ultracentrifugation, light scattering, and various spectroscopic methods. These techniques generally require that the proteins are dispersed in a form of either a single molecule or an assembly with a stoichiometric composition. It becomes often necessary to solubilize membrane proteins without disruption of their native structures in the membrane and loss of the biological activity. In this case, the solvent medium after solubilization must be able to simulate the native

environment of the proteins. A surfactant with an appropriate molecular structure can mimic the phospholipid in the bilayer.

A surfactant has a characteristic molecular structure consisting two distinct regions: one is hydrophobic moiety and the other is hydrophylic one. Namely a surfactant is "amphiphilic". Both surfactant and lipid are amphiphiles, but the former is water-soluble and the latter is generally water-insoluble. Thus phospholipids are inadequate as media for physicochemical characterization. On the other hand, aqueous solutions of appropriate surfactants can serve as adequate media for the above purpose.

The ability of surfactants to solubilize membrane proteins depends on their amphiphilic properties. Surfactants with appropriate characteristics can be synthesized intentionally. The solubilized state of membrane proteins thus can be controlled by the use of appropriate surfactants.

Surfactants can be classified into two categories according to the mode of their interaction with proteins. One is called "denaturing" surfactants which bind to proteins and subsequently denature them. The solubilization of a membrane protein consisting of two or more subunits by a denaturing surfactant usually accompanys dissociation into the subunits, and the dissociation often leads to denaturation of the subunits.

The other is called "non-denaturing" surfactants. Solubilization of membrane proteins by the non-denaturing surfactants accompanys no major conformational change and loss of activity of the proteins (Helenius and Simons, 1975). Typical surfactants belonging to the former and the latter categories are ionic surfactants and nonionic ones, respectively. The action modes of these two types of surfactants on integral membrane protein are depicted schematically in Fig.1-3. The use of surfactants for solubilization of membrane proteins and as a solvent medium for membrane proteins has been reviewed in detail by Helenius and Simons (1975) and Tanford and Reynolds (1976). Properties of surfactants used to solubilize membrane proteins have been summarized by Helenius et al. (1979).

A choice of a suitable surfactant is one of the crucial points in the characterization of membrane proteins. Characterization of membrane proteins in native-like state where original activity or some other native properties are preserved is performed in the presence of a non-denaturing surfactant. On the other hand, dissociation of membrane protein into each constituent subunit is accomplished in the presence of a denaturing surfactant. It is, therefore, very important to choose a surfactant which fits best for one's purpose.

1-1-4 Scope of this study

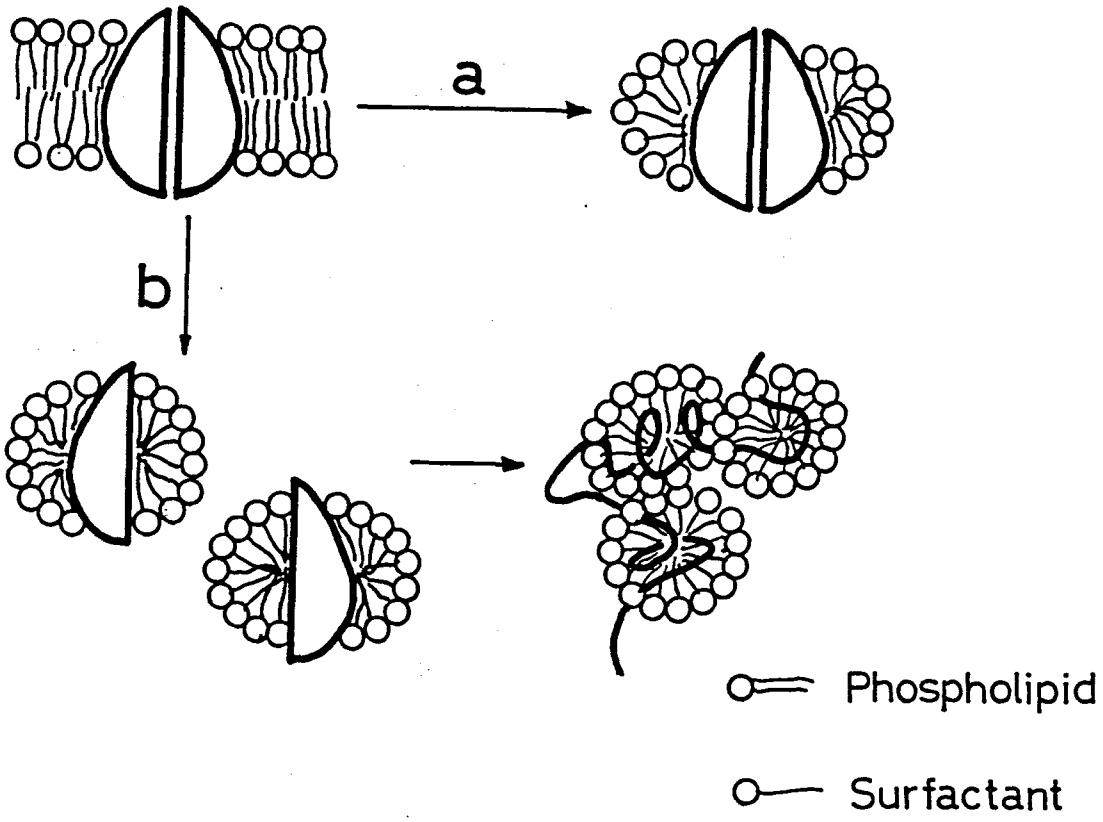


Fig.1-3 Schematic drawing of action modes of (a) non-denaturing surfactant and (b) denaturing surfactant on integral membrane proteins.

A membrane protein, particularly a transmembrane one, is often consisted of several subunits held together by non-covalent forces. It is very important in characterization of a membrane protein to clarify what kinds of and how many such subunits are assembled to construct it.

As described above, membrane proteins generally retain their intact structures when solubilized by non-denaturing surfactants, but are dissociated into individual subunits when solubilized by denaturing surfactants.

The purposes of this study are as follows: 1) establishment of a GPC/LALLS technique as a means to study molecular assembly of membrane proteins; 2) estimation of molecular assembly of the minimum functional unit of the (Na^+,K^+)-ATPase of canine kidney by the GPC/LALLS technique.

In order to achieve above purposes, it is necessary to determine not only the molecular weight of an intact membrane proteins solubilized by non-denaturing surfactants but also molecular weight of each polypeptide composing it. Accordingly it is necessary to carry out an assessment study of the use of the GPC/LALLS technique not only in the presence of non-denaturing surfactants but also in the presence of denaturing surfactants.

This thesis consists of five chapters. In the rest of this introductory chapter, the general concepts in

estimation of protein molecular weight and the principles underlying the experimental procedures employed in this thesis will be briefly summarized. In chapter 2, results of the assessment study on the use of the GPC/LALLS technique in the presence of a non-denaturing surfactant, octaethyleneglycol n-dodecyl ether, will be described using membrane proteins which are easy to handle. In chapter 3, the solubilized states of the (Na^+,K^+)-ATPase in the presence of the above non-denaturing surfactant will be discussed. In chapter 4, an application of the GPC/LALLS technique to glycoproteins will be described. In chapter 5, results of characterization of the (Na^+,K^+)-ATPase solubilized by denaturing surfactant, sodium dodecyl sulfate, will be described. Possible perturbing effects on molecular weight determination of coexisting SDS micelles will also be discussed.

1-2 Estimation of molecular weights of membrane proteins

The first step in the characterization of a membrane protein is its purification. The next step is obviously measurements of molecular weight of the protein. One can roughly estimate overall size of the protein from the molecular weight. Although one can never discuss the nature of the protein from the molecular weight value alone, the value is undoubtedly one of the most impotant properties of the protein.

Since membrane proteins are usually solubilized by surfactants and form complexes with them as mentioned in section 1-1-3, molecular weights of the proteins should be measured in surfactant solutions. Therefore, it is necessary to afford techniques by which the molecular weight of protein moiety of protein-surfactant complex can be determined.

1-2-1 Techniques using molecular sieves (Empirical methods)

Typical examples of the techniques using molecular sieves are GPC and gel electrophoresis. These techniques have been extensively used because they have an advantage that the molecular size of each solute in a mixture can be easily estimated simultaneously in a single run.

GPC and gel electrophoresis techniques yield only a single experimental parameter, i.e., retention volume and

electrophoretical mobility, respectively. Each of the two parameters is related to effective hydrodynamic size of the solute, which is a function of molecular mass, shape, and extent of solvation. Therefore, estimation of molecular weight of a protein by such a technique becomes effective only when the hydrodynamic size of each polypeptide is a unique function of its chain length. A dissociating and denaturing solvent system is the most suitable to satisfy the above requirement. Furthermore, intra-chain covalent crosslinkings (e.g., disulfide bonds) of the protein must have been cleaved to give a linear single polypeptide chain (reduced polypeptide chain).

Molecular weight of a protein can be measured by GPC in the presence of a denaturing agent such as guanidine hydrochloride, urea or sodium dodecyl sulfate (SDS) and by polyacrylamide gel electrophoresis in the presence of SDS (Shapiro et al., 1967; Weber and Osborn, 1969; Reynolds and Tanford, 1970). However, molecular weights of some proteins cannot be correctly measured by the above techniques. Proteins reduced in the presence of a denaturant do not necessarily take the same conformations.

The conformation of a protein polypeptide assumed in a denaturing condition varies due to following reasons. 1) Some proteins do not denature completely. This might arise as a result of stability against denaturation either in the sense of thermodynamics or kinetics. 2) The mode and extent of interaction with a denaturant are various.

This is manifested in the case of denaturation of a membrane protein with a surfactant. 3) Extent of cleavages of intra- and inter-molecular disulfide linkages is various. 4) Some proteins such as a glycoprotein have side chains of non-polypeptide nature. The empirical methods give satisfactory results only for denatured linear polypeptide chains. Therefore, glycoproteins are outside the range of these techniques.

The empirical methods for molecular weight estimation are only reliable in cases where hydrodynamic radii of the proteins concerned are a unique function of molecular weight.

1-2-2 Ultracentrifugal technique

Physicochemical techniques available for determination of molecular weights of proteins include ultracentrifugal analysis, light scattering photometry and osmometry. They stand on sound theoretical bases. Among the techniques, the ultracentrifugal analysis has been the only one available for determination of molecular weights of membrane proteins. The others are not applicable to this class of proteins due to experimental difficulties.

A sample solution for measurement of protein molecular weight generally contains water, protein and the third component such as a salt, and thus is a three component system. The following equation based on the

theory of three component system (Cassasa and Eisenberg, 1964) must be used:

$$(2RT/\omega^2)(d\ln c_2/dr^2) = M_2[(1-\bar{v}_2\rho_0) + (1-\bar{v}_3\rho_0)(\partial m_3/\partial m_2)_{\mu_3} M_3/M_2] \quad (1\text{-}1)$$

where c_2 is the concentration of protein experimentally determined at radial position r in the cell; ω, angular velocity of rotor; R, gas constant; T, absolute temperature; ρ_0, density of the solvent; M_2, the molecular weight of the protein; $\bar{v}_2$, partial specific volume of the protein; m_2, molal concentration of the protein; M_3, $\bar{v}_3$, and m_3 are the corresponding molecular parameters for the third component, and $(\partial m_3/\partial m_2)\mu_3$ is an expression of the interaction between the protein and the solvent system components.

The right hand side of Eq.1-1 may be simplified into a function of the effective partial specific volume, Φ_2' (Cassasa and Eisenberg, 1964).

$$\Phi_2' = \bar{v}_2 - (1/\rho_0 - \bar{v}_3)(\partial m_3/\partial m_2)_{\mu_3} M_3/M_2 \quad (1\text{-}2)$$

The above equation can be transformed into following equation

$$(1-\Phi_2'\rho_0) = (1-\bar{v}_2\rho_0) + (1-\bar{v}_3\rho_0)(\partial m_3/\partial m_2)_{\mu_3} M_3/M_2 \quad (1\text{-}3)$$

thus, the Eq.1-1 becomes

$$(2RT/\omega^2)(d\ln c_2/dr^2) = M_2(1-\Phi_2'\rho_0) \quad (1\text{-}4)$$

The effective partial specific volume of the protein moiety, Φ_2', includes the contribution of bound third component. As described in section 1-1-3, membrane proteins are insoluble in water and generally solubilized by the formation of complexes with surfactant molecules. Therefore, molecular weight estimation of membrane protein must be done in a surfactant solution. Lipids derived from the source are often included in the complex. In such a case, the quantity Φ_2' in Eq.1-4 can be measured directly by determining the density of protein solutions that have been dialyzed to equilibrium against a surfactant solution of the desired concentration. This procedure requires great care and relatively large amounts of protein. Protein concentration must be determined precisely. A more convenient procedure than measurement of Φ_2' was given by Tanford et al. (1974). It is to use a calculated buoyant density factor, as was first done for protein-surfactant complexes by Hersh and Schachman (1958). The factor $M_2(1-\Phi_2'\rho_0)$ of Eq.1-4 is replaced by

$$M_2(1-\Phi_2'\rho_0) = M_2[(1-\bar{v}_2\rho_0)+\Sigma\,\delta_i(1-\bar{v}_i\rho_0)] \qquad (1-5)$$

where δ_i is the amount of bound component i in g per g of protein and $\bar{v}_i$ is the partial specific volume of the component i when bound to the protein. The value δ_i is determined by other methods. For example, the amount of bound surfactant can be determined by equilibrium dialysis or equilibration on a chromatographic column (Tanford

et al., 1974). The value $\bar{v}_i$ for a pure component is easily determined by densitometry (Tanford et al., 1974) and has been tabulated for a large number of surfactants (Steele et al., 1978). Thus, the molecular weight of membrane protein solubilized by a surfactant can be estimated by the following equation without measurement of Φ_2', provided that the value of $\bar{v}_2$ is known.

$$(2RT/\omega^2)(d\ln c_2/dr^2) = M_2[(1-\bar{v}_2\rho_0)+\Sigma\,\delta_i(1-\bar{v}_i\rho_0)] \qquad (1\text{-}6)$$

The value of $\bar{v}_2$ can be estimated on the basis of amino acid composition (Cohn and Edsall, 1943; McMeekin and Marshall, 1952) in a good approximation. It can be assumed that the value of $\bar{v}_i$ is not altered appreciably on the binding to a protein. Thus, the value of $\bar{v}_i$ determined for free state can be used. Even if δ_i is unknown, it is possible to obtain M_2 by determining $(1-\Phi_2'\rho_0)$ as a function of solvent density using mixture of D_2O and H_2O (Reynolds and Tanford, 1976). When ρ_0 equals to $1/\bar{v}_i$, the buoyant density term due to the component i, $(1-\bar{v}_i\rho_0)$, equals to zero and M_2 is obtained from Eq.1-6 without direct knowledge of surfactant binding.

Molecular weight of a membrane protein solubilized by a nonionic surfactant thus can be estimated by the sedimentation equilibrium technique. The procedures are, however, quite laborious. For example, determination of the amount of a nonionic surfactant bound to a membrane protein is extremely difficult. In case of ionic

surfactants, another factor introduces difficulty. Namely, a protein polypeptide with a large amount of bound ionic surfactant behaves like a polyelectrolyte chain. Such a complex and supporting electrolytes, thus, share co-ions. Thus, the concentration gradient of the complex and that of the supporting electrolytes under an ultracentrifugal field are hardly independent of each other. The complex consequently becomes repelled from the lower layer in the cell. This leads to an underestimation of molecular weight of the protein polypeptide. A technique alternative to the equilibrium ultracentrifugation is required to determine molecular weights of membrane proteins without difficulty and irrespective of the nature of surfactant used.

1-3 Gel permeation chromatography (GPC)/low-angle laser light scattering (LALLS) technique

Light scattering technique is a well-established one for characterizing macromolecules in solution. Molecular weight can be estimated accurately by this technique. In addition, the size and shape of a macromolecule can be also estimated approximately, if it is large enough compared to the wavelength of incident light. In light scattering experiment, the solution and solvent used must be critically clean to be free from dusts and air-bubbles which spoil the measurements. Conventional light scattering measurements can be carried out without trouble as far as an organic solvent is used. On the other hand, measurements using aqueous solvents are generally uncomparably difficult, because of the difficulty in purification of aqueous solvents for making them free from dusts and air-bubbles. People in the protein chemistry are, therefore, not willing to use the light scattering technique.

Recently, laser became available as a light source suitable for light scattering measurement. Laser beam is highly monochromatic and has high power density. A laser is, therefore, an efficient light source for light scattering measurement. As one of the major developments in this line, Kaye et al. (1971) developed a new type of light scattering photometer equipped with an unique optics

which is quite different from that of a conventional light scattering photometer. This new type of photometer was named "low-angle laser light scattering (LALLS) photometer", in which laser was used as light source and the light scattered forward at an angle around 4° was detected.

1-3-1 Background of the light scattering technique

In 1871, Rayleigh first investigated the phenomenon of light scattering quantitatively. He evaluated the scattering from a dilute gas composed of small isotropic molecules, and could interpret why the sky was blue. Intensity of scattered light from pure liquid is very low. Smoluchowski (1908) treated this problem by means of a fluctuation theory based on the premise that the density at any particular microscopic region should fluctuate. Debye (1944) explained light scattering of macromolecular solutions using a thermodynamic theory as a result of fluctuation in concentration and showed that the molecular weight of macromolecules could be determined. Halwer et al. (1951) demonstrated that molecular weights of several water-soluble proteins could be precisely evaluated by this method. The outline of light scattering phenomenon and a complete derivation of the related equations are found in an excellent textbook by Tanford (1961).

When a monochromatic light beam passes through a solution of macromolecule, the intensity i_θ of light scattered at an angle θ can be expressed as follows,

$$\frac{i_\theta}{I_0} = \frac{2\pi^2 n_0^2 (dn/dc)^2 (1+\cos^2\theta) c}{N \lambda^4 r^2 (1/M + 2Bc + \cdots\cdots) 1/P_\theta} \tag{1-7}$$

$$\lim_{\theta \to 0} P(\theta) = 1-(16\pi^2/3\lambda^2) R_g^2 \sin^2(\theta/2) \tag{1-8}$$

where I_0 is the intensity of the incident beam; i_θ, the intensity of the scattered light; n_0, the refractive index of the solvent; dn/dc, the specific refractive index increment of the macromolecule; θ, the scattering angle; c, the weight concentration of the macromolecule; N, Avogadro number; λ, the wavelength; r, the distance between the scattering point and the observer; M, the molecular weight of the macromolecule; B, the second virial coefficient; $P(\theta)$, the particle scattering function defined as Eq.1-8; and Rg, radius of gyration of the macromolecule. Eq.1-7 is one of the most practical equations used in the study of light scattering.

In Eq.1-7, parameters related to optical constants and those to optical geometries are put together as in Eqs.1-9 and 1-10, respectively.

$$K = \frac{2\pi^2 n_0^2 (dn/dc)^2}{N \lambda^4} \tag{1-9}$$

$$R_\theta = \frac{i_\theta r^2}{I_0 (1 + \cos^2\theta)} \tag{1-10}$$

The fundamental equation of light scattering then becomes

$$\frac{K \cdot c}{R_\theta} = (1/M + 2Bc + \cdots) \frac{1}{P(\theta)} \qquad (1\text{-}11)$$

$P(\theta)$ can be considered to be 1 for the particles whose longest dimention is enough less than the wavelength of the incident light, because the electric field strength is homogeneous over the entire particle, and the particle behaves like a point dipole.

In practice, the molecular weight can be calculated from the value of $K \cdot c/R_\theta$ at infinite concentration. It is, therefore, necessary to measure R_θ in a range of concentration. A plot of $K \cdot c/R_\theta$ against c should give a straight line and extrapolate to 1/M. The slope of this line is equal to the second virial coefficient, 2B, which is the term of interaction of the macromolecule with other component.

1-3-2 Outline of the LALLS photometer

A low-angle laser light scattering (LALLS) photometer has a unique optics and is consequently free from experimental difficulties pertinent to a conventional light scattering photometer.

Fig.1-4 illustrates essential feature of the optics of the LALLS photometer we have used. Let us follow the phenomena occuring in the LALLS photometer: 1) incident

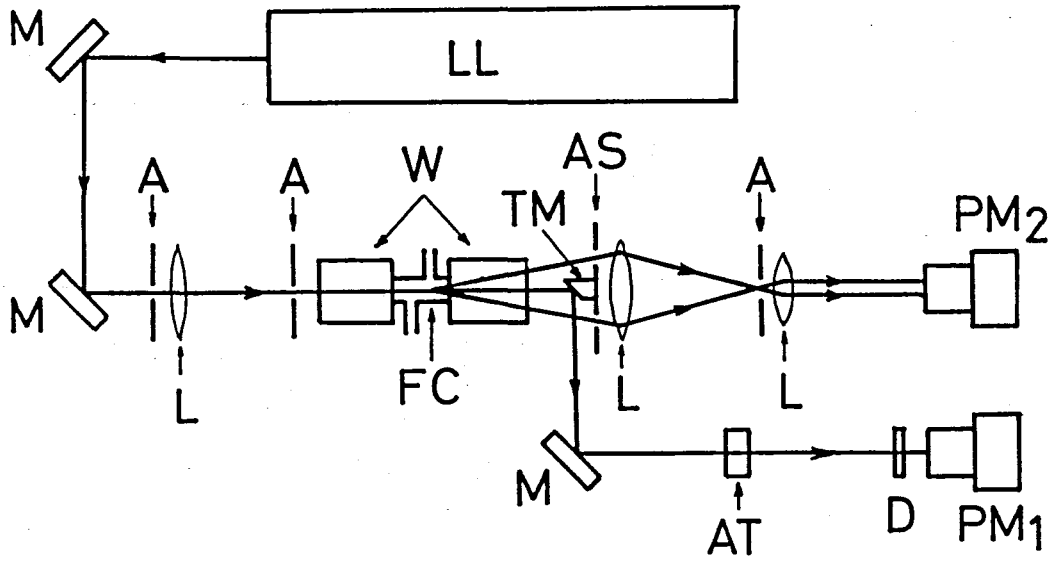


Fig.1-4 Outline of the optics of the low-angle laser light scattering photometer of TSK LS-8. LL, laser light source; M, mirror; A, aperture; L, lense; W, cell window; FC, flow-type cell; TM, trap mirror; AS, annular slit; AT, attenuator; D, diffuser; PM_1 and PM_2, photomultipliers.

light from a laser light source (LL) is well-focussed in a flow-type cell (FC); 2) straight proceeding light passing through the cell is successfully reflected by about 1 % on a trap mirror (TM) in front of an annular slit (AS) to reach a photomultiplier (PM_1); 3) light scattered at a very low scattering angle passes through the annular slit (AS) and is detected by another photomultiplier (PM_2). The annular slit facilitates to collect light scattered to the forward direction at a scattering angle near 4° in a high yield. A pair of cell windows (W) made of quartz glass is set on both sides of the flow-type cell (FC) and prevents stray light generated at air/glass boundaries from reaching the photomultiplier (PM_2). The sketch of vital part of this unique optics is shown in Fig.1-5.

LALLS photometer is equipped with a flow-type cell with a very small internal volume (ca. 30 μl) and can be used as a detector to monitor elution from GPC column. Scattering volume in the flow-type cell is as small as 0.1 μl. Therefore, even if undesirable foreign particles or air-bubbles penetrate into the cell, they merely give spike-like noizes which are easily distinguished from original signals, and immediately flow out from the cell. This photometer is well designed so that it works with high sensitivity to allow measurement for a sample solution with very low concentrations. Thus, the contribution of the second and higher virial coefficients in the parenthesis of Eq.1-7 can be ignored and

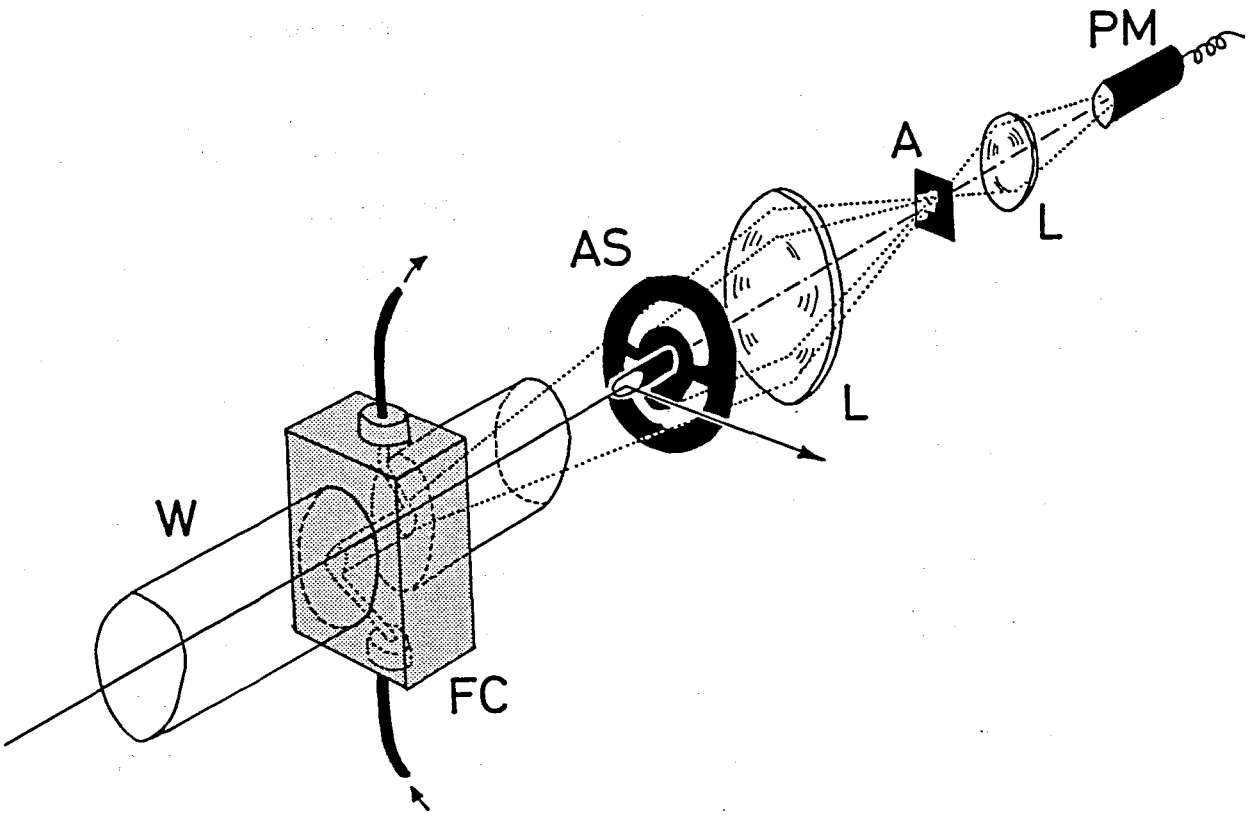


Fig.1-5 Schematic diagram illustrating how the scattering light is detected in the low-angle laser light scattering photometer of TSK LS-8.

extrapolation of $K \cdot c/R_\theta$ to zero concentration is unnecessary. The scattering angle as small as 4° is the most significant characteristics of this scattering photometer. Thus, the correction factor for the interference effect, $P(\theta)$, becomes close to unity even for a relatively large particle compared with wavelength of the incident light.

Accordingly, the Eq.1-7 can be simplified as follows

$$\frac{i_\theta}{I_0} = \frac{2\pi^2 n_0^2}{N \lambda^4 r^2} \cdot \frac{(dn/dc)^2 \; c}{1/M} \tag{1-12}$$

and by assembling the constant parameters, Eq.1-12 can be further simplified to

$$i_\theta / I_0 = k \, M \, (dn/dc)^2 \, c \tag{1-13}$$

where k can be taken as a constant depending on instrumental and experimental conditions, and can be calibrated by measurements with samples of known molecular weights. The ratio, i_θ/I_0, is proportional to the output of the scattering photometer, $(\text{Output})_{LS}$. Thus, the molecular weight can be expressed as follows,

$$M = k' \frac{(\text{Output})_{LS}}{(dn/dc)^2 \; c} \tag{1-14}$$

where k' is instrument constant.

1-3-3 Use of a differential refractometer and a spectrophotometer combined with a LALLS photometer

The molecular weight of a protein can be estimated by measuring output of the scattering photometer, $(Output)_{LS}$, with the specific refractive index increment and the concentration of the protein, as is clear from Eq.1-14. The product of the specific refractive index increment and concentration is equivalent to the difference in the refractive indices between the solution and the solvent, Δn, that is,

$$(dn/dc)\cdot c = \Delta n \tag{1-15}$$

The output of differential refractometer, $(Output)_{RI}$, is considered to be proportional to Δn, i.e.

$$(Output)_{RI} \propto \Delta n \tag{1-16}$$

Eq.1-14 can, thus, be rewritten as follows;

$$M = k'' \frac{(Output)_{LS}}{(Output)_{RI}} \cdot \frac{1}{(dn/dc)} \tag{1-17}$$

where k'' is a instrument constant.

In order to use Eq.1-17 for molecular weight determination, the sample solution is required to be in osmotic equilibrium with the buffer used. Although the equilibrium can be generally established by dialysis against the appropriate buffer solution, the equilibrium

can also be attained by gel filtration of the sample solution. When the sample is applied to GPC column and the elution is monitored by both a LALLS photometer and a differential refractometer, the molecular weight of the sample, whose dn/dc is known, can be estimated using Eq.1-17. The value k'' in Eq.1-17 must be determined in advance by performing similar experiment for standard proteins.

Specific refractive index increments are determined by a conventional batch-type differential refractometer on the solvent and solution thoroughly equilibrated with the former. Measurement of dn/dc by the conventional technique as mentioned above is a time-consuming process and accompanied by loss of samples. When the elution from the GPC column is monitored by a spectorophotometer, the output, $(\text{Output})_{UV}$, is given as follow,

$$(\text{Output})_{UV} \propto c / E \tag{1-18}$$

where E is the extinction coefficient at the wavelength used, generally, 280 nm. Specific refrative index increment is, thus, expressed as follows;

$$dn/dc = k''' \; E \; (\text{Output})_{RI} / (\text{Output})_{UV} \tag{1-19}$$

where k''' is constant. Substituting Eq.1-19 for dn/dc in Eq.1-17, following equation is obtained;

$$M = K \frac{(\text{Output})_{LS} \; (\text{Output})_{UV}}{(\text{Output})_{RI}^{2} \; E} \tag{1-20}$$

where K is constant.

Accordingly, the molecular weight of a protein can be determined easily by monitoring elution from a GPC column by a LALLS photometer, a flow-type precision differential refractometer and a spectrophotometer connected in series. Outline of this measuring system is shown in Fig.1-6. Fig.1-7 shows three elution curves obtained for bovine serum albumin by the measuring system shown in Fig.1-6. They may be helpful to illustrate features of the output of the LALLS photometer in comparison with those of the others. Displacement observed among positions of the corresponding peaks is due to the difference in positions of the detectors along the flow path and the pens on the recorder charts. For proteins without molecular weight distribution, the height of each peak can be taken as a measure of the output of the corresponding detector.

Peaks designated as "2" and "3" can be assigned to dimer and monomer of bovine serum albumin, respectively. Obviously they should have the same values of dn/dc and extinction coefficient. For either of the tracings by the refractometer and the spectrophotometer, the ratio of the height of peak 2 to peak 3 is 1:10. On the other hand, the ratio in the tracing of the scattering photometer is 1:5. The above difference in the output ratios clearly exemplifies that the LALLS photometer functions as a detector giving output proportional to the product of molecular weight and weight concentration. Peak 1 can be

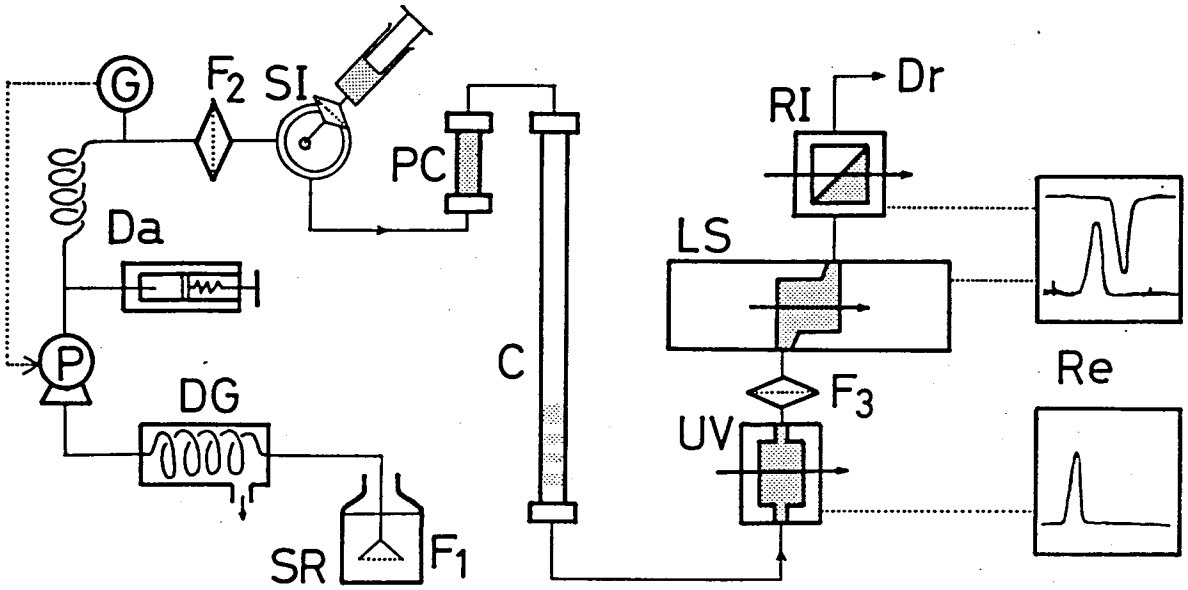


Fig.1-6 Schematic drawing of the arrangement of instruments used in the GPC/LALLS technique. SR, solvent reservoir (3.5 l); F_1, sintered stainless-steel filter (Umetani Seiki, Model SFY); DG, degasser (Elma Optical, Model ERC-3310); P, high pressure pump (Milton-Roy, Model S-100 and helically coiled stainless-steel tube(2m x 0.2mm I.D.)); G, pressure gauge with a provision for switching off the pump when the pressure exceeds the value permissible for the column; F_2, sintered stainless-steel filter (Umetani Seiki, Model SLF); SI, syringe-loading sample injector (Rheodyne, Model 7125) with a loop of volume 100 µl; PC, pre-column (Toyo Soda, TSK-GEL GSWP, 10 x 0.75 cm); C, column (Toyo Soda, TSK-GEL G3000SW, 60 x 0.75 cm); F_3, ultrafilter with a pore size of 0.5 µm (Millipore, type FHLP 01300); UV, ultraviolet spectrophotometer (Toyo Soda, TSK UV-8, Model II); LS, low-angle laser light scattering photometer (Toyo Soda, TSK model LS-8, He-Ne laser); RI, precision differential refractometer (Toyo Soda, TSK Model RI-8); Re, double-pen recorder; Dr, drain.

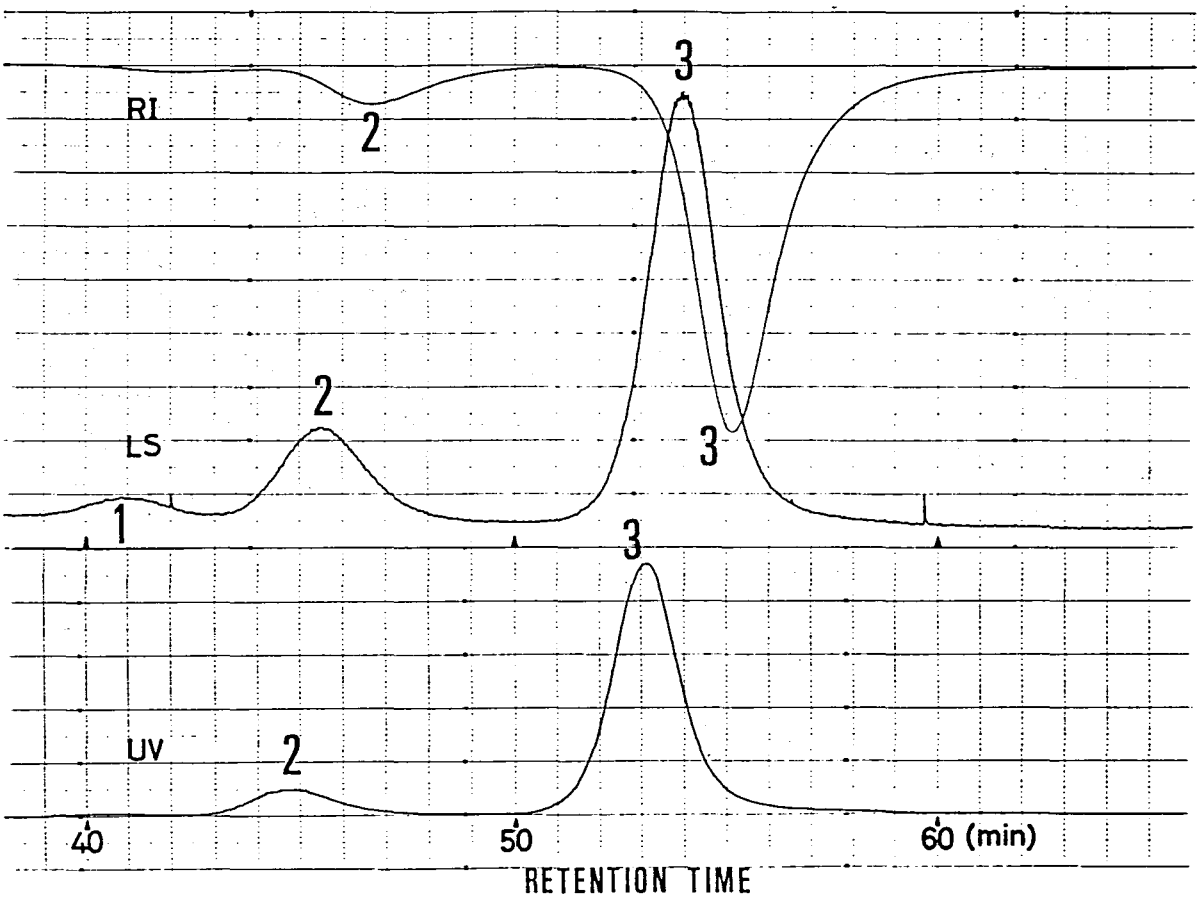


Fig.1-7 Elution patterns of bovine serum albumin obtained using measuring system shown in Fig.1-6. The three curves are, from the bottom to top, the tracings obtained by the spectrophotometer (UV), the LALLS photometer (LS) and the differential refractometer (RI), respectively. The sample contained 400 µg of bovine serum albumin in 100 µl of eluant. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4 (pH 7) containing 3 mM sodium azide. Gain setting: spectrophotometer, 0.64 fs; LALLS photometer, 32; refractometer, 64. Flow rate: 0.31 ml/min. Temperature: 25°C.

assigned to a mixture of materials eluted at the void volume of the column. Large aggregates of bovine serum albumin may be eluted at this position. However, the deflection in the refractometer output more sensitive than that in the spectrophotometer suggests that the peak 1 includes materials (often called "micro gel") other than the aggregates. Performance of the measuring system will be discussed in chapter 4.

In dealing with membrane proteins, one must use a surfactant in order to dissolve the given protein in aqueous media. Non-denaturing surfactants are often used to solubilize membrane proteins in biologically active forms. On the other hand, denaturing surfactants are used to investigate the properties of the constituent subunits. In both cases, surfactant molecules obviously bind to the protein in the solubilizing process to form complexes with it.

Molecular weight and concentration of the protein-surfactant complex, denoted as M_c and c_c, are represented as follows (Takagi, et al., 1980);

$$M_c = M_p(1+\delta) \tag{1-21}$$

$$c_c = c_p(1+\delta) \tag{1-22}$$

where M_p and c_p are the molecular weight and concentration of the protein moiety of the complex, respectively. δ is the amount of surfactant bound per g of the protein (g/g).

In this case, Eq.1-13 can be rewritten to the following equation,

$$\frac{i_{\theta,c}}{I_0} = k\, M_p (1+\delta)\,(dn_c/dc_c)^2\, c(1+\delta) \qquad (1\text{-}23)$$

where $i_{\theta,c}$ is the intensity of scattered light from protein-surfactant complex. On the other hand, dn_c/dc_c is represented as follows,

$$dn_c/dc_c = (dn_c/dc_p)/(1+\delta) \qquad (1\text{-}24)$$

Therefore, Eq.1-23 is rewritten to

$$M_p = k''' \frac{i_{\theta,c}/I_0}{(dn_c/dc_p)^2\, c} \qquad (1\text{-}25)$$

where

$$i_{\theta,c}/I_0 \propto (\text{Output})_{LS} \qquad (1\text{-}26)$$

$$(dn_c/dc_p)\, c_p \propto (\text{Output})_{RI} \qquad (1\text{-}27)$$

Thus, the molecular weight of protein moiety of the complex can be calculated from the following equation.

$$M_p = K \frac{(\text{Output})_{LS}}{(\text{Output})_{RI}} \frac{1}{(dn_c/dc_p)} \qquad (1\text{-}28)$$

In ultracentrifugal analysis, a sample protein must have been highly purified. When a sample solution contains several molecular species, it is hardly possible to

estimate their molecular weights precisely by the technique. On the other hand, even such a sample solution is within the range of the GPC/LALLS technique, owing to fractionation by the high performance GPC column.

This technique is not only as convenient as the molecular sieving techniques but also as accurate as classical physicochemical techniques, and seems to be practical in the field of protein chemistry.

2 Application of the GPC/LALLS technique for the characterization of membrane proteins solubilized by a nonionic surfactant

Summary

An assessment study was carried out to evaluate the LALLS technique combined with high performance gel chromatography in the presence of a non-denaturing surfactant, octaethyleneglycol n-dodecyl ether ($C_{12}E_8$), the precision differential refractometry and the ultraviolet photometry. It was found that the combined technique is very powerful as a method for the determination of the molecular weight of a membrane protein solubilized by the surfactant. For trial, molecular weights of the following membrane proteins of Escherichia coli both solubilized in oligomeric forms were meausred; porin that forms the transmembrane diffusion pore in the outer membrane and λ-receptor protein that facilitates the diffusion of maltose-maltodextrins across the outer membrane. The result obtanied indicates that both porin and λ-receptor protein exist as trimers in the surfactant solution.

2-1 Introduction

It is often necessary to characterize a membrane protein in a native-like state. Non-denaturing surfactants are used for such purpose. It has been considered that nonionic surfactants, bile salts and zwitterionic surfactants do not denature membrane proteins. A number of nonionic surfactants have polyoxyethylene chain as polar group. Nonionic surfactant with n-alkyl chain as non-polar group is supposed to simulate natural phospholipid with respect to the structure of the hydrophobic region. From this point of view, it can be understood the reason why alkyl polyoxyethylene ethers have been extensively used in the field of biochemistry. The surfactant have the general formula $C_nH_{2n+1}(OCH_2CH_2)_xOH$ and will be abbreviated to C_nE_x.

Although such surfactants are available commercialy, they are heterogeneous with respect to the chain length of polyoxyethylene. The hydrocarbon parts of the surfactant are also heterogeneous. Recently, it has became possible to obtain C_nE_x in completely pure form specified with respect to both n and x. Octaethyleneglycol n-dodecyl ether ($C_{12}E_8$) is the most widely-used surfactant for characterization of membrane proteins among them. Use of surfactants of the type of C_nE_x is very effective since the solubilized states of membrane proteins can be altered at ease by the change of either or both of the lengths of

hydrocarbon chain and the polyoxyethylene chain.

Most membrane proteins consist of two or more subunits. In characterization of such membrane proteins, following approaches are carried out:

1. Determination of the number and kinds of subunits
2. Elucidation of the properties of each subunit
3. Investigation of the subunit conformation in native state
4. Analysis of the association-dissociation of subunits
5. Investigation of the interaction between subunits.

Determination of the number and kinds of subunits constituting a membrane protein is thus the first step of physicochemical characterization. When a membrane protein consists of a single kind of subunits, the number of subunits can be determined from the ratio of molecular weight in the native state to that in the denatured state. Native and denatured states of membrane proteins can be brought about by solubilization by non-denaturing surfactants and denaturing surfactants, respectively. On the other hand, when the subunit composition is heterogeneous, it becomes necessary to determine not only the molecular weight of each kind of subunit but also its molar ratio to others. In both of the cases, accurate determination of the molecular weight in native state is essential. Whether the subunit composition is accurately evaluated or not depends on the precision of the process.

Porin and λ-receptor protein are transmembrane

proteins extracted from outer membrane of Escherichia coli. These membrane proteins consist of a single kind of subunits, and form hydrophilic pore to allow diffusion of various solutes with low molecular weight across the outer membrane of E.coli. It has been presumed that they exist as trimers based on the results of sedimentation equilibrium experiments in the presence of SDS with ambiguous assumptions (Nakae et al., 1979).

This chapter describes the results of the assessment study on the application of the GPC/LALLS technique to the measurements of molecular weights of membrane proteins solubilized by a nonionic surfactant, $C_{12}E_8$. The number of subunits constituting the proteins could be unambiguously determined to be three to indicate the efficiency and high performance of the present technique.

Application of the GPC/LALLS technique to determiantion of molecular weights of membrane proteins solubilized by ionic surfactant, sodium dodecyl sulfate (SDS) and sodium deoxycholate, have been reported (Kameyama et al., 1982, Imamura et al., 1982). In those studies, however, the refractive index increments of protein-surfactant complexes were measured in separate batch-type experiments requiring much time and labor or determined depending on some assumptions. In this study, a simplified procedure to estimate specific refractive index increments was proposed. Advantages of the technique will be described. We will also mention several points which

must be taken into consideration in experiments in the applicaiton of the technique for such a purpose.

2-2 Experimental procedures

Molecular weight was measured with a system consisted of a high performance gel chromatography column, a low-angle laser light scattering photometer and a precision differential refractometer. Outline of the system is illustrated in Fig.2-1 and their specification was described in the legend. Separation based on molecular sieving effect was carried out using a TSK-G3000SW column packed with porous modified silica gel particles. For measurements of refractive index increments by the simplified procedure proposed in this chapter, the scattering photometer was removed and an spectrophotometer (Pharmacia UV-1) was installed before the refractometer.

Measurements using the above system were carried out at a room temperature maintained at $25 \pm 2^{\circ}C$. Specific refractive index increments of hen's ovalbumin and $C_{12}E_8$ were measured by a batch-type precision differential refractometer (Union Giken Co., model RM-102) at 633 nm and $25 \pm 0.03^{\circ}C$. Ovalbumin was equilibrated with a buffer solution containing $C_{12}E_8$ using gel chromatography through a column of Sephacryl S-300 Superfine (1.6 x 80 cm).

Bovine serum albumin, hen's ovalbumin, bovine erythrocyte carbonic anhydrase and bovine pancreatic ribonuclease A were the best commercially available products. A mixture of four kinds of highly purified proteins in a lyophilized state was donated from Oriental

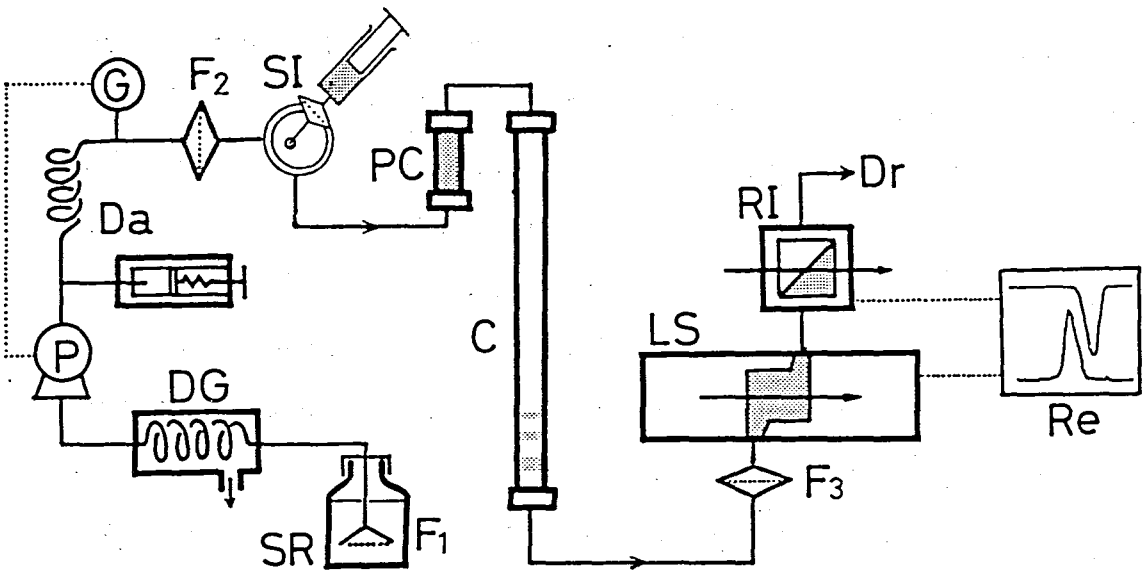


Fig.2-1 Outline of instrumentation used in this study. For notation, see Fig.1-6.

Yeast Co. Ltd. and had the following composition: 250 μg of yeast glutamate dehydrogenase, 360 μg of pig heart lactate dehydrogenase, 390 μg of yeast enolase and 520 μg of yeast adenylate kinase in a sealed vial. The mixture had been blended in the ratio according to our suggestion.

Porin and λ-receptor protein of the E.coli outer membrane were prepared using the method described by Nakae (1979). Octaethyleneglycol n-dodecyl ether ($C_{12}E_8$) was obtained from Nikko Chemicals Co., Tokyo (Japan) and used without further purification. Other reagents were the best commercially available products. A buffer solution with a pH of 7.1 having the following compositon was used: 1.9 mM (0.1 %) $C_{12}E_8$, 18mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 7.0 mM imidazole, 1.0 mM EDTA, 3 mM sodium azide and 200 mM sodium acetate.

Experiments with λ-receptor protein were carried out using another measuring system which is essentially similar to that mentioned above and has been described by Ishii et al. (1983). In the experiments, a buffer solution with a pH of 7.0 having the following composition was used as the elution solvent; 5 mM $C_{12}E_8$, 10mM HEPES, 3 mM sodium azide and 100 mM sodium chloride.

2-3 Results

2-3-1 Standard procedure

Following is the standard procedure adopted in the present study to determine molecular weight of a membrane protein solubilized by $C_{12}E_8$: 1) the chromatographic system (Fig.2-1) was well equilibriated with the buffer solution containing $C_{12}E_8$; 2) a membrane protein was solubilized by $C_{12}E_8$ and applied to the system; 3) the elution from the column was monitored by a low-angle laser light scattering photometer and a precision differential refractometer; and 4) the step of 2) was repeated and the elution was monitored by the tandem array of detectors in which the scattering photometer has been replaced by the spectrophotometer. The molecular weight of a protein can be calculated by the following equation (Takagi, 1981; Kameyama et al., 1982):

$$M = k\ (dn/dc)^{-1}\ (Output)_{LS}/(Output)_{RI} \qquad (2\text{-}1)$$

where M, k, dn/dc, $(Output)_{LS}$ and $(Output)_{RI}$ are molecular weight of the protein, the instrument constant, the specific refractive index increment the protein, the output of the scattering photometer and the output of the refractometer, respectively. The value of dn/dc in Eq.2-1 can be calculated by the following equation:

$$dn/dc = k'\ E\ (Output)_{RI} / (Output)_{UV} \qquad (2\text{-}2)$$

where k', E and $(Output)_{UV}$ are the constant, the extinction coefficient of the protein expressed in terms of its weight concentration and the output of the spectrophotometer. Peak height was adopted as the measure of output of the detectors in the present study. The instrument constants, k and k', must be determined in advance. As has been described in the previous papers (Takagi et al., 1980; Kameyama et al., 1982), Eq.2-1 can be used to determine molecular weight of a membrane protein solubilized by a surfactant.

2-3-2 Elution behavior of standard proteins

Figs.2-2 and 2-3 show elution patterns of two kinds of mixture of purified proteins both obtained using the HEPES buffer solution containing $C_{12}E_8$. Although these are not membrane proteins, the elution curves are sufficient to demonstrate the high performance of the TSK-G3000SW column in the presence of $C_{12}E_8$. A small hump was always detected at a retention time of about 34 min only for the tracing of the scattering photometer (mention of retention time in this chapter refers to that observed for the tracing of the scattering photometer). It might be assignable to large foreign particles or small air-bubbles contaminating the sample solution or those released from the sliding valve at the time of sample injection. Another small peak was observed at 43 min in the lower curve in Fig.2-2, and might be assigned to oligomeric aggregates of

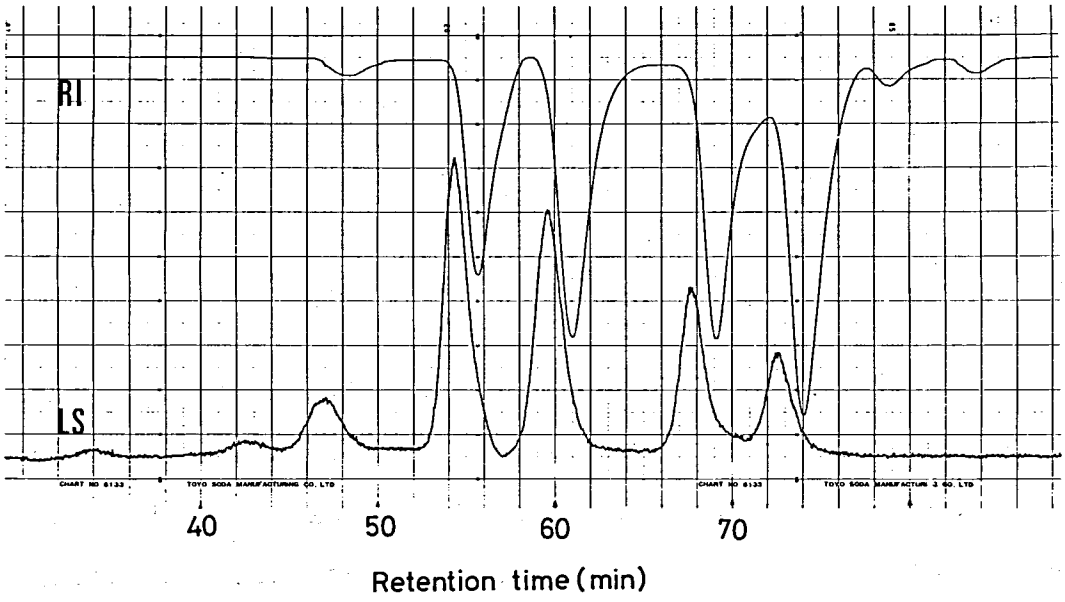


Fig.2-2 Elution patterns of the mixture of four water-soluble standard proteins obtained using the elution buffer containing 0.1% $C_{12}E_8$. The curves indicate the elution patterns obtained by the LALLS photometer (LS) and the differential refractometer (RI). The sample contained bovine serum albumin (330 µg), ovalbumin (400 µg), carbonic anhydrase (500 µg) and ribonuclease A (610 µg) in 100 µl of the elution solvent. Gain setting: LALLS photometer, 32; refractometer, 128. Flow rate: 0.33 ml/min.

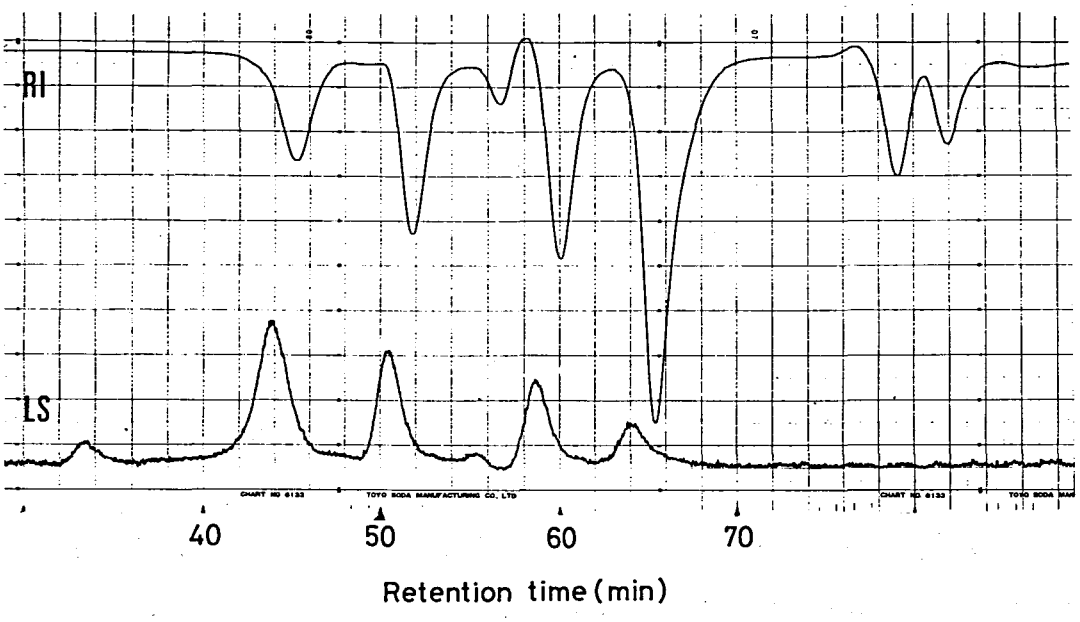


Fig.2-3 Elution patterns of the mixture of four water-soluble standard proteins recorded by the LALLS photometer (LS) and the differential refractometer (RI). The sample contained glutamate dehydrogenase (100 μg), lactate dehydrogenase (140 μg), enolase (160 μg) and adenylate kinase (210 μg) in 100 μl of the elution solvent. Gain setting: LALLS photometer, 32; refractometer,32. Flow rate: 0.32 ml/min.

bovine serum albumin. Five peaks were then observed in both of the curves, and can be assigned to the dimer of bovine serum albumin, bovine serum albumin, ovalbumin, carbonic anhydrase and ribonuclease A from left to right. Four pairs of major peaks in Fig.2-3 can be assigned to glutamate dehydrogenease, lactate dehydrogenase, enolase and adenylate kinase from left to right. There is a difference in elution positions between those recorded by two detectors for each protein. The difference is attributable to the difference in pen position of recorder and location of the detectors.

Outside the range shown in Figs.2-2 and 2-3, the tracings of the two detectors were flat to allow unambiguous interpolation of the base-line. Behind the last protein peak, the refractometer gave positive and negative peaks which could be assigned to the perturbation of solvent composition as the result of sample injection. In advance, control experiments had been carried out in the absence of the surfactant. The elution patterns of standard proteins shown in Fig.2-3 were shown in Fig.2-4 together with those obtained in the presence of the surfactant. Elution positions of all proteins were not affected by the presence or absence of $C_{12}E_8$. The result indicates that the performance of the column is not affected by the presence of $C_{12}E_8$. The results also indicate that the solution states of the proteins among which three (two dehydrogenases and enolase) are

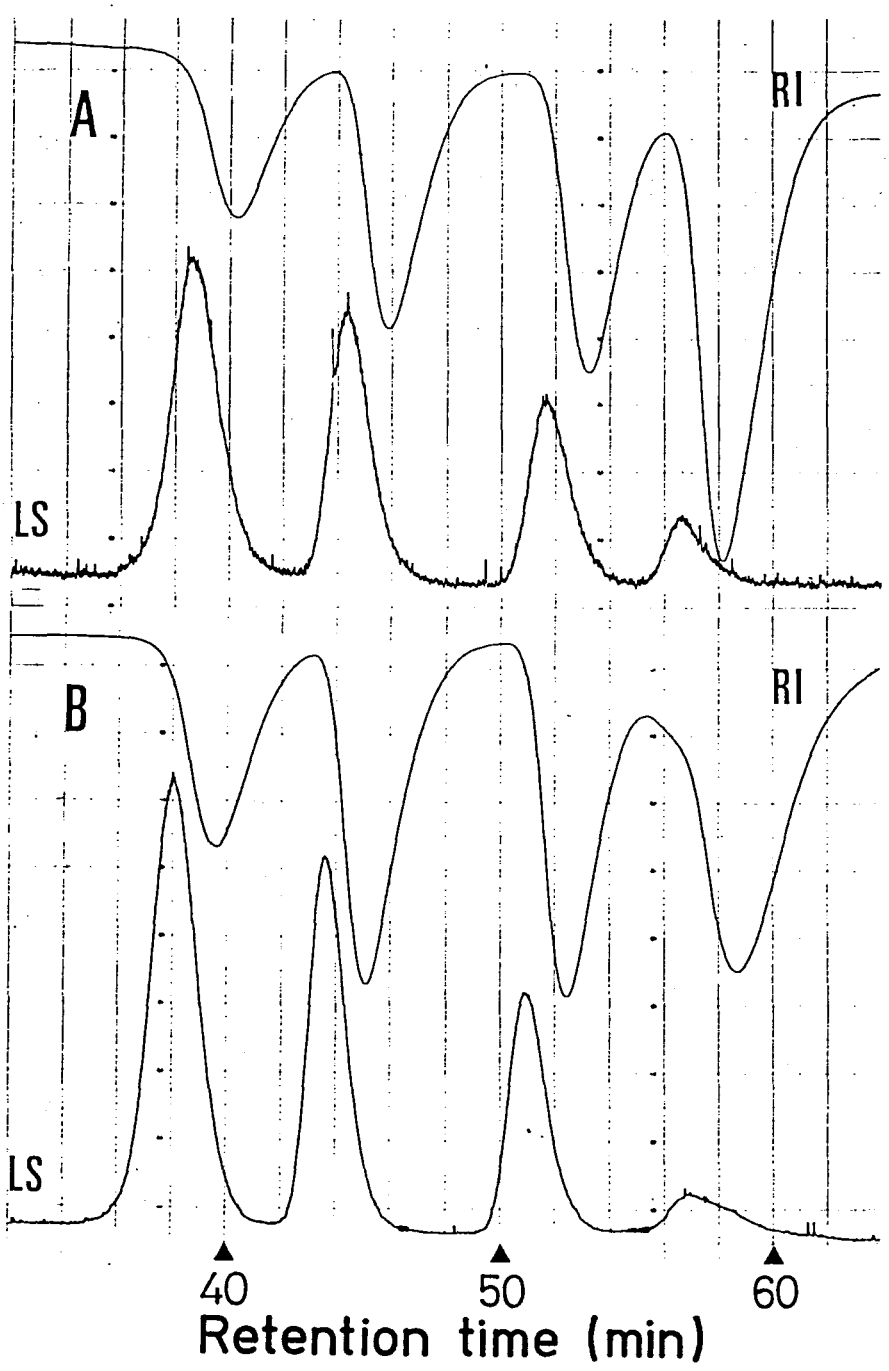


Fig.2-4 Elution patterns of four standard proteins (A) in the presence of $C_{12}E_8$ and (B) in the absence of $C_{12}E_8$. These proteins are the same as those shown in Fig.2-3.

oligomeric proteins were not perturbed by the presence of $C_{12}E_8$.

The ratios of the output of the scattering photometer to that of the refractometer are plotted in Fig.2-5 for the data shown in Figs.2-2 and 2-3. The plot gave a straight line going through the origin. Repeated experiments gave quite reproducible results. According to Eq.2-1, the linear plot indicates that the values of dn/dc are virtually the same for the proteins.

The specific refractive index increment of ovalbumin in the presence of $C_{12}E_8$ was measured absolutely by batch-type differential refractometer (Fig.2-6). The value is obtained to be 0.183 ml g^{-1} and the same as that in the absence of $C_{12}E_8$. This fact suggests that $C_{12}E_8$ scarcely binds to ovalbumin. Moreover, the result, together with the elution behavior unperturbed by the presence of $C_{12}E_8$, strongly suggests that all of the proteins scarcely bind $C_{12}E_8$. Makino et al. (1973) have reported that bovine serum albumin maximally binds only less than 3 moles of Triton X-100 per molecule and ovalbumin binds none of the surfactant. Variation in the values of dn/dc for most globular proteins are very small in the long wavelength side of the visible region (Perlmann and Longsworth, 1948). The average value of dn/dc is 0.187 ml g^{-1} for such proteins. We have assigned the value to the proteins shown in Fig.2-5. The ordinate was divided taking the dn/dc value into consideration on the left side. Fig.2-5 thus

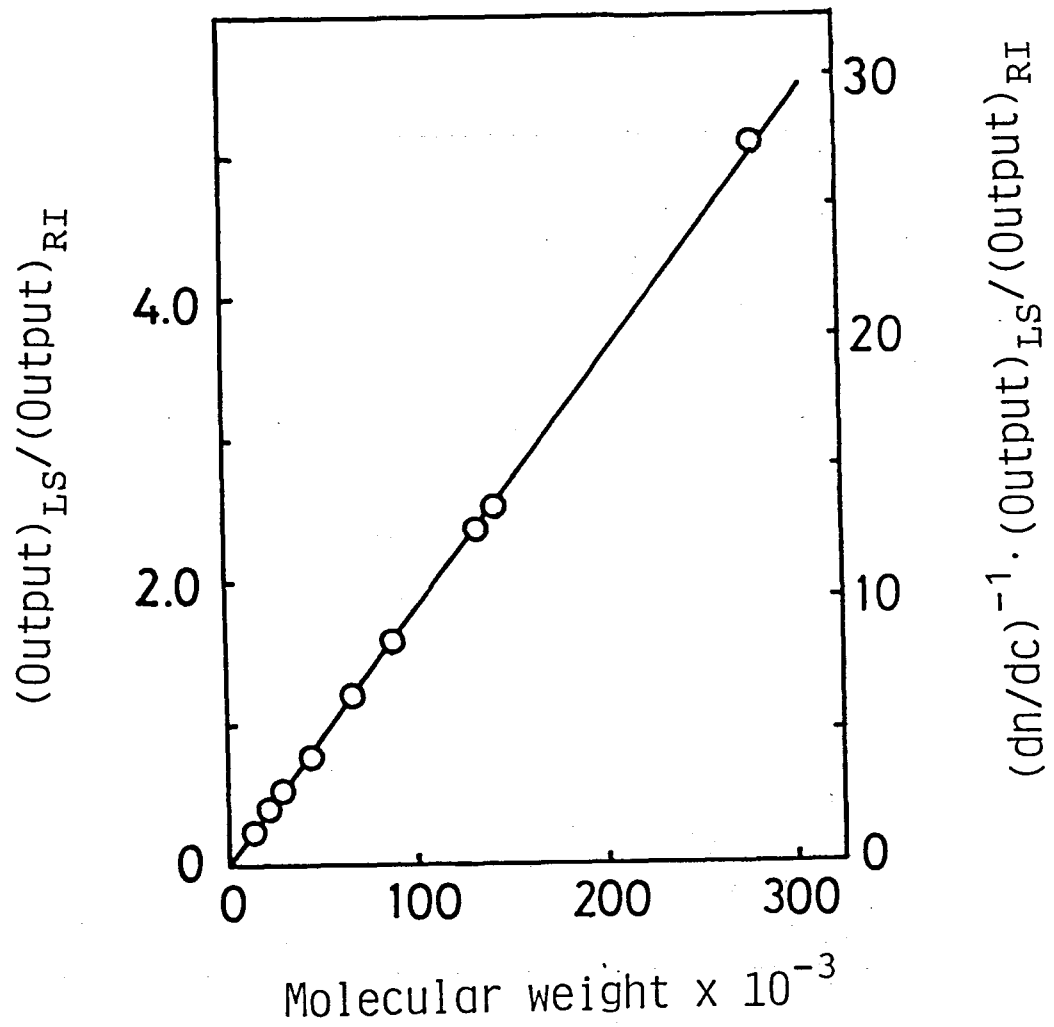


Fig.2-5 Calibration line obtained by the plot of the ratio of the output of the LALLS photometer to that of the refractometer against molecular weight of the respective water-soluble standard proteins shown in Figs.2-2 and 2-3. On the right side, the ordinate is the above ratios devided by 0.187, the value of dn/dc assumed for the proteins in common. The proteins and their molecular weights are, from the bottom to the top, ribonuclease A (13,700), adenylate kinase (21,500*), carbonic anhydrase (29,000), ovalbumin (44,000), bovine serum albumin (66,300), enolase (88,000), the dimer of bovine serum albumin (132,600), lactate dehydrogenase (142,000) and glutamate dehydrogenase (280,000*). The value with asterisks were determined by the present technique due to the lack of authentic values.

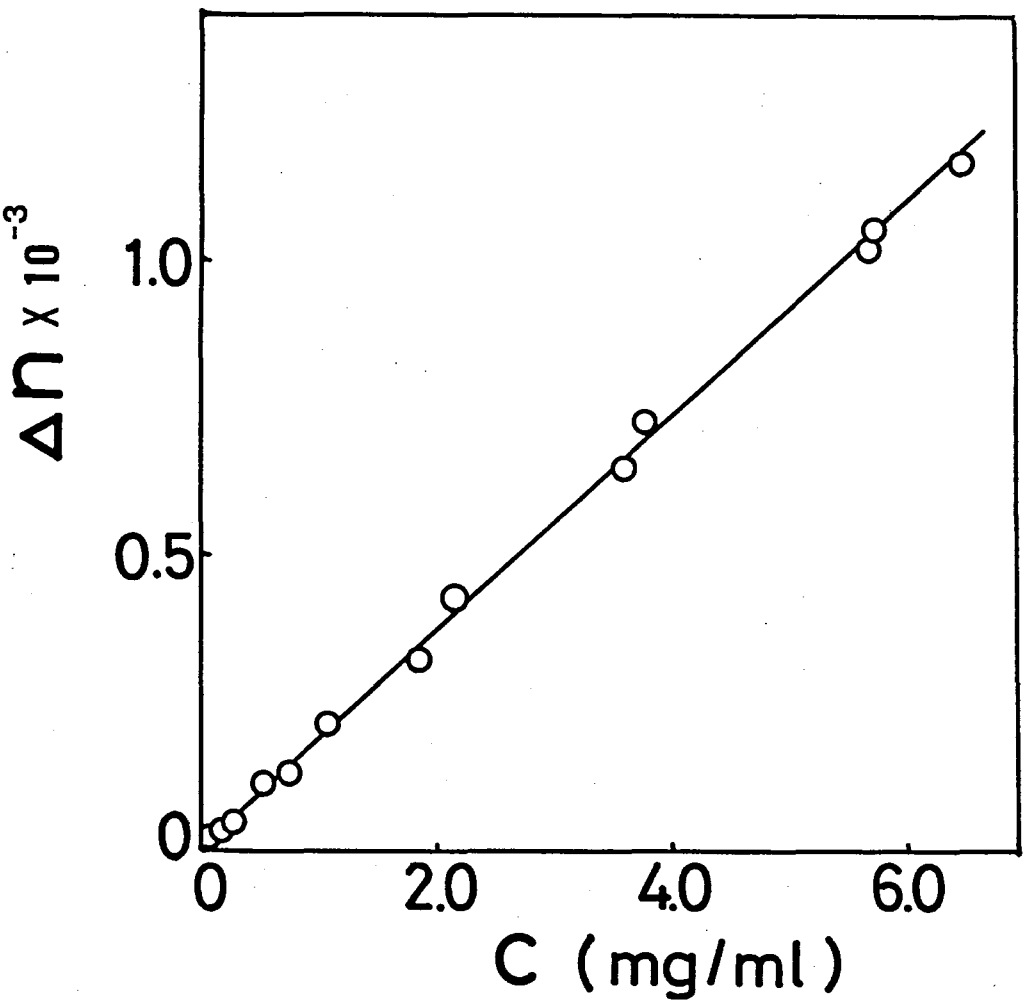


Fig.2-6 Determination of the specific refractive index increment of ovalbumin in the presence of $C_{12}E_8$. Each point was measured with several fractions of the peak from Sephacryl S-300 column.

can be used as a calibration line to determine molecular weight of a protein with any value of dn/dc.

2-3-3 Elution behavior of $C_{12}E_8$ micelles

It is to be noted that there are a hump and a depression in succession in both of the tracings in Fig.2-3 in front of the peak of enolase. Similar anomaly can be observed also in Fig.2-2, although only a small depression is appreciable due to the overlap of a possible hump with the trailing edge of bovine serum albumin.

Fig.2-7 shows the elution patterns obtained when a solution of $C_{12}E_8$ was applied to the column. Since the solvent contained $C_{12}E_8$ in a concentration above the critical micelle concentration, 7×10^{-5} M (Meguro, et al., 1981), the peak in Fig.2-7 can be assigned to the micelles of $C_{12}E_8$.

The above-mentioned anomalies in Figs.2-2 and 2-3 were found at the elution position of the micelles. Presumably the injection of a sample solution of a mixture of proteins perturbs the distribution of $C_{12}E_8$. When $C_{12}E_8$ is contained in a concentraiton above the critical micelle concentration, any change in concentration of $C_{12}E_8$ leads to the change in micelle concentration. It is, therefore, reasonable that the anomalies were observed at the elution postion of $C_{12}E_8$ micelles.

2-3-4 Elution behavior of porin and λ-receptor protein

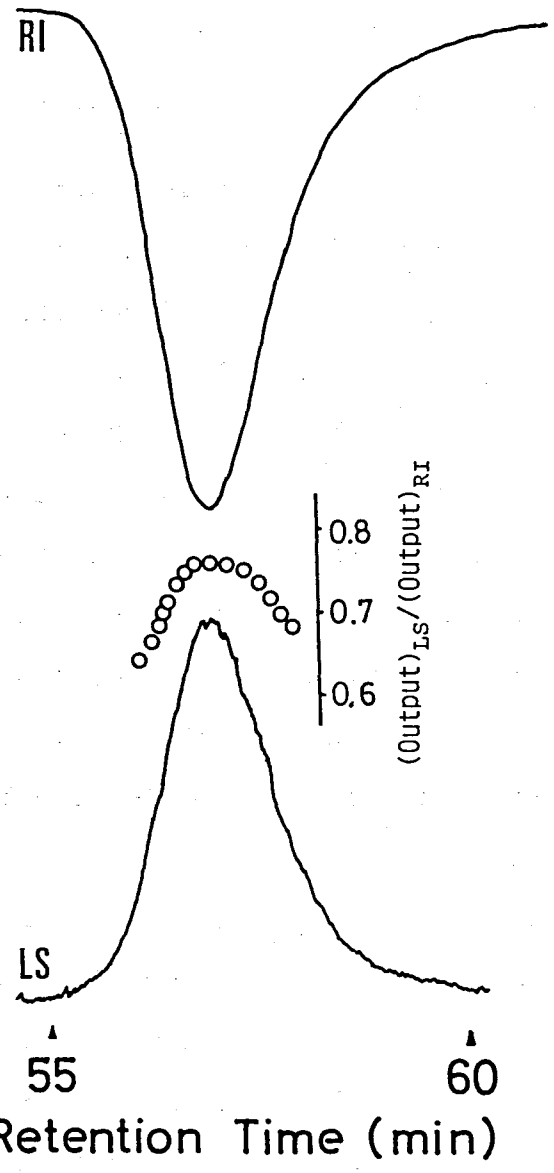


Fig.2-7 Elution patterns of $C_{12}E_8$ micelle recorded by the LALLS photometer (LS) and the differential refractometer (RI). The sample contained 440 µg of $C_{12}E_8$ in 100 µl of HEPES-imidazole buffer solution. The tracing by the latter is displaced forward a distance identical to that observed between the peaks when a protein without molecular weight distribution was used as a sample. Points show the ratios of the output of the two detectors, $(Output)_{LS}/(Output)_{RI}$, for each point of the tracings. Gain setting: LALLS photometer, 32; refractometer, 128. Flow rate: 0.32 ml/min.

Fig.2-8 shows a pair of elution patterns of porin. The pair of major peaks at the retention time of 44 min can be assigned to porin. The peak appeared only in the lower curve at 34 min is of the same nature as those apparent at an identical retention time in Figs.2-2 and 2-3. The big anomalies observed with both of the curves after 55 min can be assigned to the $C_{12}E_8$ micelles. Trailing edges of the micellar peaks were overlapped with a big negative contribution. Such anomaly could not be observed when only $C_{12}E_8$ was injected into the column (Fig.2-7).

Fig.2-9 shows elution patterns of λ-receptor protein obtained by a measuring system essentially similar to that shown in Fig.2-1 (see Experimental section). As described in the legend to Fig.2-9, the sample solution contained λ-receptor protein solubilized by SDS. However, SDS was not detected in the eluate containing the solubilized protein by calorimetric analysis of the methylene blue-SDS complex extracted with chloroform. Accordingly, SDS bound to the protein must be completely replaced by $C_{12}E_8$ during the passage through the column.

2-3-5 Specific refractive index increments

The values of dn/dc of porin and λ-receptor protein were measured using the spectrophotometer and the refractometer as described in the beginning of this section using Eq.2-2. The instrument constant, k', was

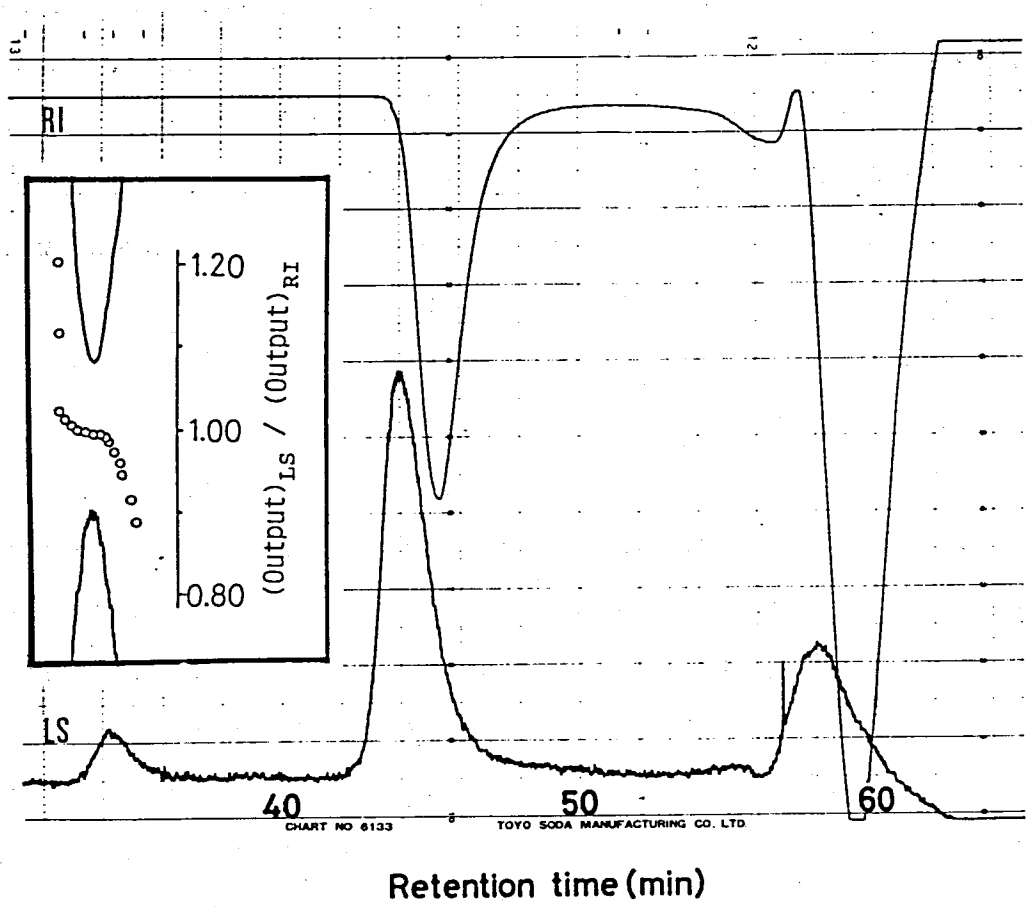


Fig.2-8 Elution patterns of porin of the E.coli outer membrane solubilized by $C_{12}E_8$ recorded by the LALLS photometer (LS) and the differential refractometer (RI). The sample contained 45 μg of porin in 100 μl of $C_{12}E_8$ HEPES-imidazole buffer solution. The inset shows the output ratios point-by-point for the peak region of the elution curves. The tracings are adjusted in the same manner as described in the legend to Fig.2-7. Gain setting: LALLS photometer, 32; refractometer, 32. Flow rate: 0.33 ml/min.

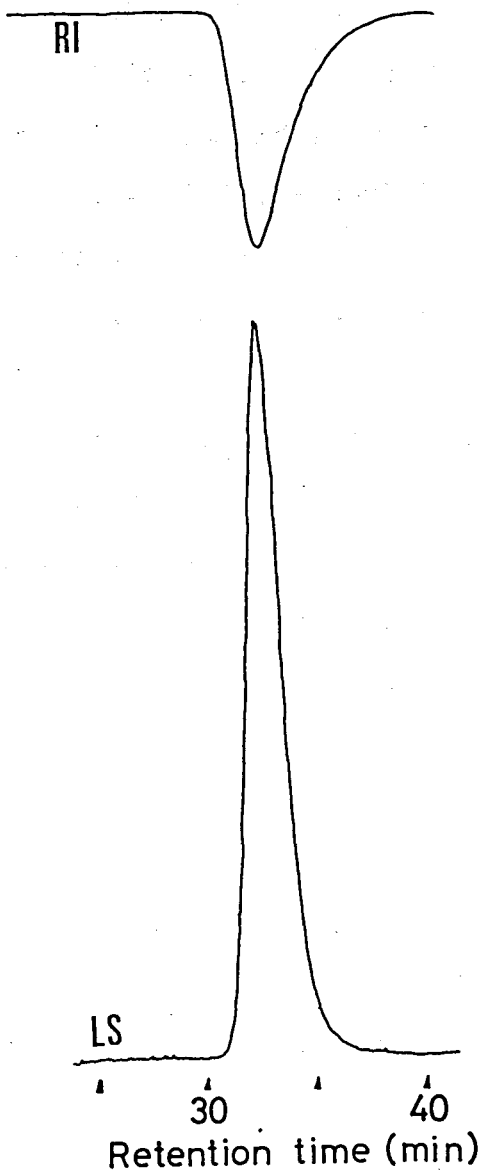


Fig.2-9 Elution patterns of λ-receptor protein of the E.coli outer membrane solubilized by $C_{12}E_8$ recorded by the LALLS photometer (LS) and the differential refractometer (Shodex RI-11) (RI). The sample contained 250 μg of λ-receptor protein in 100 μl of 50 mM Tris-HCl buffer solution (pH 7,0) containing 0.25% sodium dodecyl sulfate, 0.4 M sodium chloride, 5 mM EDTA and 0.05% β-mercaptoethanol. Gain setting: LALLS photometer, 4; refractometer, 64. Flow rate: 0.40 ml/min.

determined using the dimer of bovine serum albumin as a standard protein. The values of dn/dc and extinction coefficient expressed in absorbance of 1 mg ml^{-1} protein solution at 280 nm for 1 cm light path were assumed to be 0.189 ml g^{-1} and 0.67 (Fasman, 1976), respectively, for bovin serum albumin. The procedure was tentatively applied to proteins of known dn/dc values, ovalbumin and ribonuclease A. The values obtained were 0.187 and 0.191 ml g^{-1} for the former and the latter, respectively, and were in good agreement with the reported values, 0.188 and 0.192 ml g^{-1} (Fasman, 1976), for the respective proteins. The extinction coefficients of ovalbumin and ribonuclease A were assumed to be 0.735 ml mg^{-1} cm^{-1} and 0.706 ml mg^{-1} cm^{-1}, respectively, at 280 nm (Fasman, 1976).

The values of dn/dc of porin equilibrated with the HEPES buffer solution containing 1.9 mM $C_{12}E_8$ was estimated to be 0.303 ml g^{-1} assuming the extinction coefficient at 276 nm to be 1.41 ml mg^{-1} cm^{-1} (Rosenbusch, 1974) using Eq.2-2. The value was also estimated for λ-receptor protein to be 0.259 ml g^{-1} assuming the extinction coefficient at 280 nm to be 2.47 ml mg^{-1} cm^{-1} (Ishii et al., 1981). The value of dn/dc of $C_{12}E_8$ was estimated to be 0.135 ml g^{-1} using the batch-type precision differential refractometer. The value is in good agreement with that reported by Becher (1961).

2-3-6 Molecular weights of the membrane proteins

Molecular weight of porin was estimated to be 113,900 ± 1,500 (n=5) according to Eq.2-1. The molecular weight of the monomeric unit of porin has been determined to be 37,200 (Chen et al., 1979) by the amino acid sequencing study. Porin solubilized by $C_{12}E_8$ can, therefore, be concluded to exist as a trimer just in the same manner as solubilized by SDS without heating (Kameyama et al., 1982). Fig.2-8 also includes the plot of the output ratios at various retention times in the region of the peak. A plateau region was observed and can be taken to indicate that the porin oligomer has a stoichiometric composition, as will be discussed later.

Molecular weight of λ-receptor protein was estimated to be 149,000 (± 4,500) in the HEPES buffer solution containing 100 mM sodium chloride and 5 mM $C_{12}E_8$ by essentially the same procedure as in the study of porin. Another experiment carried out using the HEPES buffer solution lacking sodium chloride and containing 2 mM $C_{12}E_8$ gave an essentially the same value, 143,000. Molecular weight of the monomeric unit of this protein has been determined to be 47,400 by the DNA sequencing study (Clement & Hofnung, 1981). λ-Receptor protein solubilized by $C_{12}E_8$ thus can be concluded also to exist as trimer.

2-3-7 Micellar weight of $C_{12}E_8$

The micellar weight of $C_{12}E_8$ was estimated to be 52,400 ± 1,500 (n=5). Fig.2-7 also includes the plot of the output ratios at various retention times. The plot gave a flat region around the peak top. As has also been shown in Fig.2-8, the flat region is restricted to the region around the peak top. Plots of similar nature were obtained for several proteins with no molecular weight distribution. Such a deformation in the frontal and tail region seems to be inevitable at the present time especially when a sample loop of a rather small internal volume (100 μl) is used. The presence of a plateau in the plot should be taken, therefore, as an indication of homogeneity with respect to size of micelles.

2-4 Discussion

A membrane protein often consists of two or more polypeptide chains. In general, such a membrane protein is solubilized by a nonionic surfactant without suffering from denaturation and thus retaining its intact molecular composition. We have been lacking a reliable method for estimation of the molecular weight of a membrane protein thus solubilized, excepting the sedimentation equilibrium technique requiring elaborate experimental procedures (Tanford et al., 1974). The technique is inapplicable to a sample solution containing several molecular species of solubilized membrane proteins. The GPC/LALLS technique using the measuring system shown in Fig.2-1 is quite effective for such samples. We have used $C_{12}E_8$ as a typical example of nonionic surfactants. A series of nonionic surfactants homologous to $C_{12}E_8$, and other kinds of nonionic surfactant can also be used in place of $C_{12}E_8$ for the purpose (Nakae, T. and Ishii, N, J., personal communication).

Both porin and the λ-receptor protein of the E.Coli outer membrane selected as test materials were shown to exist as trimer when solubilized by $C_{12}E_8$. Outer membranes of bacteria differ in their properties from cytoplasmic membranes. Membrane proteins present in the formers naturally differ from those in the latters. Porin and λ-recepter protein mentioned above are not exceptional.

These proteins resist against denaturing action of SDS at room temperature, and are denatured only when heated at 100°C in the presence of SDS. The oligomeric structure of the proteins is thus quite stable, and can be retained at room temperature at least for three months in the presence of SDS. In the presence of $C_{12}E_8$, the enzymes are also quite stable. The nonionic surfactant, however, lacks solubilizing power against denatured forms of the proteins, and the proteins are precipitated when heated at 100°C in its presence. The characteristics of the proteins mentioned above are not shared by most membrane proteins of cytoplasmic membranes. The stability of the oligomeric structure of the proteins, however, makes them quite suitable as test materials for assessment of the performance of the measuring system of molecular weights of membrane proteins solubilized by surfactants.

Kameyama et al. (1982) have shown that the present technique can be used to estimate the molecular weight of the subunit when porin is solubilized by SDS. The present technique thus can be used to estimate molecular weight of an oligomeric membrane protein as well as its subunit molecular weight(s).

A nonionic surfactant such as $C_{12}E_8$ neither denatures a globular protein nor binds to it in large quantities. These characteristics allow one to use globular protein as standards to obtain calibration line as in Fig.2-5 assuming that the proteins share a common value of dn/dc

as a first approximation. The presence of $C_{12}E_8$ micelles in the buffer solution is an important prerequisite in the application of a solubilized membrane protein to a gel chromatographic column. No appreciable effect of the presence of $C_{12}E_8$ micelles was observed on the performance of the TSK-GEL column. It is to be noted, however, that the excess amount of $C_{12}E_8$ micelles contained in a sample solution behaves as a species with a molecular weight of 52,400 and eluted before enolase with a molecular weight of 88,000. The value, 52,400, is not in agreement with the value of 65,000 reported by Tanford et al. (1977) and Corti et al. (1982), but is in good agreement with the value of 50,000 reported by Fujimatsu and Kuroiwa (1982). A small membrane protein solubilized by $C_{12}E_8$ might have a size comparable to that of the micells. Molecular weight of such a protein can not be correctly estimated due to the overlap with the peak of $C_{12}E_8$ micelles. The difficulty can be overcome by the use of other surfactants analogous to $C_{12}E_8$ and with different micellar size.

It is also to be noted that application of a sample solution produced surplus and shortage of $C_{12}E_8$ micelles in a pair as is shown in Figs.2-2 and 2-3. The center of the anomaly corresponds to the elution position of $C_{12}E_8$ micelles. The anomaly is very significant in Fig.2-8. In this case, the sample contained a large amount of salt. The anomaly seems to be the effect of osmotic unbalance on the distribution of $C_{12}E_8$ micelles.

The introduction of the low-angle laser light scattering technique combined with the high performance gel chromatography made it possible significantly to save the time and the amount of a sample protein necessary for determination of its molecular weight. The accurate determination of the specific refractive index increment for each of the protein components is the prerequisite to the present method for estimation of molecular weight. In the orthodox procedure, a solution of protein solubilized by a surfactant must be thoroughly dialyzed against the solvent containing surfactant until reaching equilibrium before measuring the refractive indices of the internal and the external solutions. It is hardly possible to attain dialysis equilibrium in the presence of a surfactant with low critical micelle concentration. In the present study, the determination of the value of dn/dc was also carried out using a kind of "on-line" procedure, that is, the dialysis was replaced by the high performance gel chromatography to minimize the amount of a sample necessary. The eluate from the column was monitored by a spectrophotometer at 280 nm, and then by a precision differential refractometer. The specific refractive index increments of the protein components eluted can be correctly estimated from the two elution patterns thus obtained. Linear arrangement of the three kinds of detector, namely a spectrophotometer, a LALLS photometer and a refractometer, makes the determination of molecular

weight more efficient and time- and sample-saving. Performance of this measuring mode will be demonstrated in chapter 4. The limited performance of the spectrophotometer used in the present study did not allow this trial.

When the extinction coefficient of a protein is unknown, the amount of the sample required for determination of the coefficient will exceed that for the main operation. The only possible way to minimize the sample necessary in this step seems to be the application of the quantitative amino acid analysis (Craig & Kyte, 1980; Peters et al., 1981a, 1982). Example of the application of this technique to determination of extinction coefficients of membrane protein will be shown in chapter 5. The flow-type differential refractometer used in the present study, as well as most commercially available ones, is equipped with a tungthten lamp. According to our experience, this causes no serious problem as regards studying a protein with no absorption band in the visible region. To apply the present technique to a colored sample, the light source must be replaced by one emitting monochromatic light of 633 nm. Although the above problem are left to be solved, the low-angle laser light scattering technique in combination with the high performance gel chromatography, the ultraviolet photometry and the differential refractometry seems to be unrivaled as a way to determine molecular weights of membrane

proteins solubilized by surfactants.

In this chapter, application of the GPC/LALLS technique to molecular weight measurement of membrane proteins consisted of one kind subunit was assessed. In next chapter, this technique will be applied to estimation of subunit composition of membrane protein consisting of two kinds of subunits.

3 Molecular assembly of canine kidney (Na^+,K^+)-ATPase solubilized by a nonionic surfactant

Summary

The (Na^+,K^+)-ATPase of canine renal outer medulla was solubilized by a non-denaturing surfactant, octaethyleneglycol n-dodecyl ether ($C_{12}E_8$), in the presence of sodium ion. Molecular species of the solubilized ATPase were analyzed by the GPC/LALLS technique. The elution patterns thus obtained can best be interpreted by assuming the presence of at least four kinds of protein components. Among them, those with molecular weights 286,000 ± 30,000 and 123,000 ± 8,000 were the major components. A stoichiometric composition of 1:1 molar ratio for the α and β polypeptide chains was obtained for the two major components as well as for the intact ATPase. The (Na^+,K^+)-ATPase was thus indicated to be solubilized by $C_{12}E_8$ to give the $\alpha\beta$-protomer and its dimer, $\alpha_2\beta_2$, as the main components.

3-1 Introduction

Concentration of sodium ion is lower inside a cell than in its outside, while distribution of potassium ion is the reverse. The concentration gradients are maintained by a pumping mechanism. Adenosine triphosphatase activated by sodium and potassium ions, (Na^+,K^+)-ATPase [EC 3.6.1.3], is the entity of the pump. The enzyme is transmembrane protein and catalyzes the outward transport of three sodium ions and the inward transport of two pottassium ions across the membrane, ultilizing energy derived from hydrolysis of one molecule of ATP (Dahl & Hokin, 1974; Jørgensen, 1975; Skou, 1975). This is called "active transport".

(Na^+,K^+)-ATPase has been purified from various sources, mammalian kidney (Jorgensen, 1975), the eel electric organ (Yoda & Yoda, 1981), the duck salt gland (Hopkins et al., 1976) and the shark rectal gland (Hokin et al., 1973) in which the enzyme is present in high concentration. (Na^+,K^+)-ATPase is composed of larger subunit and smaller one, called under the names of α and β subunits, respectively. The α subunit spans the membrane and contains the ATP hydrolysis site on the cytoplasmic side and binding site for specific inhibitor, ouabain, on the outside of the cell. The β subunit is a glycoprotein and its function is not clear, although it is definitely an indispensable part of the whole enzyme complex.

The mechanism of the active transport by (Na^+,K^+)-ATPase are poorly understood. Kyte (1975) demonstrated that (Na^+,K^+)-ATPase is a transmembrane protein and also suggested that at least a noncovalent dimer of α subunits existed in the native state. He interpreted the mechanism of the active transport as follows; channel was formed by the juxtaposition of two identical asymmetric units and sodium and pottasium ions passed through the channel along the dyad axis. Whether this proposal is actually the case or not must be determined by experiments. Firstly, it is important to identify the smallest functional unit necessary for the active transport.

When (Na^+,K^+)-ATPase are solubilized by SDS, the α and β subunits separate into each other. The molecular weight of the α subunit of ATPase from the shark rectal gland was estimated to be 106,000 (Hasting & Reynolds, 1979; Esmann et al., 1980) and that of the β subunit was estimated to be 36,600 (Hasting & Reynolds, 1979) and 39,700 (Esmann et al., 1980) by the sedimentation equilibrium technique in the presence of SDS. The molecular weight of the α and β subunits for the enzymes from kidney were estimated to be 93,000 and 32,300, respectively, by the same technique in the presence of SDS or guanidine hydrochloride (Freytag & Reynolds, 1981).

The (Na^+,K^+)-ATPase often retains the enzymatic

activity when solubilized by a non-denaturing surfactant. Molecular weight determination of the enzyme thus solubilized, therefore, can give insight into the issue of the minimum functional unit. Various molecular weight values of (Na^+,K^+)-ATPase solubilized by non-denaturing surfactants have been reproted, and accordingly subunit composition of the enzyme proposed are diverse. The sedimentation equilibrium technique gave molecular weight of 380,000 ± 21,000 (Hasting & Reynolds, 1979) and 257,000 ± 16,000 (Esmann et al., 1979) to the active and soluble protein components of (Na^+,K^+)-ATPase obtained from shark rectal gland by solubilization by non-ionic surfactants, Lubrol (Hastings & Reynolds, 1979) and octaethylene glycol n-dodecyl ether ($C_{12}E_8$) (Esmann et al., 1980), respectively. Their values suggested $\alpha_2\beta_4$ (Hastings & Reynolds, 1979) or $\alpha_2\beta_2$ (Esmann et al., 1980) as the subunit structure of the minimum active protein unit of (Na^+,K^+)-ATPase. In contrast with these results, Brotherus et al. (1981) showed by sedimentation velocity analysis that the molecular weight of the active major protein unit in a solution of pig kidney (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ was 170,000 ± 9,000. They suggested that the minimum active protein unit was $\alpha\beta$ or $\alpha\beta_2$. Similar molecular weights ranging between 140,000 and 189,000 were estimated for ATPase solubilized by Triton X-100 (Clarke, 1975) or digitonin (Winter & Moss, 1979) which, however, lacked enzymatic activity. These results

have provoked much controversy over the size of the minimum unit of active enzyme and its subunit structure.

The heterogeneity of protein components in the solubilized (Na^+,K^+)-ATPase and the time-dependent aggregation of the $\alpha\beta$-protomeric unit detected as the major component were demonstrated by sedimentation velocity analysis (Brotherus et al., 1981). The presence of various oligomers such as $\alpha\beta$, $\alpha_2\beta_2$, $\alpha_3\beta_3$ and $\alpha_4\beta_4$ was also suggested by Craig (1982a,b) from the results of SDS-polyacrylamide gel electrophoresis of the products of cross-linkage with glutaraldehyde of (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$. These results raised serious questions about the application of the sedimentation equilibrium technique to the characterization of solubilized ATPase preparations, though the technique is effective when a preparation conatains a single species.

It has been shown that the LALLS technique can be effectively applied to the estimation of molecular weights of membrane proteins (Takagi et al., 1980; Miyake & Takagi, 1981). The technique has also been shown to be very convenient if it is used in combination with GPC column and the precision differential refractometer (Takagi, 1981). It has been demonstrated that this technique is very reliable for the purpose of elucidating subunit composition of membrane protein solubilized by SDS (Kameyama et al., 1982) or $C_{12}E_8$ (shown in chapter 2).

In this study, (Na^+,K^+)-ATPase purified from canine

kidney was solubilized by $C_{12}E_8$ and the molecular weight and the subunit composition were determined by the GPC/LALLS technique. There were several species in the solution of solubilized ATPase as Brotherus et al. pointed out (1981). The GPC/LALLS technique is most suitable for such sample. Namely, this technique has advantage that molecular weights of all species in such a mixture can be correctly determined in a single run. On the other hand, subunit composition of each species in the eluate from GPC column was determined by GPC in the presence of SDS. The results obtained showed that the two major protein components were the $\alpha\beta$-protomer with a molecular weight of 123,000 $\pm$ 8,000, and its dimer ($\alpha_2\beta_2$), while highly aggregated materials were present as minor components.

3-2 Experimental procedures

3-2-1 Preparation of microsome from canine kidney

Preparation of microsome from canine kidney was performed as follows: 1) kidney stored at -20°C were thawed and cut in round slices, and the outer medulla were whittled down by a surginal knife, (on an average, 4.7 g of the outer medulla was obtained from one kidney of about 40 g); 2) the isolated outer medulla were then minced with scissors in the preparation medium (0.25 M sucrose, 31 mM imidazole, 30 mM HEPES, 1 mM EDTA, pH 7.1) and homogenized using a Polytoron homogenizer (Kinematica, PT 10) for 40 seconds; 3) the homogenate was centrifuged at 10,000 rpm for 35 min; 4) the pellet was suspended in the same preparation medium and homogenized for 20 seconds again; 5) the homogenate was centrifuged in the same condition as before and the supernatant was centrifuged again at 20,000 rpm for 30 min; 6) the resultant pellet was suspended in 5 ml of the preparation medium per 100 g of the outer medulla by homogenization with Teflon pestle homogenizer, then frozen quickly in a solid CO_2/aceton mixture, and stored at -20°C. On an average, 10 mg of proteins were obtained from 1 g of outer medulla. Concentration of proteins was measured by Bradford method (Bradford, 1976).

3-2-2 Purification of (Na^+,K^+)-ATPase by SDS-treatment and

sucrose density gradient centrifugation in a zonal rotor

Microsome in which proteins were contained at the concetration of 1.40 mg/ml was incubated with 50 mM imidazole-HCl (pH 7.5), 3 mM ATP, 2 mM EDTA and 2 mM DTE, and SDS was added to a final concentration of 0.700 mg/ml by a peristalic pump to remove peripheral protein and loosely-membrane-bound protein and to disrupt lipid bilayer. A linear sucrose gradient was formed by a programmed gradient pump from 15 % to 45 % (w/v) sucrose in 25 mM imidazole-HCl, 1 mM EDTA and 2 mM DTE, and delivered into the zonal rotor with the rotation speed of 2,000 rpm at a rate of 40 ml/min. The microsome suspension treated with SDS (200 ml) was injected by the pump (Gilson, minipuls 2) at a rate of 5 ml/min and subsequently the overlay solution (120 ml of 25 mM imidazole-HCl, 1 mM EDTA and 2 mM DTE) was injected at a rate of 20 ml/min into a zonal rotor of Beckman Ti-15. Centrifugation was continued for 4 hrs at 32,000 rpm with a Beckman L5-65 ultracentrifuge.

Overlay solution was injected at a rate of 40 ml/min into the zonal rotor and 34 fractions of 40 ml were collected and analyzed for protein concentration and ATPase activity. Eluted solutions of fraction number 3 to 14 were collected and centrifuged at 30,000 rpm for 12 hrs. The pellet was washed once with 50 mM imidazolium

chloride buffer (pH 7.0) containing 5 mM $MgCl_2$ and then twice with 1 mM 1,2-cyclohexylenedinitrilotetraacetate (CDTA), to minimize contamination of the enzyme preparation with sodium and potassium ions, and finally suspended in 1 mM EDTA neutralized with imidazole to pH 7.2 (20°C). The specific ATPase activity of the preparation ranged from 42 to 50 units/mg protein (units refer to μmol P_i/min) at 37°C. Only 0.1 % of the activity was ouabain-insensitive. The enzyme preparation suspended could be stored unfrozen at 0°C without any inactivation for at least 100 days, and called "membrane-bound (Na^+,K^+)-ATPase".

3-2-3 Determination of concentration of membrane-bound (Na^+,K^+)-ATPase

Concentration of the membrane-bound (Na^+,K^+)-ATPase was determined by the standard method described in Bio-Rad Technical Bulletin 1051 (April, 1977) which was originally reported by Bradford (1976). This method is based on the shift of absorption maximum of Coomassie Brilliant Blue G250 from 465 to 595 nm when it is bound to a protein.

A suspension of the membrane-bound ATPase containing known amount of the protein was solubilized in 0.2 M sodium phosphate buffer (pH 7.0) containing 1 % SDS or in 0.1 M imidazole - 0.12 M HEPES buffer (pH 7.4) containing 4 mg/ml $C_{12}E_8$ to give a final protein concentration

between 0.165 and 0.345 mg/ml. The rusultant solution was clear and not subjected to any centrifugation. The absorbance at 280 nm of the protein solution was corrected for light scattering in the same manner as described by Reddi (1957). The extinction coefficients at 280 nm thus obtained were 1.23 ± 0.05 ml mg^{-1} cm^{-1} (n=10) in the SDS solution and 1.21 ± 0.07 ml mg^{-1} cm^{-1} (n=5) in the $C_{12}E_8$ solution. The average value of 1.22 ± 0.05 (n=15) was used to estimate the protein concentration of the (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$.

3-2-4 Solubilization of membrane-bound (Na^+,K^+)-ATPase by $C_{12}E_8$

The membrane-bound (Na^+,K^+)-ATPase suspended in 1 mM EDTA neutralized with imidazole to pH 7.2 was solubilized by $C_{12}E_8$ with the following final composition unless otherwise stated; 2 mg/ml protein, 6 mg/ml $C_{12}E_8$, 0.2 M sodium acetate, 1 mM EDTA, 0.02 % sodium azide and 7 mM imidazole-18 mM HEPES buffer (pH 7.0). The centrifugation was started 5 min after the incubation at 23°C, and carried out for 11 min after reaching 140,000 x g at 23°C in order to remove foreign particles. The supernatant was carefully collected as the solubilized ATPase, and stored at 23°C until used for chromatography. The supernatant contained mainly protein-$C_{12}E_8$ complex and lipid-$C_{12}E_8$ mixed micelles.

3-2-5 Simulation of elution curve by the curve fitting method

When bovine serum albumin was applied to the column and the elution was monitored using the measuring system shown in Fig.2-1, each peak in the elution patterns obtained by the three types of detector could be excellently regarded as a Gaussian distribution curve, except the trailing edge. Fig.3-1 shows the elution patterns of dimer of bovine serum albumin and the simulated Gaussian curves for the elution patterns. Accordingly, we assumed that the peak in an elution pattern of a monodisperse species can be simulated by a Gaussian distribution curve.

We tried to simulate the patterns measured as a summation of a set of Gaussian distribution curves expressed by the following equation:

$$\text{Output} = \Sigma H_i \exp[-2.7726 (x - X_i)^2 / W_i^2] \qquad (3\text{-}1)$$

where the suffix "i" denotes the value for a component i; and x, the position on the abscissa. A distribution curve was specified by three parameters, peak top position (X), peak top height (H) and half-width at half-maximum height (W).

An elution pattern could be resolved into components each with a form of Gaussian distribution curve by the computer program SALS (Statistical Analysis with Least

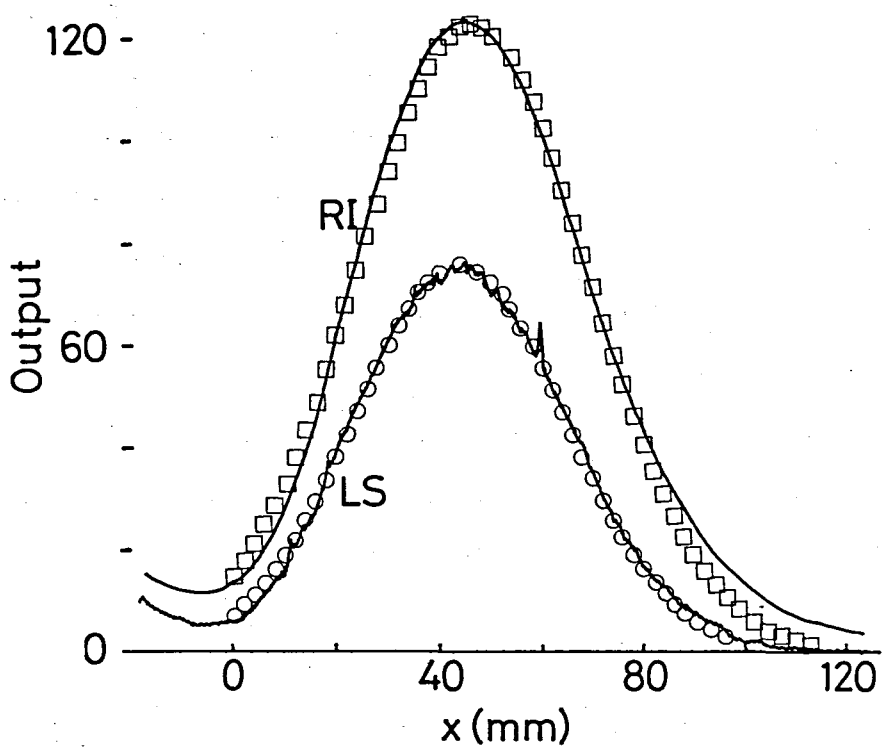


Fig.3-1 Elution patterns of dimer of bovine serum albumin (solid lines), and the simulated Gaussian curves (-○- , -□-). LS, tracing of the LALLS photometer; RI, that of the differential refractometer. Eluant, 18 mM HEPES buffer containing 7.0 mM imidazole, 1.0 mM EDTA, 0.02% sodium azide and 200 mM sodium acetate and 0.1% $C_{12}E_8$ (pH 7.1).

Squares fitting) (Nakagawa and Oyanagi, 1980).

3-2-6 Determination of specific refractive index increment

The specific refractive index increment of the (Na^+,K^+)-ATPase complexed with $C_{12}E_8$, dn_c/dc_p, expressed in terms of the weight concentration of the protein moiety, c_p, was determined according to the following equation, which was shown in chapter 1.

$$dn_c/dc_p = k_1 \, E \, (Output)_{RI} / (Output)_{UV} \qquad (3\text{-}2)$$

Here, n_c is the refractive index of protein-$C_{12}E_8$ complex; k_1, the instrument constant; E, the extinction coefficient expressed in the absorbance of 1 mg/ml protein at 280 nm for 1 cm light path; and $(Output)_{RI}/(Output)_{UV}$, the ratio of the output of the refractometer at a given gain to that of the spectorophotometer at 280 nm and a given full scale. The instrument constant, k_1, could be determined by the measurement on a protein with known values of dn_c/dc_p and E, e.g., bovine serum albumin, as follows. One hundred μl of bovine serum albumin solution (7 mg/ml protein) in the buffer containing 1 mg/ml $C_{12}E_8$, was applied to the chromatography column. The elution was monitored by the spectrophotometer and the precision differential refractometer. This elution pattern was shown in Fig.3-2. The ratio of the maximum height of the peak for the dimer of bovine serum albumin in the refractive index pattern to

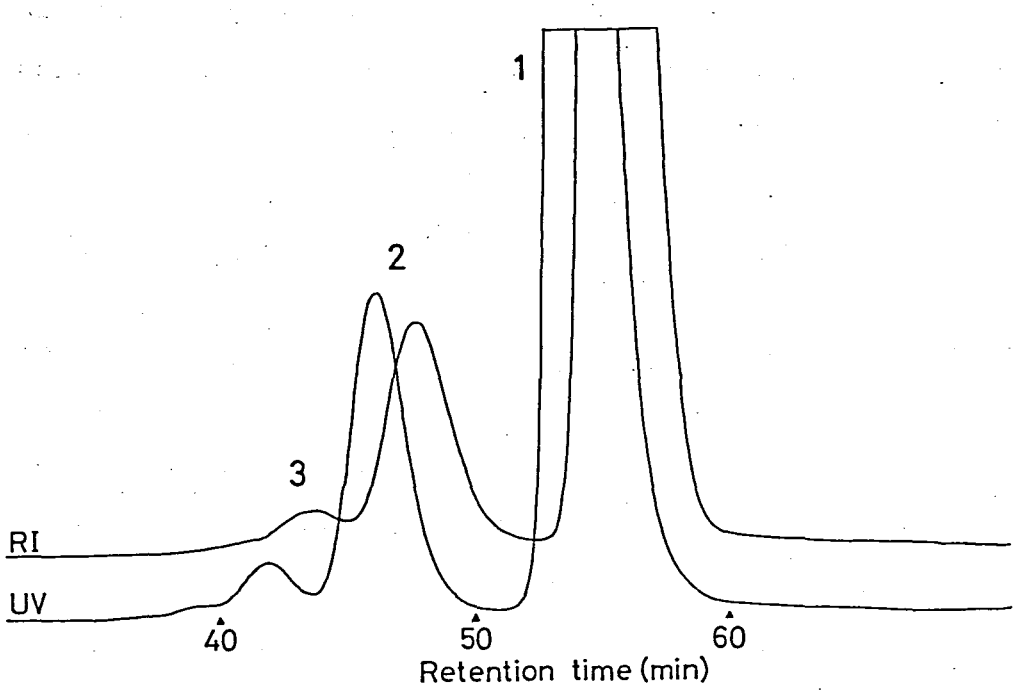


Fig.3-2 Elution patterns of bovine serum albumin obtained by the spectrophotometer (UV) and the differential refractometer (RI). Aliquots (100 µl) of bovine serum albumin (7 mg/ml) were applied to the column. Gain setting: spectrophotometer, 0.1 fs; refractometer, 32. Flow rate: 0.307 ml/min. 1, monomer of the albumin; 2, dimer of it; 3, trimer of it. Eluant, the same as that in Fig.3-1.

the corresponding one in the ultraviolet absorption pattern was taken as the values of $(Output)_{RI}/(Output)_{UV}$. The value of k_1 was obtained on the assumption that dn_c/dc_p and E were 0.187 ml/g and 0.670 ml mg^{-1} cm^{-1}, respectively, for dimer of bovine serum albumin.

The dimer of bovine serum albumin was selected as the standard due to the following reason: namely, although the monomer of bovine serum albumin overlaps with the micelles of $C_{12}E_8$ in the elution pattern, the dimer is free from the trouble.

Fig.3-3 shows a pair of the elution patterns of solubilized (Na^+,K^+)-ATPase obtained by a spectrophotometer and a differential refractometer. Fig.3-4 also shows the elution patterns rewritten so that the displacement in the patterns due to the differences both in positions of detector-cells and recorder-pens (in Fig.3-3) was adjusted to show the patterns in a manner as if they had been obtained using the detectors sharing a single cell and a single pen. The ratio of the peak top height in the refractive index pattern to that in the ultraviolet absorption pattern was calculated for each pair of Gaussian distribution curves to obtain $(Output)_{RI}/(Output)_{UV}$ for the corresponding component. Symbols II to IV and A are assigned to the components obtained by the computer program SALS. The extinction coefficient was assumed identical for the components II, A, III and IV of the (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$

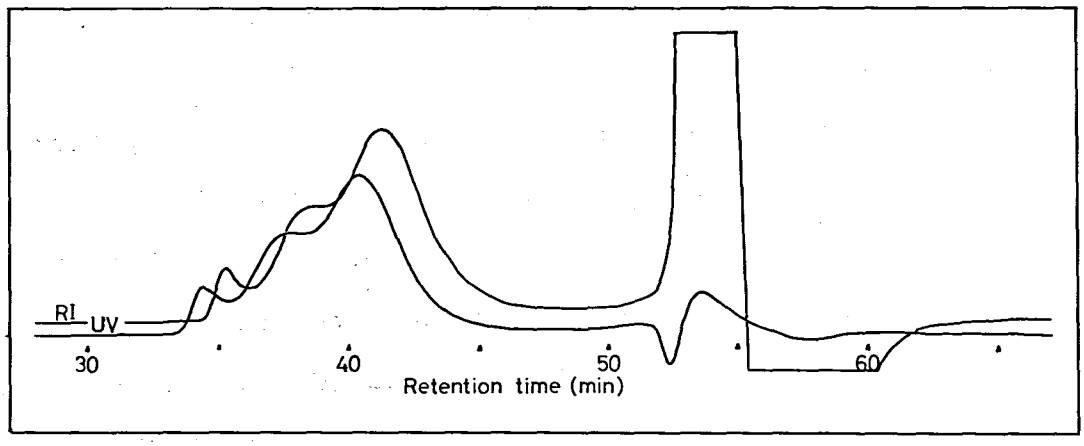


Fig.3-3 Elution patterns of solubilized (Na^+,K^+)-ATPase obtained by the spectrophotometer (UV) and the differential refractometer (RI). Aliquots (100 µl) of (Na^+,K^+)-ATPase (1.8 mg/ml) solubilized by $C_{12}E_8$ were applied to the column. Gain setting: spectrophotometer, 0.2 fs; differential refractometer, 32. Flow rate: 0.31 ml/min.

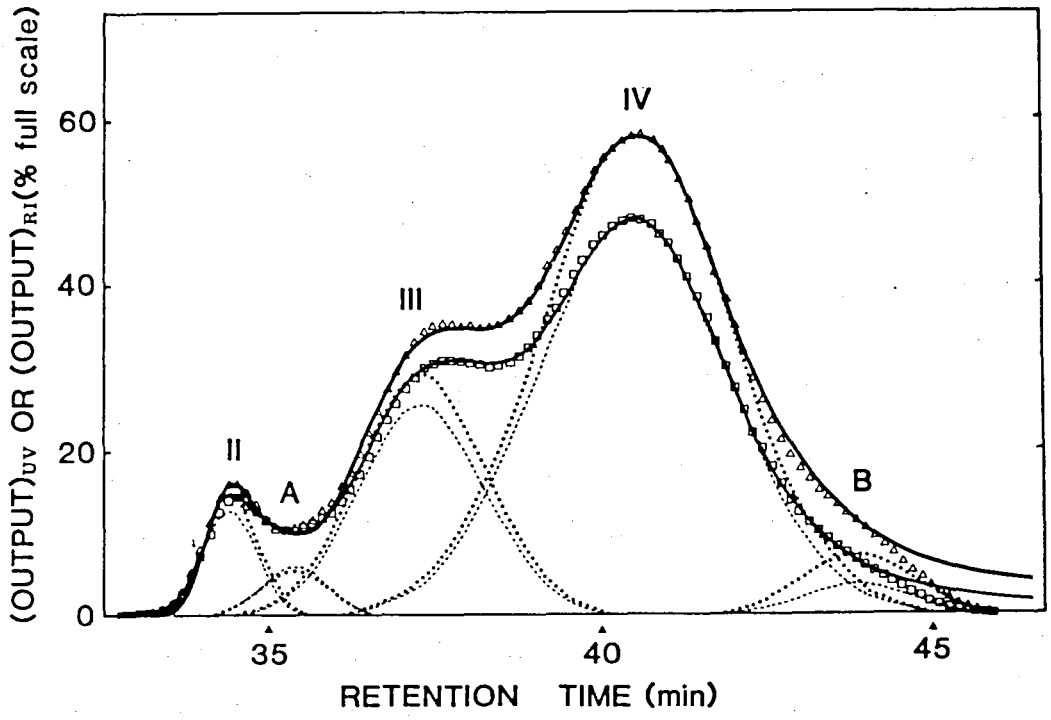


Fig.3-4 Elution patterns of (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ obtained by a spectrophotometer and a differential refractometer. Each of the two elution patterns is resolved in a set of Gaussian curves designated as II, A, III, IV and B, and is displayed by broken and dotted line for outputs of the spectrophotometer and refractometer, respectively. The simulated patterns as summation of Gaussian curves for the spectrophotometer and refractometer are plotted by the symbols of □ and △, respectively, overlapping solid lines of the observed patterns. Gain setting: spectrophotometer, 0.2 fs; refractometer, 32.

and to be 1.22 ± 0.05 based on the assumption that the composition of the polypeptide chains of these components would be same with that of the membrane-bound (Na^+,K^+)-ATPase.

3-2-7 Monitoring by a LALLS photometer and a differential refractometer

The molecular weight, M_p, of the protein moiety of each component was obtained according to the following equation:

$$M_p = k_2 \, (dn_c/dc_p)^{-1} \, (\text{Output})_{LS} / (\text{Output})_{RI} \qquad (3\text{-}3)$$

where k_2 is the instrumental constant; $(\text{Output})_{LS}$, the output of the scattering photometer; and $(\text{Output})_{RI}$, the output of the differential refractometer. The elution from the chromatography column was monitored successively with the LALLS photometer and the differential refractometer. The ratio of peak top heights, $(\text{Output})_{LS}/(\text{Output})_{RI}$, was calculated for each couple of peaks of corresponding component i. The instrument constant, k_2, was determined by the measurement of a mixture of highly purified proteins of known molecular weight, containing glutamate dehydrogenase (molecular weight, 280,000), lactate dehydrogenase (142,000), enolase (88,000) and adenylate kinase (21,500). Since virtually no $C_{12}E_8$ binds to these typical water-soluble proteins, the values of specific

refractive index increment, dn/dc, could be used for these proteins in Eq.3-3 in place of dn_c/dc_p which was used to take bound surfactant into consideration. Detailed experimental procedure has been described in chapter 2.

3-3 Results

3-3-1 Solubilization of membrane-bound (Na^+,K^+)-ATPase by $C_{12}E_8$

Preliminary experiments were carried out to find an appropriate condition for solubilization of the (Na^+,K^+)-ATPase which had been purified from canine kindney in a form bound to fragments of the membrane of the renal medulla. Fig.3-5A shows the increase of protein concentration in the supernatant when the membrane-bound ATPase was treated with an increasing amount of $C_{12}E_8$ at a constant protein concentration of 0.6 mg/ml in 0.2 M sodium acetate containing 1 mM EDTA and 0.02 % sodium azide at pH 7.0 and 23°C. As much as 87 % of the total protein in the enzyme preparation could be maximally solubilized at 2 mg/ml $C_{12}E_8$ or above. Fig.3-5B shows the result of another experiment in which the protein concentration of (Na^+,K^+)-ATPase was changed while the concentration of $C_{12}E_8$ was fixed at 1 mg/ml. Maximum solubilization of about 85 % was observed when the ratio of surfactant to protein was more than 3:1. In a similar experiment, the membrane-bound (Na^+,K^+)-ATPase of 2 mg/ml protein could be solubilized in a yield of 88 % by treatment with 6 mg/ml $C_{12}E_8$. In the following experiments, therefore, the membrane-bound (Na^+,K^+)-ATPase was solubilized by $C_{12}E_8$ in the condition of the weight

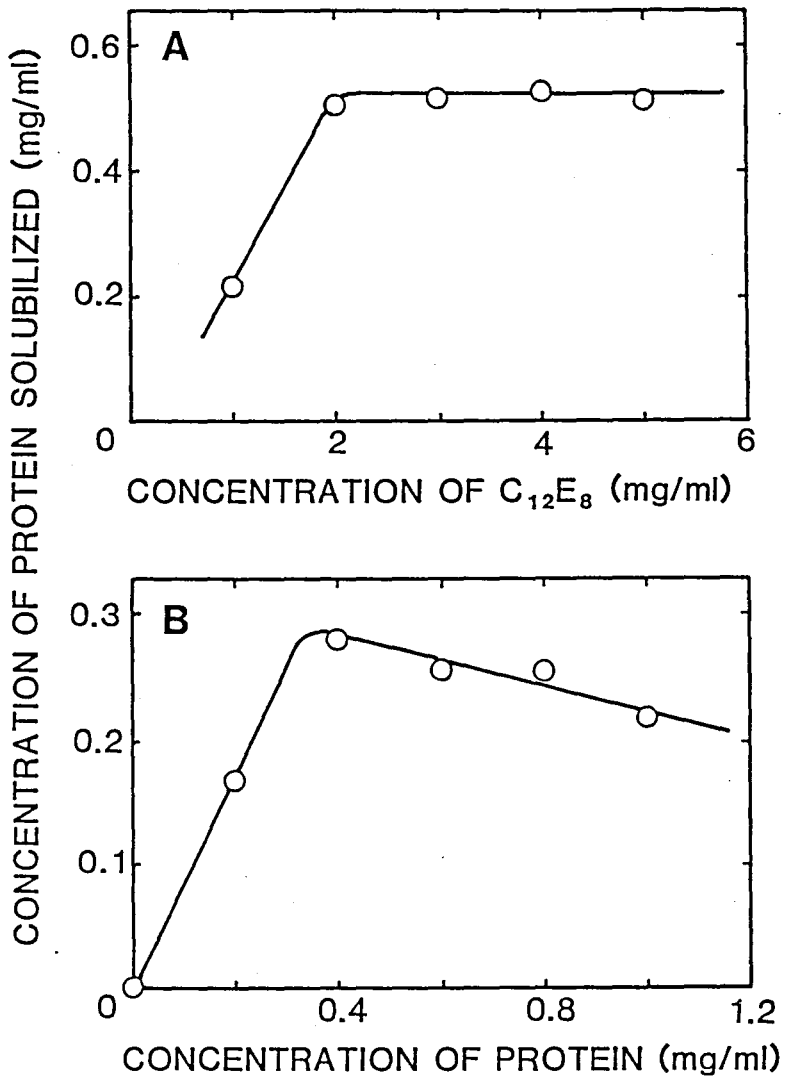


Fig.3-5 Plots of the protein concentration of (Na^+,K^+)-ATPase solubilized against concentrations of $C_{12}E_8$ or protein used. The purified membrane-bound (Na^+,K^+)-ATPase was solubilized at fixed concentrations of (A)0.6 mg/ml protein or (B) 1 mg/ml $C_{12}E_8$ in a solution containing 0.2M sodium acetate, 1 mM EDTA, 0.02 % sodium azide, 7 mM imidazole - 18 mM HEPES buffer (pH 7.0) and various concentrations of (A)$C_{12}E_8$ or (B)protein as indicated, at 23°C.

ratio of ATPase to $C_{12}E_8$ was 1:3.

3-3-2 Gel permeation chromatography

The (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ was applied to a TSK-G3000 SW column. The column had been equilibrated and eluted at 23 ± 3°C with the same solvent as that used for the solubilization of the enzyme except that it contained 1 mg/ml $C_{12}E_8$. Fig.3-6 shows typical elution patterns obtained by the spectrophotometer at 280 nm. Symbols, II to V and A are assigned to the major peaks and shoulder. The elution patterns thus obtained were the same as those obtained with the precision differential refractometer as far as the major peaks were concerned (see Fig.3-4).

Fractions corresponding to the respective peaks mentioned above were analyzed for the α and β subunits by SDS-polyacrylamide gel electrophoresis. Only the fraction corresponding to the peak V lacked the α and β subunits (Hayashi, personal communication). The ratio of the height of the peak V detected by the refractometer to that by the spectrophotometer was about hundred times larger than that observed for other peaks shown in Fig.3-4. The peak V could be detected by the refractometer even when a sample solution containing $C_{12}E_8$ but not ATPase was applied to the column. Moreover, Hayashi showed that all the phospholipids contained in a sample solution were eluted

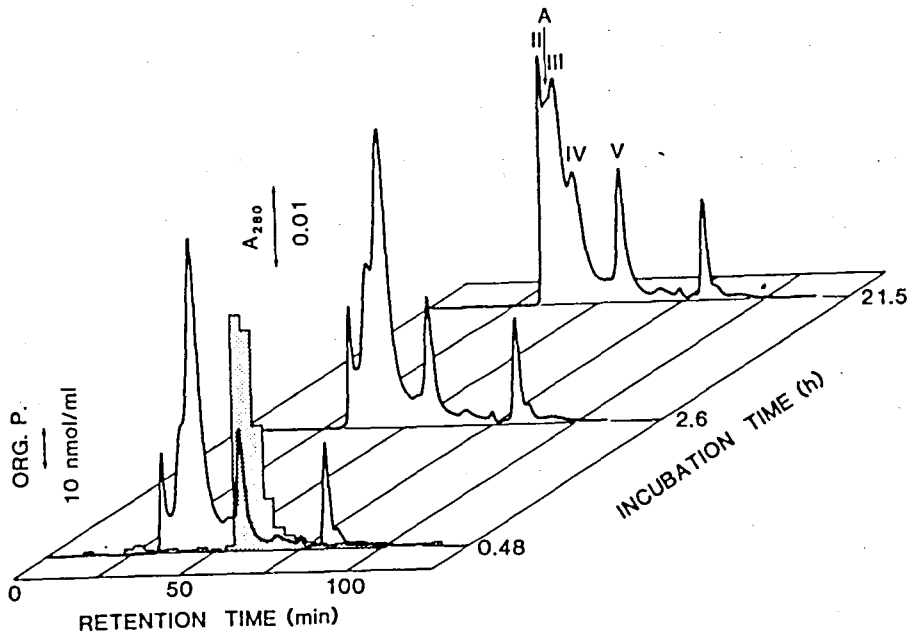


Fig.3-6 Elution patterns of the solubilized (Na^+,K^+)-ATPase obtained by the spectrophotometer (Pharmacia, model UV-2). The sample contained 180 µg of the enzyme in 100 µl of the solvent. The sample was applied to the column at the time indicated after solubilization. The content of organic phosphorus from the column was 92 % . Flow rate: 0.34 ml/min. ORG.P., organic phosphorus.

at the position of the peak V (Fig.3-6). The peak V thus can be assigned to the micelles of $C_{12}E_8$ in which phospholipids and other materials having absorption in the ultraviolet region are incorporated.

As is clear from Fig.3-6, the elution pattern changed significantly with time after solubilization of the (Na^+,K^+)-ATPase. Peaks II and III were amplified at the expense of peak IV, while a small shoulder, A, appeared between peaks II and III after prolonged incubation. The protein component corresponding to peak IV seems to aggregate progressively to form larger components.

Behind peak V, the spectrophotometer and the differential refractometer gave quite different elution patterns as shown in Fig.3-3. Since no protein component was eluted in this region, no further attention will be paid to these components.

3-3-3 Specific refractive index increment

Fig.3-3 shows a typical example of a pair of elution patterns obtained by the two kinds of monitors mentioned above. Both of the elution patterns could be simulated using the computer program, "SALS", as the summation of five Gaussian distribution curves shown by dotted or broken lines, and the curves are designated as II, A, III, IV and B as shown in Fig.3-4. The specific refractive index increment, dn_c/dc_p, of each component could be

calculated by the simpified procedure described in chapter 2. The extinction coefficient, E, was assumed to be 1.22 ± 0.05 ml mg^{-1} cm^{-1} at 280 nm for every protein components eluted. The assumption could be justified for protein components III and IV by the fact that their compositions of polypeptide chains were essentially identical with that of the ATPase solubilized before fractionation, as described later. The value of dn_c/dc_p thus obtained for the components, II, III and IV were essentially the same, while that of the component A was exceptionally low (see Table I). Although the elution pattern of the (Na^+,K^+)-ATPase changed with time after solubilization as has been shown in Fig.3-6, no change was observed with time after solubilization for the values of dn_c/dc_p estimated for each component. In order to evaluate the validity of the simplified procedure, the specific refractive index increment of bovine carbonic anhydrase was estimated by this procedure. The value obtained was 0.194 ± 0.010 ml/g, which is reasonable for a typical globular protein in the presence of $C_{12}E_8$. This fact suggests the validity of this procedure for estimation of specific refractive index increment in the present study.

These values of dn_c/dc_p allow one to estimate the amount of $C_{12}E_8$ bound to each of the protein components, if all proteins in each component of the ATPase are assumed to show identical value of specific refractive index increment, 0.187 ml/g, which is appropriate for a

Table I Molecular Weights of Protein Components of (Na^+,K^+)-ATPase Estimated at Various Incubation Times after Solubilization by $C_{12}E_8$, Together with Specific Refractive Index Increments.

components	dn_c/dc_p (ml/g)	molecular weights					
		incubation time (h)				average(± s.e.)	relative
		0.5	2.5	7.8	21.5		
II	0.254 ± 0.027	1,500,000	2,010,000	1,820,000	1,600,000	1,740,000 ± 230,000	14 ± 2
A	0.202 ± 0.038	726,000	921,000	860,000	836,000	836,000 ± 82,000	6.8 ± 0.8
III	0.255 ± 0.033	293,000	320,000	284,000	246,000	286,000 ± 30,000	2.3 ± 0.3
IV	0.264 ± 0.028	126,000	132,000	118,000	115,000	123,000 ± 8,000	1

simple protein.

3-3-4 Molecular weights of the major components

Molecular weights of the major protein components were estimated by monitoring the elution from the column by a LALLS photometer and a differential refractometer connected in series. The elution patterns thus obtained are shown in Fig.3-7. The LALLS photometer could detect a peak in front of peak II. The new one was designated as peak I. The elution patterns obtained by the LALLS photometer could also be resolved into Gussian distribution curves in the same manner as described above. Fig.3-8 shows the typical elution curves measured with the LALLS photometer together with the corresponding curves with the refractometer. Elution experiments similar to those shown in Fig.3-7 were carried out for samples incubated for various times after solubilization. Elution patterns obtained with the ATPase incubated for 30 min and 21.5 hrs after the solubilization are shown in Fig.3-8A and B, respectively. As is clear from the figures, the two pairs of the pattern could be resolved into Gaussian distribution curves by the same set of the two parameters specifying curves, namely, position of peak top (X) and half width at half maximum height (w), except for the component I which was not detected by the differential refractometer.

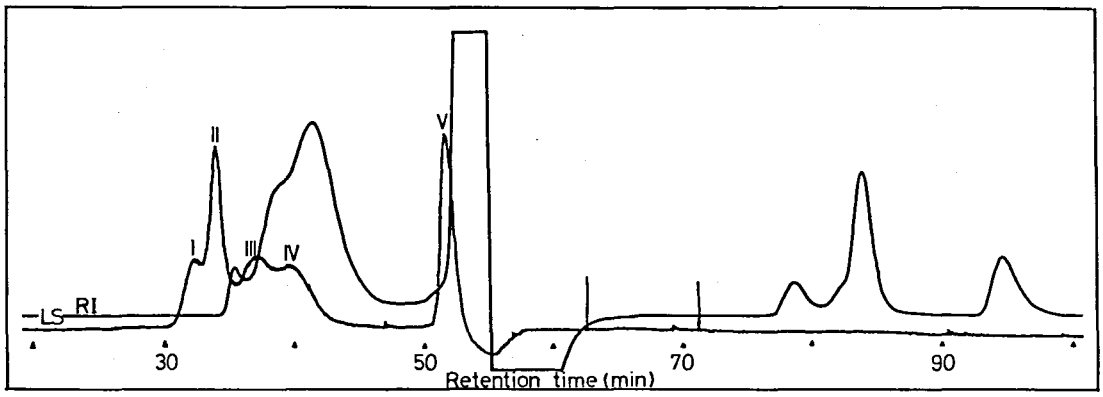
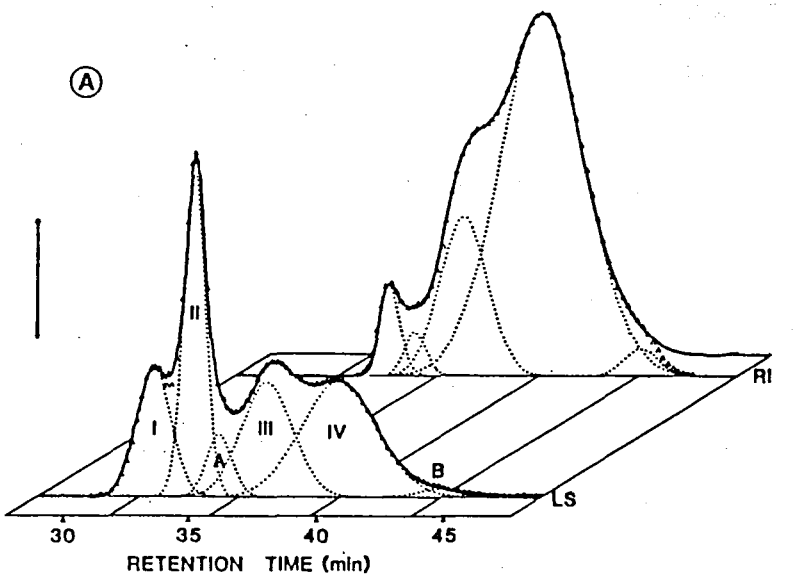


Fig.3-7 Elution patterns of solubilized (Na^+,K^+)-ATPase obtained by the LALLS photometer (LS) and the differential refractometer (RI). The sample contained 180 µl of the enzyme in 100 µl of the solvent. Gain setting: LALLS photometer, 8; differential refractometer, 32. Flow rate: 0.31 ml/min.



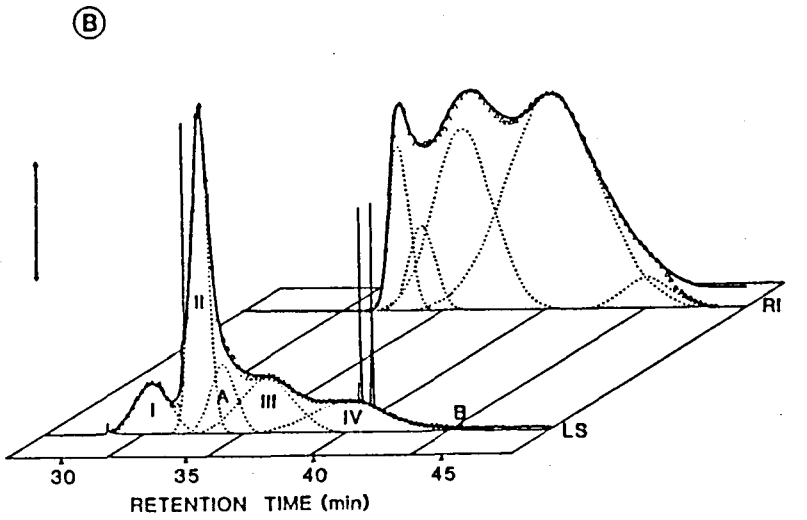


Fig.3-8 Elution patterns of solubilized (Na^+,K^+)-ATPase recorded by the LALLS photometer and the differential refractometer, and their simulation as summation of each set of Gaussian curves. At (A) 30 min and (B) 21.5 hrs after solubilization, each 100 µl solution was applied to the column. Two abscissas corresponding to two kinds of detectors are adjusted so that the position giving the half height of the peak II in each elution pattern coincides with each other. The elution patterns are drawn by solid lines, and the constituent Gaussian curves are shown by dotted lines. The value obtained by the simulation are displayed by the symbol of △ . Gain setting: (A) LALLS photometer, 8; refractometer, 32. (B) LALLS photometer, 4; refractometer, 32. The length of the bar with arrows corresponds to one-fifth of the full scale. Flow rate: 0.31 ml/min.

The instrument constant, k_2, in Eq.3-3 was determined by using four kinds of typical water-soluble globular proteins according to Eq.3-3. The values of $(\text{Output})_{LS}/(\text{Output})_{RI}$ obtained for these proteins were plotted against their known molecular weights. As shown in Fig.3-9, a good linear plot was obtained indicating that these proteins share a virtually common value of specific refractive index increment. This would be a reflection of the fact that $C_{12}E_8$ is scarcely bound to these proteins. Accordingly a value of 0.187 ml/g was adopted to determine k_2 from the slope of the linear plot.

Molecular weight was estimated for each protein component of the ATPase solubilized by $C_{12}E_8$ at various times after solubilization according to Eq.3-3. Results obtained are listed in Table I. No systematic change of molecular weight was observed with incubation time before application to the column. The average of the molecular weights obtained at various times was therefore included in the table. In the last column, the molecular weights of the components are compared with that for component IV. Peak I does not seem to come from protein because the LALLS photometer always detected a similar peak no matter whether a sample contained protein or not. Peak I was not detected with the refractometer as described above (see Fig.3-8). For component B, the outputs of the refractometer and the LALLS photometer were too low to obtain a reliable value. Accordingly, no further attention

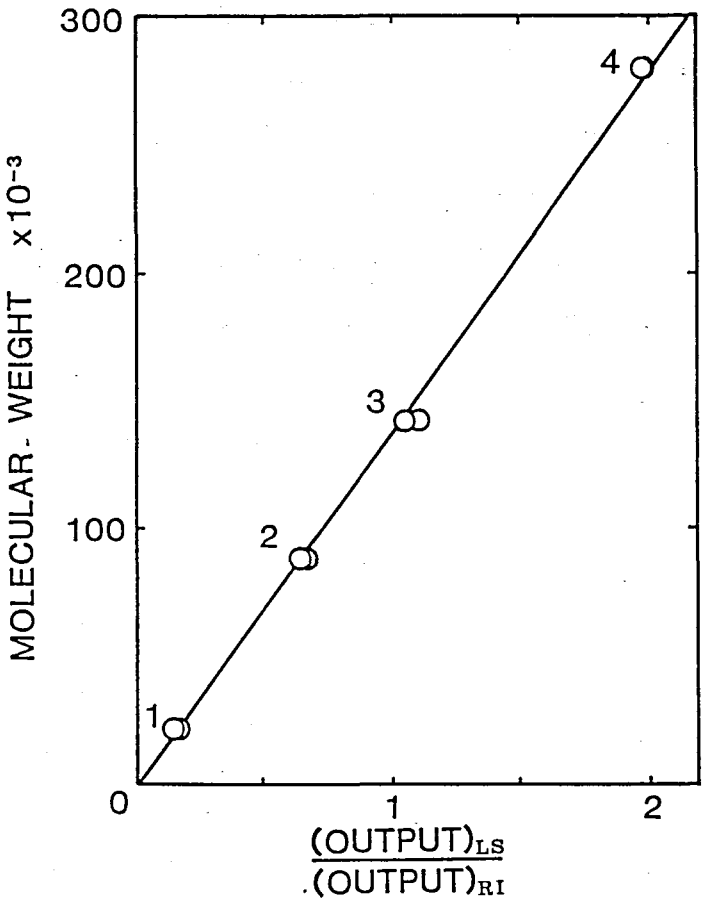


Fig.3-9 Plots of the molecular weights of four standard proteins versus the ratio of output of the LALLS photometer to that of the refractometer. A mixture of the four standard proteins was applied to the column in the same manner as in Fig.3-8. The peak heights were adopted as the measure of the corresponding outputs. Standard proteins: 1, adenylate kinase; 2, enolase; 3, lactate dehydrogenase; 4, glutamate dehydrogenase.

will be paid to these two components.

3-3-5 Change in composition with incubation time of the solubilized (Na^+,K^+)-ATPase

As is clear from Figs.3-6 and 3-8, the ratios of the components found for the ATPase solubilized by $C_{12}E_8$ changed with incubation time. Change in the composition was analyzed by measuring the area under each of the Gaussian curves obtained by the resolution of an elution pattern recorded by the refractometer. Because the amount of bound $C_{12}E_8$ was almost the same for each of the major components, the tracing of the Gaussian curve obtained by the refractometer gives the most reliable estimate of the quantity of protein on the basis of weight. The time course of the change in the quantity of each protein thus analyzed is shown in Fig.3-10. Components III and IV were found to increase and decrease progessively in their amounts, respectively, with increase of incubation time. On the other hand, components II and A increased only slightly but steadily in their contents and the component B showed no change. These results suggest that component IV associated to produce components II, A and III.

3-3-6 Effect of concentration of $C_{12}E_8$ on the population of molecular species

Preliminary experiments were carried out to

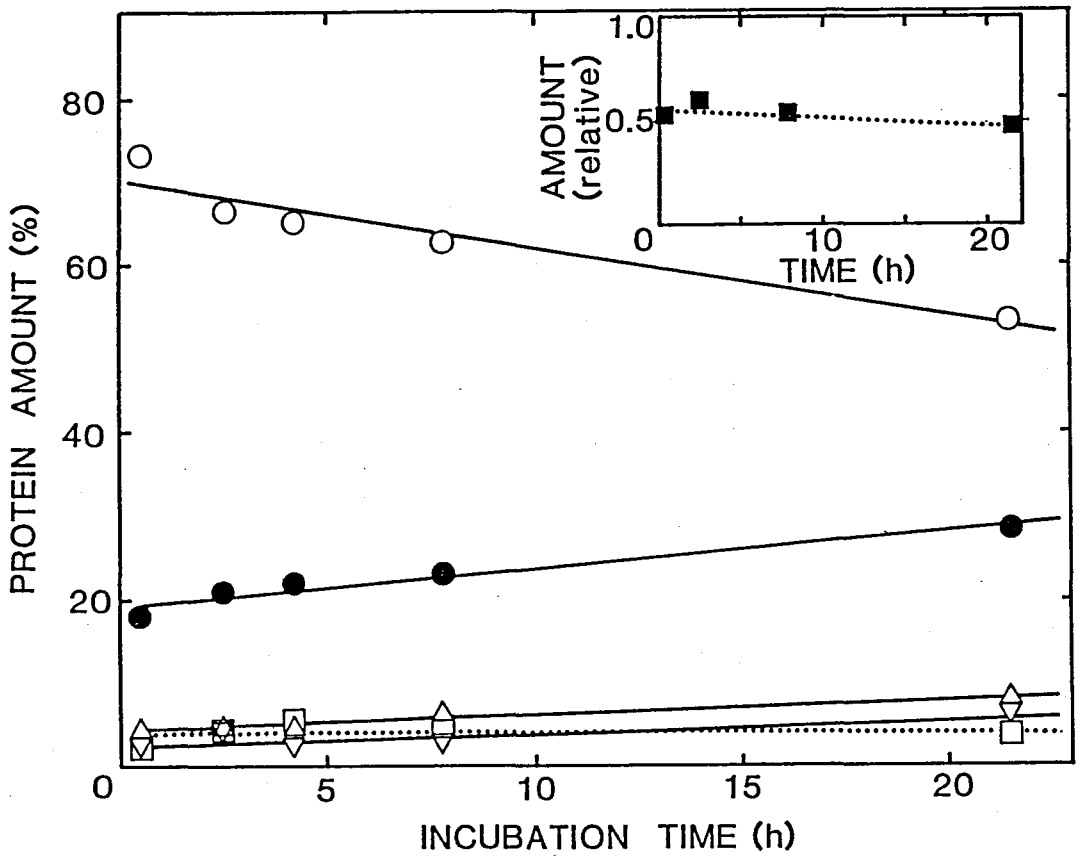


Fig.3-10 Change of the protein components of the solubilized (Na^+,K^+)-ATPase in the content during incubation time at 23°C after the solubilization. An amount of each protein component was estimated by integration of area under the corresponding Gaussian curves obtained for the tracing of the refractometer (see Fig.3-8). Percentage of each protein component to the total amount is plotted against the time after the solubilization of enzyme. Components IV, ○ ; III, ● ; II, △ ; A, ▽ ; B, □ . Relative amounts of component I were obtained as areas under the corresponding Gaussian curves obtained for the tracing of the LALLS photometer, and plotted in the inset (■) where the ordinate is arbitrary.

investigate the effect of concentration of $C_{12}E_8$ in elution buffer solution on the population of molecular species arising as result of solubilization of the membrane-bound (Na^+,K^+)-ATPase by $C_{12}E_8$.

Membrane-bound (Na^+,K^+)-ATPase was solubilized under the same condition as in the experiments described above. The concentration of $C_{12}E_8$ in the elution buffer was variously changed, and elution pattern was obtained in each condition. The elution was monitored by a spectrophotometer at 280 nm, a LALLS photometer and differential refractometer connected in series using the measuring system shown in Fig.1-6. Elution patterns thus obtained are shown in Fig.3-11(A)-(D). The elution patterns were resolved into several Gaussian distribution curves by computer program "SALS", similar to Figs.3-4 and 3-8.

Fig.3-12 shows the change of the elution pattern obtained by a spectrophotometer when ATPase incubated for various times after solubilization was applied to the column equilibrated with $C_{12}E_8$ of the concentraiton 0.1 mg/ml. Table II shows the molecular weights and dn_c/dc_p values of all species estimated in the various elution conditions. Components IV and III could be assigned to the $\alpha\beta$-protomer and its dimer, $\alpha_2\beta_2$, respectively in all the elution conditions.

The elution patterns obtained by the UV spectrophotometer are compared in Fig.3-13. Fig.3-14 shows

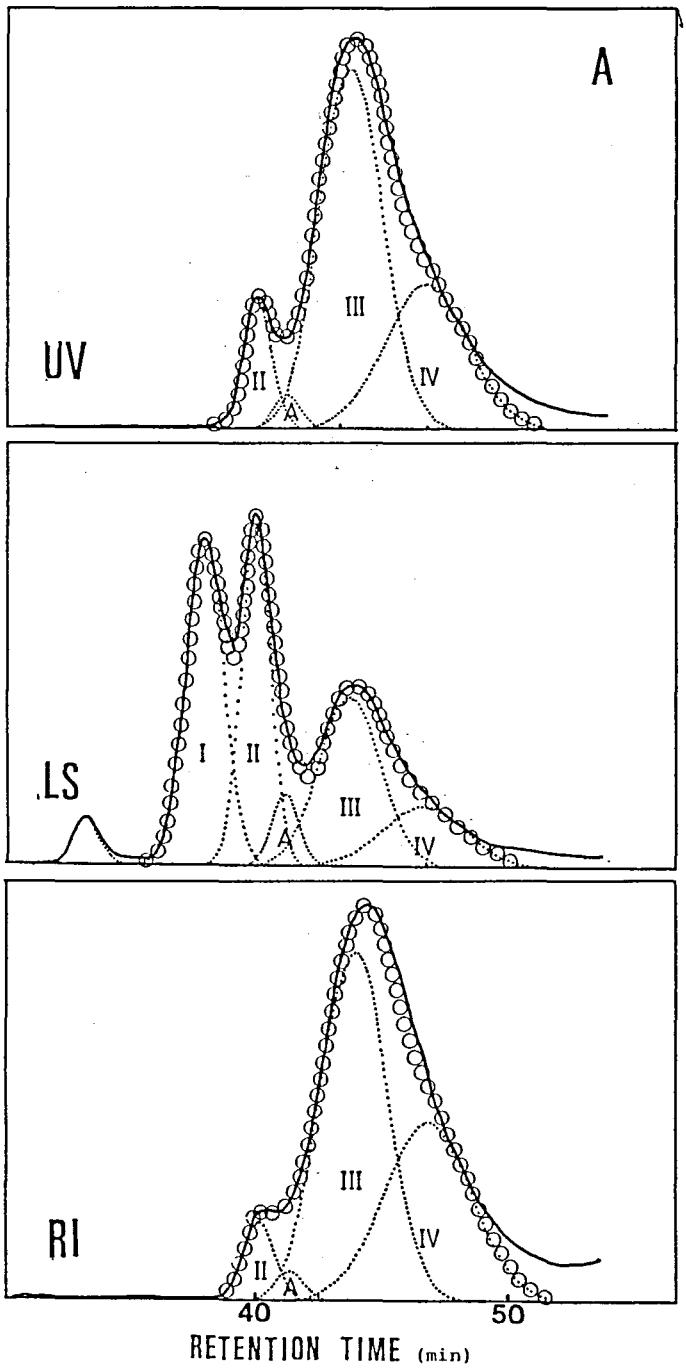


Fig.3-11 Elution patterns of (Na^+,K^+)-ATPase incubated for 35-40 min after solubilization. Concentration of $C_{12}E_8$ in elution buffer was (A) 0.1 mg/ml, (B) 0.5 mg/ml, (C) 1.0 mg/ml and (D) 2.0 mg/ml. The elution patterns obtained by the spectrophotometer (UV), the LALLS photometer (LS) and the differential refractometer (RI) are drawn by solid lines, and the constituent Gaussian curves are shown by dotted lines. The values obtained by the simulation are displayed by the symbols of O . Gain setting: spectrophotometer, 0.16 fs; LALLS photometer, 8; differential refractometer, 32.

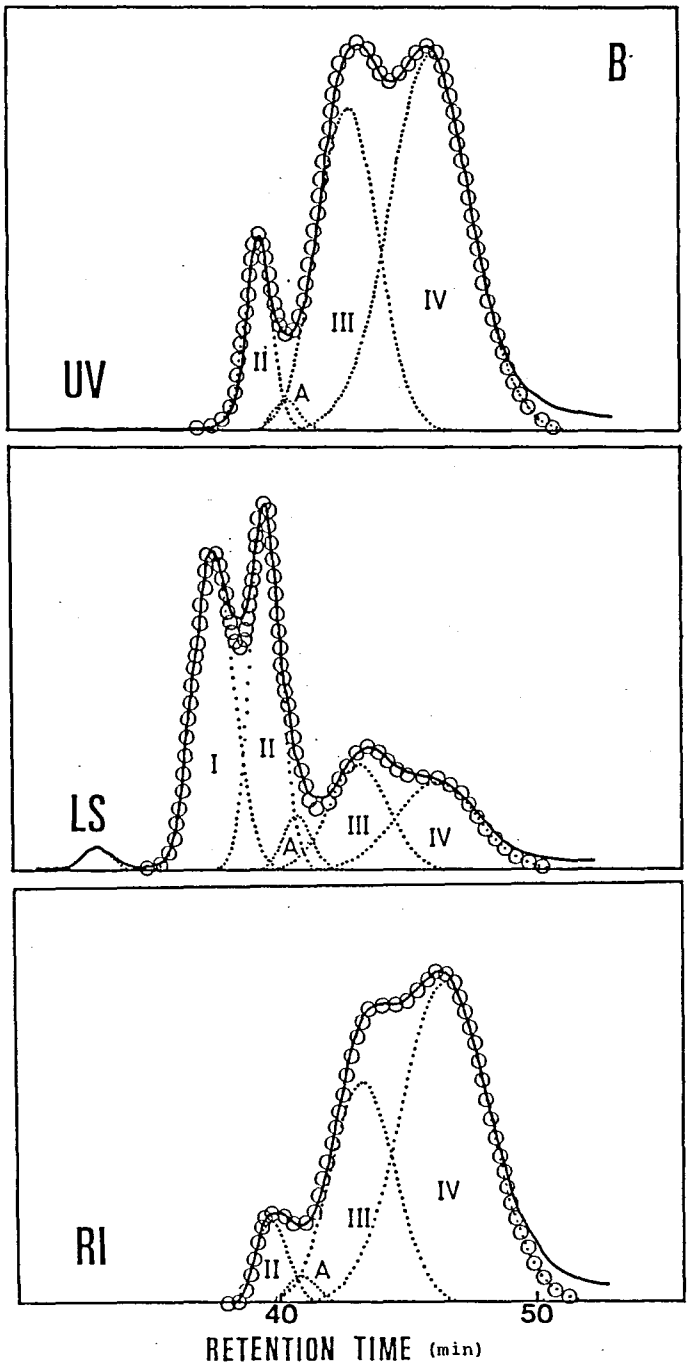


Fig.3-11 (continued)

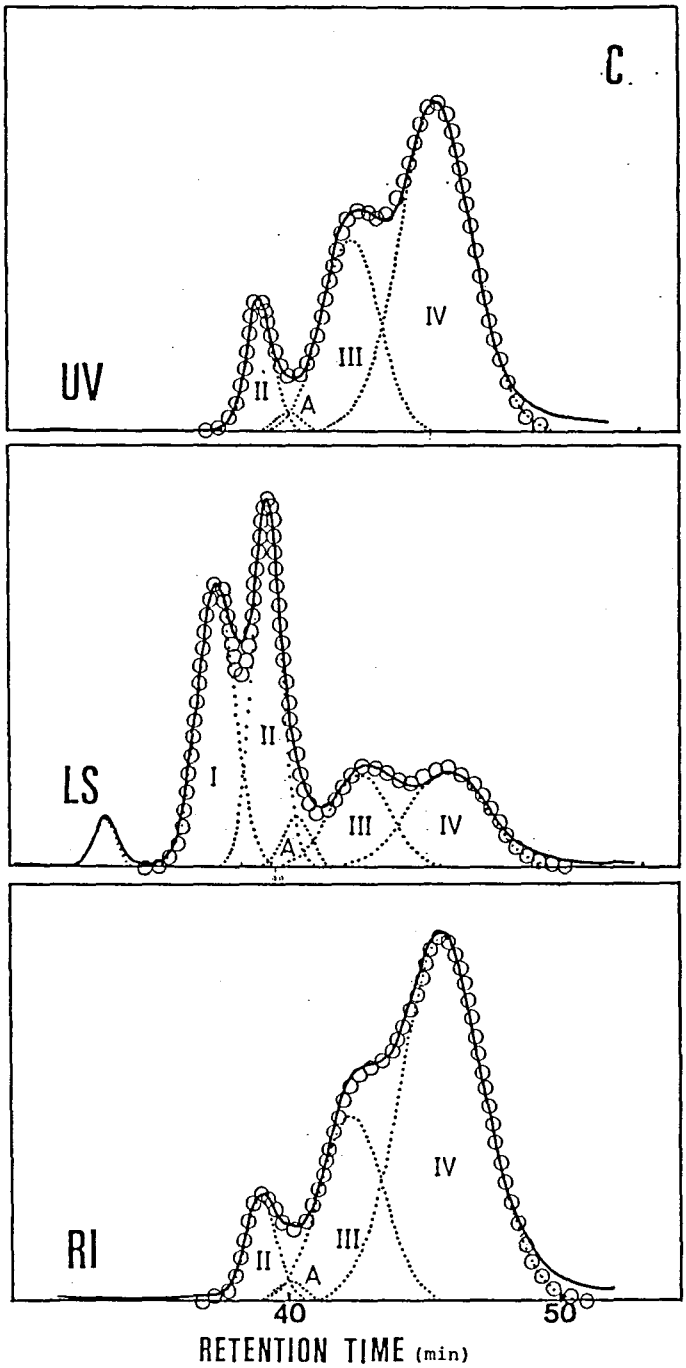


Fig.3-11 (continued)

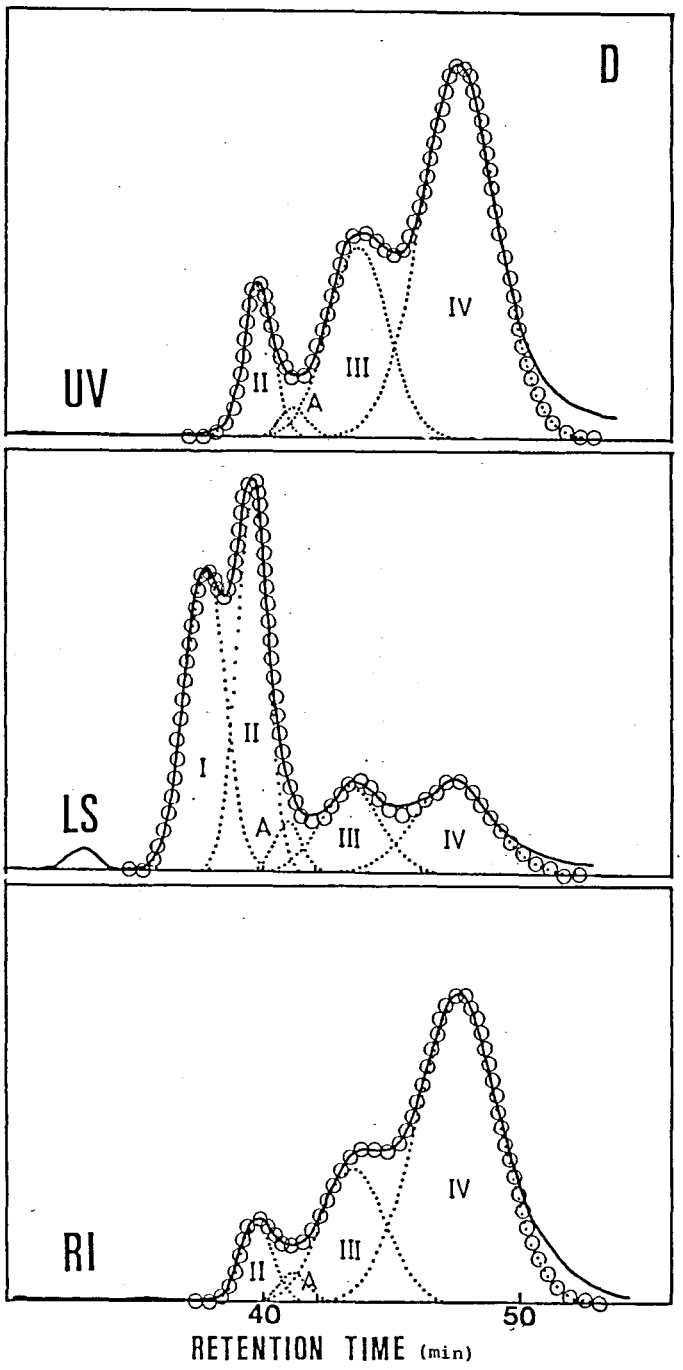


Fig.3-11 (continued)

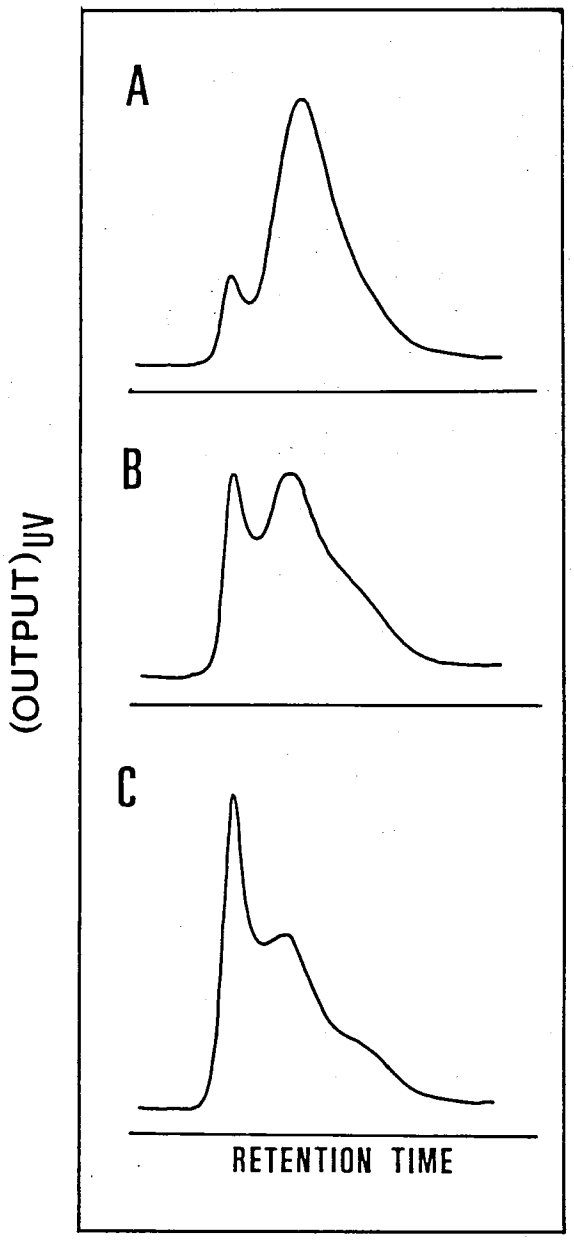


Fig.3-12 Change of elution pattern of (Na^+,K^+)-ATPase obtained by the spectrophotometer with the incubation time after solubilization. Incubation time was (A)35 min, (B)7 hrs and (C)23 hrs. Concentration of $C_{12}E_8$ in the elution buffer was 0.1 mg/ml in all cases.

Table II Molecular Weights of Solubilized (Na^+,K^+)-ATPase Estimated in the Presence of Various Concentrations of $C_{12}E_8$.

$[C_{12}E_8]$ (mg/ml)		35 - 40 min				5.5 - 16.5 hr			
		IV	III	A	II	IV	III	A	II
2	MW(K)	120± 6	295±15	554±28	2,900±150	130± 5	234±12	285±15	1,680±90
	dn_c/dc_p*	0.295	0.250	0.362	0.183	0.332	0.291	0.306	0.197
1	MW(K)	97± 6	210±12	889±51	1,810±100	115± 7	228±13	303±17	1,220±70
	dn_c/dc_p	0.361	0.307	0.336	0.247	0.352	0.303	0.320	0.236
0.5	MW(K)	127±11	277±24	839±74	3,670±320	137±12	297±26	426±38	2,260±200
	dn_c/dc_p	0.313	0.252	0.327	0.163	0.311	0.258	0.331	0.153
0.1	MW(K)	142± 8	259±15	1,350±80	3,280±180	130± 7	258±14	334±19	1,940±110
	dn_c/dc_p	0.335	0.261	0.236	0.173	0.339	0.273	0.298	0.176
Average	MW(K)	122±19	260±37	908±330	2,930±800	121±15	254±31	337±63	1,780±440
	dn_c/dc_p	0.326 ±0.028	0.268 ±0.027	0.315 ±0.055	0.192 ±0.038	0.334 ±0.017	0.281 ±0.020	0.314 ±0.015	0.191 ±0.035
MW relative to 123(±8)K		0.99±0.17	2.1±0.3	7.4±2.7	23.8±6.7	0.98±0.14	2.1±0.3	2.7±0.5	14.5±3.7

* (ml/g)

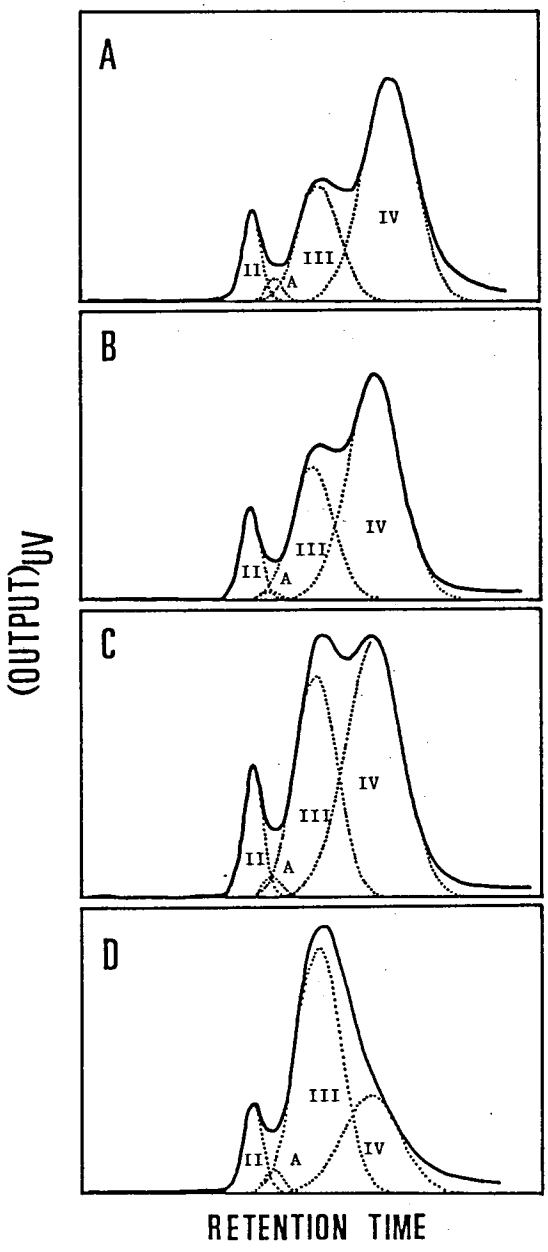


Fig.3-13 Difference of elution patterns obtained by the spectrophotometer with the concentration of $C_{12}E_8$ in elution buffer. The concentration of $C_{12}E_8$ in elution buffer was (A)2.0 mg/ml, (B)1.0 mg/ml, (C)0.5 mg/ml and (D)0.1 mg/ml. Incubation time after solubilixation was 35 - 40 min in all cases.

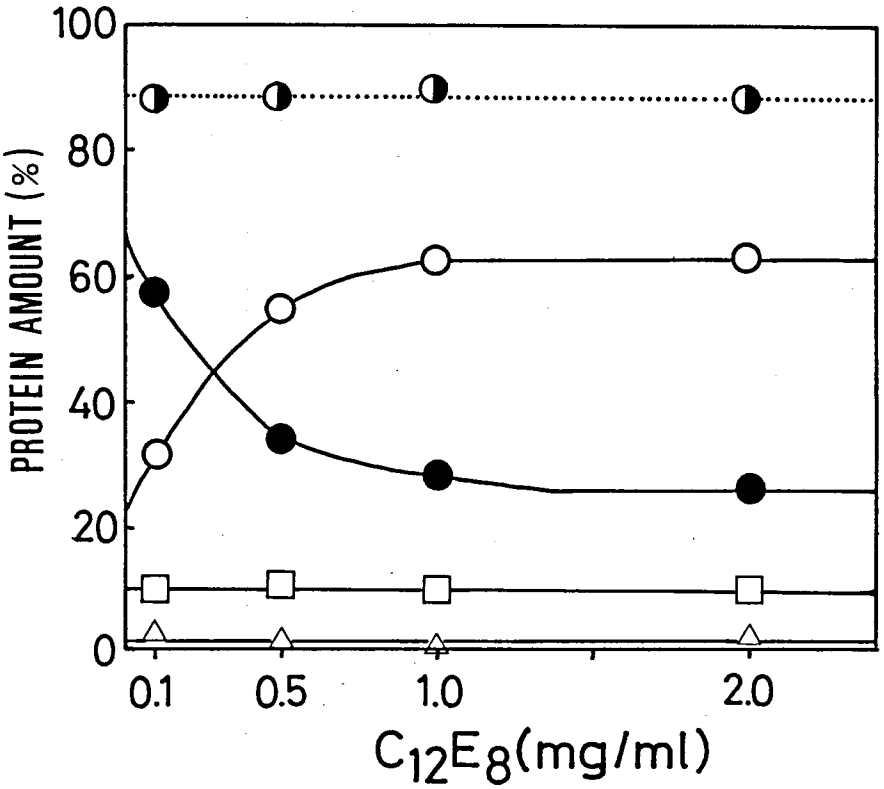


Fig.3-14 Change of the composition of the protein components of solubilized (Na^+, K^+)-ATPase when concentration of $C_{12}E_8$ in elution buffer was variously changed. The amount of each protein was estimated by the same manner as described in the legend of Fig.3-10. Percentage of each protein component to the total amount is plotted against the concentration of $C_{12}E_8$ in elution buffer. Components IV, ○ ; III, ● ; II, □ ; A, △ . Sum of the amounts of components III and IV is plotted as symbol of ◑ .

the effect of concentration of $C_{12}E_8$ in the elution buffer on the population of the components for experiments carried out 35-40 min after solubilization. At 0.1 mg/ml of concentration of $C_{12}E_8$, the amount of $\alpha_2\beta_2$ is much larger than that of $\alpha\beta$-protomer, but this relation was reversed above 0.3 mg/ml. The sum of the amount of these two components was indenpendent of the concentration of $C_{12}E_8$ in the elution buffer. The amount of the species named A and II did not change, but these species seem to be inhomogeneous, because the molecular weights estimated were variable.

As the result of the change of concentration of $C_{12}E_8$, no new molecular species appeared, but the population of the species observed in the previous experiments was influenced.

3-3-7 Constituent polypeptides of (Na^+,K^+)-ATPase

Membrane proteins consisted of subunits are usually dissociated into their constituents in the presence of a denaturing surfactant. The membrane-bound (Na^+,K^+)-ATPase was solubilized by a denaturing surfactant, SDS, and the product was subjected to the high performance gel chromatography in the presence of SDS. These experiments were mainly carried out by Hayashi, our co-worker. Fig.3-15 shows elution patterns obtained by the spectrophotometer. The ATPase preparation was found to

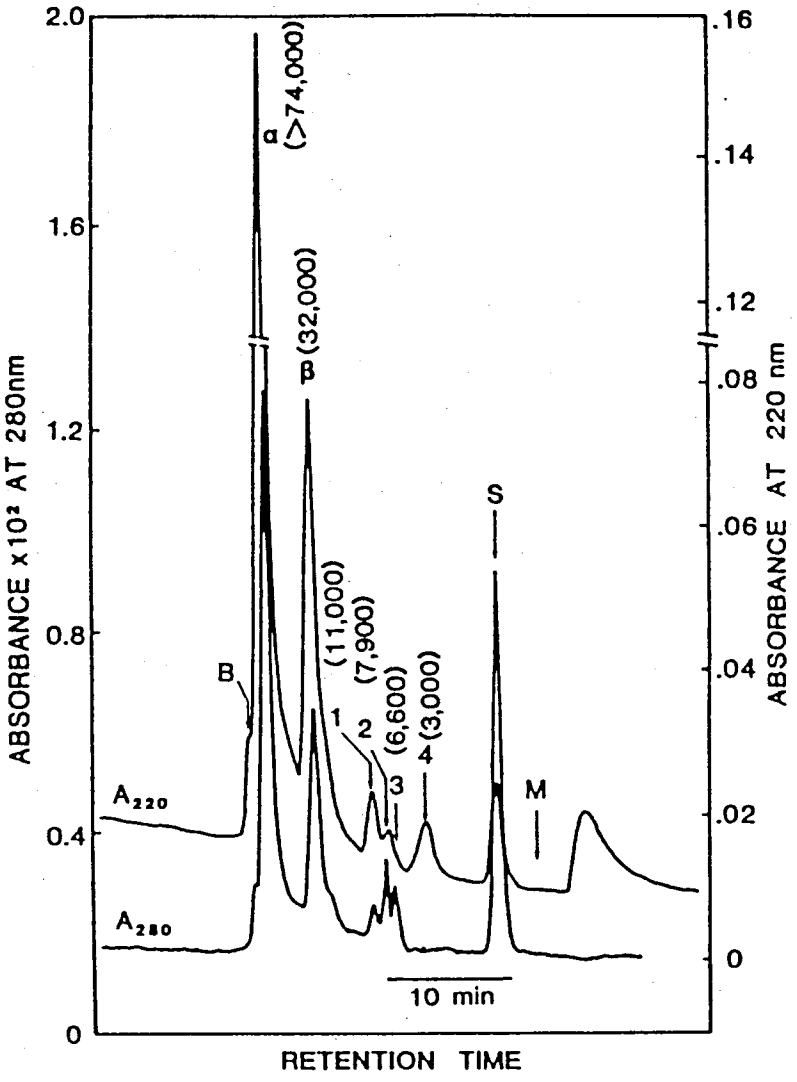


Fig.3-15 Elution patterns of the whole (Na^+,K^+)-ATPase from two columns in the presence of sodium dodecyl sulfate as detected by the spectrophotometer at 280 and 220 nm. The membrane-bound ATPase was dissolved in 0.2 M sodium phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate. The aliquot of 25 µl was applied to the columns equilibrated with the same buffer containing 0.2% sodium dodecyl sulfate, and the columns were eluted with the buffer at a flow rate of 1 ml/min. A double-beam spectrophotometer model UV-140 (Shimadzu Manufac. Co. Ltd.) was used as a monitor at 220 nm. The components other than the α and β chains were designated 1 to 4. Their molecular weights were estimated according to the method reported by Imamura et al. (1981), and shown in parentheses. The elution positions of Blue Dextran, sodium dodecyl sulfate and β-mercaptoethanol are indicated by the arrows labelled B, S and M, respectively.

contain several minor components besides the α and β polypeptide chains. Their molecular weights were estimated according to the correlation between the retention time and the molecular weight which was reported by Imamura et al., (1981) and the value obtained were represented in the parentheses in Fig.3-15. From its absorption characteristics, component 1 was judged to be a polypeptide with a molecular weight of 11,000 and its content was about 5 % of that of α chain based on the absorption at 220 nm. This component seems to correspond to the γ chain, the presence of which has been reported by several groups (Forbush et al., 1978; Reeves et al., 1980 and Hardwicke & Freytag, 1981).

Hayashi has investigated the subunit composition of each component (II to IV in Fig.3-8) by GPC in the presence of SDS. The aliquot of the elution corresponding to components II and IV was collected in a test tube containing solid SDS, and this eluted solution was kept at room temperature overnight. The solution obtained was applied to the GPC column in the presence of SDS. Elution patterns thus obtained are shown in Fig.3-16A. An enormously large peak was observed behind the peak of β chain. It was overlapped by a negative peak which appeared just before it. The composite peak was observed even when a sample solution containing $C_{12}E_8$ but no ATPase was applied to the column. The peak, therefore, can be assigned to mixed micelles probably composed of both $C_{12}E_8$

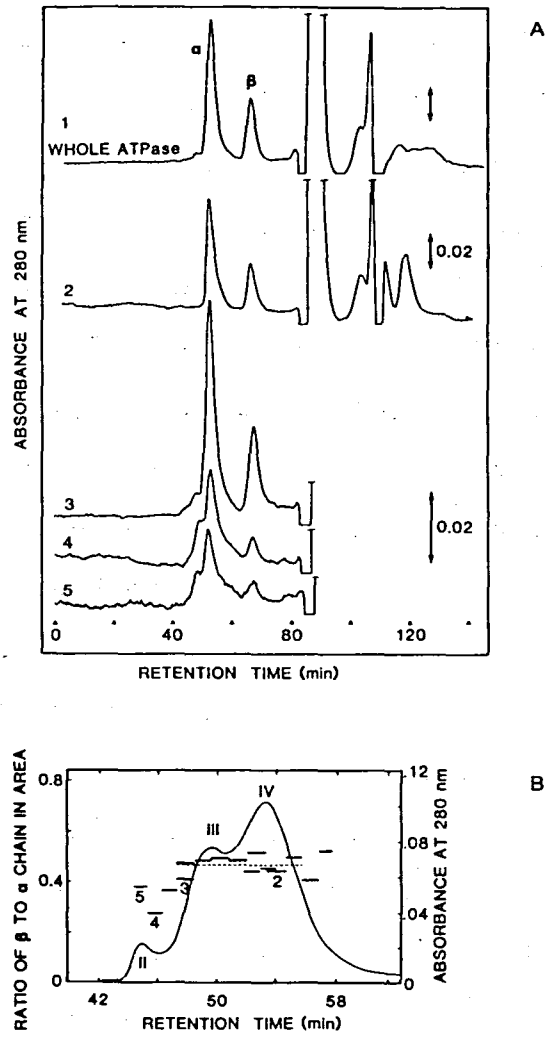


Fig.3-16 Ratio of the β to α chain constituting the protein components of (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$. (A) (Na^+, K^+)-ATPase solubilized by $C_{12}E_8$ was applied to the column in the presence of $C_{12}E_8$ in the same way as described in Fig.3-8. After the chromatography, each fraction of eluate was mixed with solid sodium dodecyl sulfate and then applied to the column in the presence of sodium dodecyl sulfate. The numbers 2 to 5 labelling the observed elution patterns correspond to those for the fractions of eluate shown in (B). Whole (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ was treated with sodium dodecyl sulfate and directly applied to the column in an identical manner. Its elution pattern is shown as number 1 in the top position. (B) Area under peaks of the α and β chains for the fractions were estimated from their patterns obtained in (A). The β/α area ratios were plotted against the fractions of eluate. The results obtained from two separate experiments are illustrated together. The dotted line represents the average ratio (0.471 ± 0.032, n=10) for protein components III and IV, corresponding to the retention time indicated by line length.

and SDS. Both the high refractive index and the attenuation of light intensity due to light scattering and ultraviolet absorption seem to be responsible for the abnormal shape of the peak. The elution profiles of component III (Fig.3-16A(3)) and component IV (Fig.3-16A(2)) were virtually identical to that for the whole enzyme (Fig.3-16A(1)). Ratio of areas of the α to β chains for both components III and IV was 1 : 0.471 ± 0.032 (S.E., n=10) (Fig.3-16B) which was the same as that (0.468 ± 0.023, n=2) for the whole enzyme. Although the elution patterns for components II and A (Fig.3-16A(5) and (4), respectively) were similar to those of components III and IV, the β/α ratios obtained for components II and A became inaccurate because of the increase of areas under peaks other than the α and β peaks.

As shown in Table III, the α/β mass ratios of components III and IV as well as that of the origial membrane-bound enzyme were calculated to be 2.96 from both values of 2.13 for the α/β area ratio (Fig.3-16) and 0.72 for the α/β extinction coefficient ratio at 280 nm (Freytag and Reynolds, 1981; Hayashi et al., 1977). From this weight ratio, the α/β molar ratio of components III and IV as well as that of the original enzyme is concluded to be 1:1 based on the molecular weights of 93,900 for the α chain and 32,300 for the β chain reported for the pig kidney (Na^+,K^+)-ATPase by Freytag and Reynolds (1981)

(Table III). Furthermore, the subunit compositions of components III and IV could be concluded unambiguously to be $\alpha_2\beta_2$ and $\alpha\beta$, respectively, based on the molecular weights of the components determined in the present study (Table I). The subunit compositions of components A and II are suggested to be $(\alpha\beta)_{6-8}$ and $(\alpha\beta)_{12-16}$, respectively, by comparing their molecular weights with that of component IV, assuming that the α/β area ratio under peaks of ultraviolet absorption at 280 nm is essentially the same as that for components III and IV.

Table III Stoichiometric Composition of α and β Polypeptides Constituting the Major Components (IV and III) of (Na^+,K^+)-ATPase Solubulized by $C_{12}E_8$.

Ratio of α to β polypeptide			Molecular weights[b] of α and β polypeptides			
			Component IV (123,000 ± 8000)		Component III (286,000 ± 30,000)	
in area under A_{280} peak[a]	in E_{280}	in weight	α	β	α	β
2.13 ± 0.14[c]	0.72[d]	2.96 ± 0.12	91,900±9,500	31,100±2,500	214,000±28,000	72,200±8,300
Relative[e]			0.98 ± 0.10	0.96 ± 0.08	2.28 ± 0.30	2.24 ± 0.26
Polypeptide stoichiometry concluded			$\alpha_1\beta_1$		$\alpha_2\beta_2$	

[a] Determined in this work (Fig.3-16).

[b] The molecular weights were calculated by multiplying the molecular weight of each of the components by weight fraction of the α or β polypeptides.

[c] All the errors except for this were calculated based on the propagation of the observed standard errors in the estimations of area ratios of α to β polypeptide and of molecular weights of components IV and III.

[d] Average value of the ratios obtained for dog kidney enzyme (0.60) [Hayashi et al.(1977)] and for pig kidney enzyme (0.83) [Freytag and Reynolds (1981)].

[e] The relative amounts of polypeptide are shown in comparison with molecular weights of 93,900 and 32,300 for the α and β chains, respectively [Freytag and Reynolds (1981)].

3-4 Discussion

Since a membrane protein solubilized by a nonionic surfactant is expected to retain its oligomeric structure in the native state (Helenius & Simons, 1975), the first step for elucidation of the subunit composition is the molecular weight determination of the protein complexed with the surfactants. We have succeeded in application of the GPC/LALLS technique to molecular weight estimation of membrane protein solubilized by nonionic surfactant, $C_{12}E_8$ (details were described in chapter 2).

In order to investigate the subunit composition of (Na^+,K^+)-ATPase of canine kidney, molecular weight measurement of the enzyme solubilized by $C_{12}E_8$ was carried out with the GPC/LALLS technique. As reported by Brotherus et al. (1981), various species were obtained by solubilization of membrane-bound (Na^+,K^+)-ATPase and the population of the species changed with the incubation time after solubilization. Since a high performance GPC column is used in the measuring system of the GPC/LALLS technique, the sample solution containing several species could be fractionated during the passage through the column. Accordingly, the molecular weights of all species contained in a sample solution of sulubilized ATPase could be estimated simultaneously by the GPC/LALLS technique. It is a great advantage of this technique that molecular weight measurement for such sample can be effectively

carried out.

The elution patterns obtained when solubilized ATPase was applied to the measuring system of the GPC/LALLS technique are shown in Fig.3-7. Since the separation of components was imcomplete, we were forced to resolve the elution patterns into a set of Gaussian distriubtion curves each corresponding to the contribution of a component using the computer program SALS (Fig.3-8).

The smallest number of Gaussian distribution curves thus obtained are shown in Fig.3-8 as dotted lines. As seen from the Gaussian distribution curves in the tracing of the refractometer shown in Fig.3-8A, the component III and IV were major products obtained by the solubilization of ATPase. The total amounts of these component correspond to 70 % of whole products. In addition to these two components, components I, II, A and B were obtained.

The ratios of the output of the LALLS photometer to that of the refractometer for components II-IV could be determined definitely. Therefore, reliability of the molecular weight estimated from Eq.3-3 is dependent on the accuracy of the value of specific refractive index increment determined from Eq.3-2. The output ratio of $(\text{Output})_{RI}/(\text{Output})_{UV}$ in the equation could also be determined definitely. Thus, the reliability of molecular weight ultimately depends on the accuracy of the extinction coefficient for the components of the solubilized ATPase. The two major components IV and III,

the first and the second in abundance on the basis of weight were concluded to be solely composed of the α and β chains in an equal molar ratio (Hayashi, personal communication). We have not yet been able to determine the extinction coefficient for each of the two chains, and were forced to adopt the value, 1.22 ± 0.05 (ml mg^{-1} cm^{-1}) determined for the whole enzyme solubilized. The whole enzyme contains other minor components 1, 2, 3 and 4 in addition to the α and β chains (Fig.3-15). If the area under each of the peaks in the elution pattern obtained by monitoring at 220 nm was assumned to be proportional to the weight fraction of the component, the presence of the minor components having ultraviolet absorption would bring about an error of at most 9 % in the determination of the extinction coefficient for the protein unit composed of the α and β chains. The above extinction coefficient is in close agreement with the values scattered around 1.26 (ml mg^{-1} cm^{-1}) reported for the pig kidney enzyme (Brotherus et al. , 1981) and the rabbit kidney enzyme (Peter et al., 1981a) by the weight-averaged summation of the coefficients of the α and β chains of the respective enzyme which were determined on the basis of quantitative amino acid analysis.

Among the six components resolved, the parameter for component B could not be estimated quantitatively due to the small amplitude of the corresponding peak. This component may arise from the α and β chains dissociated

from αβ-complex since each chain isolated was eluted at the nearly same retention time as that of component B when each of the polypeptide chains was subjected to the chromatography in an identical manner (Hayashi et al., unpublished data).

Component A, like component B, was not directly observed as an actual peak. However, all elution patterns given by the LALLS photometer, the differential refractometer and the spectrophotometer could not be simulated very well by the computer program SALS until the presence of component A was assumed. This fact seems to show that component A is not an artificial peak but a reflection of the presence of protein(s) which cannot be identified now. The value of dn_c/dc_p for component A, however, was exceptionally low. The parameters specifying component A are greatly influenced depending on the sizes of trailing and preceding edges of the components II and III respectively. These circumstances would make the parameters of the component A less reliable than those of the other major components. The exceptionally low value of dn_c/dc_p for component A may be explained by the circumstances mentioned above. The component IV is most abundant and least overlapped with neighbors. The Gaussian distribution curve presumed for the component IV is, therefore, most reliable among those for the major components.

Stoichiometry in the binding of specific ligands to

(Na^+,K^+)-ATPase have been determined on the weight basis of the protein determined by quantitative amino acid analysis. The stoichiometry gave one ATP site (Moczydlowski & Fortes, 1981; Matsui et al., 1983), one ouabain site, three Na^+ sites and two K^+ sites (Matsui et al., 1983) or two Rb^+ sites (Jorgensen & Petersen, 1982) per 160,000 to 175,000 daltons of protein. One phosphorylated intermediate was produced from ATP (Peters et al., 1981b) or inorganic phosphate (Hayashi,Y. et al., unpublished results) per 170,000 to 180,000 daltons of protein. The size thus estimated for the smallest ligand-binding protein unit is a little larger than the molecular weight of 123,000 $\pm$ 8,000 estimated for the $\alpha\beta$-protomer in the present study. The discrepancy might be ascribed both to the presence of polypeptide(s) other than the α and β chains such as the γ chain which is included in the membrane-bound enzyme, but not in the enzyme exposed to nonionic surfactant such as Brij 58 (Hardwicke & Freytag, 1981), and to the contamination of the samples by the inactivated enzyme (Hayashi & Post, 1980). Therefore, it would be concluded that the $\alpha\beta$-protomer is the smallest protein unit capable of stoichiometrically binding ligands as described above, or is the main portion of the membrane-bound enzyme.

The ATPase solubilized by $C_{12}E_8$ under the present conditions contained the $\alpha\beta$-protomer and its dimer as the major components. Higher oligomers composed of $(\alpha\beta)_{6-8}$ and

$(\alpha\beta)_{12-16}$ could also be detected and accounted for about 10 % of the total protein. These oligomers other than the $\alpha\beta$-protomer increased in amount with the storage time after the solubilization of ATPase (Fig.3-10). These results are consistent with those reported by Brotherus et al. (1981). They showed by sedimentation velocity analysis that the molecular weight of the major protein component of the solubilized ATPase was 17,000 ± 9,000 and that the component aggregated with storage time in a solution of $C_{12}E_8$. Pappert and Schubert (1982) reported that Band 3 protein of erythrocyte membrane solubilized by a surfactant, nonaethyleneglycol dodecyl ether, homologous to $C_{12}E_8$, was in an association equilibrium of monomer and oligomers, and that the protein was converted into stable dimer during storage with the surfactant. They attributed the aggregation to the formation of cross-linkages by the action of peroxides (Lever, 1977) formed from the surfactants (Pappert & Schubert, 1982). The results for (Na^+,K^+)-ATPase in the present study and that for Band 3 protein (Pappert & Schubert, 1982) are very similar with respect to the aggregation of protomeric protein units in nonionic surfactant derived from polyether. The mechanism of aggregation suggested for the Band 3 protein might be true also in the case of (Na^+,K^+)-ATPase.

Craig (1982a,b) showed by cross-linking of protein units followed by gel electrophoresis that various oligomers with $(\alpha\beta)_n$ composition (n=1 to 5) were produced

in kidney (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ or Lubrol. His results were consistent with the present ones with respect to the presence of various oligomeric proteins in the solubilized ATPase. However, the content and kind of oligomers differ significantly depending on the analytical technique used. The present method is more reliable than the corss-linking method used by Craig (1982a,b), since the former is more direct and non-destructive than the latter. The ionic environments of the solubilized enzyme, however, are not exactly the same. Therefore, the possibility cannot be denied that the discrepancy reflects the dependence of the oligomer composition on the salt composition in the solvent used, as suggested by Nakao et al. (1983a).

Hayashi has analyzed ATPase activity of component IV and III, namely the αβ-protomer and its dimer. From his results, both of these two components retained no ATPase activity. This result seems to be at variance with recent claims that the protomeric (Brotherus et al., 1981; Craig, 1982b) or dimeric unit (Hasting & Reynold, 1979; Esmann et al., 1980) of the solubilized ATPase retains its activity. However, Hayashi also showed that ATPase solubilized by $C_{12}E_8$ significantly retained the activity over a period of at least 8 hrs before the fractionation by the GPC (personal communication). It is known that the phospholipids have been shown to be essential for the enzymatic activity of (Na^+,K^+)-ATPase (Tanaka &

Strickland, 1965; Fenster & Copenhaver, 1967; Taniguchi & Tonomura, 1971; Ottolenghi, 1979; Hegyvary et al., 1980). As shown in Fig.3-6, components IV and III contained less phospholipid. The lack of ATPase activity in these component is presumably explained as the result of elimination of phospholipid from them. The almost complete delipidation may be ascribed to the chromatographic fractionation under the conditions adopted in the present study (higher temperature, higher ionic strength, treatment and fractionation using higher concentration of $C_{12}E_8$, and lack of any stabilizing agents, such as glycerol and potassium chloride), which are severer than those adopted by other investigators (Hastings & Reynold, 1979; Esmann et al., 1979; Esmann et al., 1981; Nakao et al., 1983a)

Nakao et al. (1983b) have investigated subunit composition of solubilized (Na^+,K^+)-ATPase from pig kidney by the GPC/LALLS technique and reported, without measuring molecular weight, that αβ-protomer and its trimer were obtained when the enzyme was solubilized by $C_{12}E_8$. In their experiments, 100 mM N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) - Tris buffer solution containing 0.1 mg/ml of $C_{12}E_8$ and 25 mM sodium chloride was used as an elution buffer. We thought that the difference of their result from ours was due to the difference of the concentration of $C_{12}E_8$ in elution buffer which related to the extent of delipidation during the

passage through the column. As seen in Fig.3-6, most phospholipids separate from the enzyme to form mixed micelles with $C_{12}E_8$. The results of the preliminaly experiment that the concentration of $C_{12}E_8$ in the elution buffer is variously changed, namely, 0.1, 0.5 or 0.2 mg/ml, show that the components of ATPase obtained are common in all cases and only a change of the population of the components was observed (Table II). We could not confirm the presence of the component, $\alpha_3\beta_3$, which had been reported to be the major one in a preparation of ATPase solubilized by $C_{12}E_8$ (Nakao et al., 1983b).

It can be concluded safely from the results of the present study that the basic structural unit of (Na^+,K^+)-ATPase is the αβ-protomer with a molecular weight of 123,000 ± 8,000. This unit accounted for 70 % of the total protein in the solution of (Na^+,K^+)-ATPase solubilized under the present conditions. As much as three quarters of the ATPase activity of intact enzyme could be restored by dilution of the solubilized ATPase into 250 volumes of the assay medium. It would not always be correct, however, to conclude from these results that the minimum functional unit is the αβ-protomer rather than the $\alpha_2\beta_2$-dimer. This is because there are possibilities that the protomer would be significantly transformed into the dimer and higher oligomer(s) by a reduction in the concentration of $C_{12}E_8$ and/or by contact with substrates, and that the resultant oligomer(s) would manifest the

activity, as suggested by Craig (1982a). Therefore, it cannot be determined from the present results whether the minimum functional unit for the ATP-hydrolysis is the αβ-protomer or its dimer.

The results obtained in the present study clearly demonstrate the unrivaled efficiency of the measuring system composed of a high performance gel chromatography column, a LALLS photometer, a precision differential refractometer and a spectrophotometer each equipped with flow-type cells, for investigating the molecular composition of membrane proteins solubilized by a surfactant.

4 Application of the GPC/LALLS technique to the estimation of molecular weight of glycoprotein

Summary

The molecular weights of human α_1-acid glycoprotein and chicken ovomucoid, both with high carbohydrate contents, were determined by an improved GPC/LALLS technique. The molecular weights thus obtained were in excellent agreement with those determined by the amino acid sequential and carbohydrate compositional analyses. It was recognized that monitoring elution from a GPC column by a spectrophotometer, a LALLS photometer and a precision differential refractometer is a versatile way to determine protein molecular weight.

4-1 Introduction

Proteins are often associated with non-protein substance covalently or non-covalently. Glycoproteins are formed by conjugation of protein polypeptide with carbohydrate through covalent bonds. They occur in cells, both in soluble and membrane-bound form, as well as in intercellular matrix and in extracellular fluids. Actually, most membrane proteins are glycoproteins. The carbohydrate content of glycoproteins varies from less than 1 % to over 85 % of the total weights.

In spite of remarkable advances in our knowledge of metabolism of glycoproteins, little is known about the contribution of the carbohydrate moieties to functions of the proteins. As the first step in understanding the functional importance of the covalently bound carbohydrate, a glycoprotein must be accurately characterized chemically and physically.

Difficulties are often encountered in the physicochemical characterization of glycoproteins. First is the question of molecular homogeneity. Although simple proteins are generally monodisperse, it is not always the case with glycoproteins, which very often exhibit molecular heterogeneity with respect to the carbohydrate moiety. Such glycoproteins migrate on SDS-polyacrylamide gel electrophoresis as a diffuse band, making it impossible to assess their molecular size. Second is the

pitfall in estimation of their molecular weight by empirical methods. Estimation of protein molecular weight was generally carried out by convenient methods depending on the molecular sieving effect, such as gel chromatography and gel electrophoresis, because these methods enable estimation of molecular weights of several or more proteins by a single run using very small amounts of samples. As mentioned in section 1-2, there are great difficulties in estimation of the molecular weight of a glycoprotein by such methods even in cases where polydispersity does not occur. On the other hand, classical physicochemical methods give the molecular weight accurately, but can not satisfy the demand of both time and sample saving.

Glycoproteins containing a substantial amount of carbohydrate behave anomalously during gel chromatography and SDS-polyacrylamide gel electrophoresis. On gel chromatography, most proteins give a linear relationship between their elution volumes and the logarithm of their molecular weights. Glycoproteins do not conform to the above relationship. On SDS-polyacrylamide gel electrophoresis, glycoproteins migrate at a rate slower than that would be expected from their molecular weights.

Primary requirements for estimation of protein molecular weight by these empirical methods is that the effective hydrodynamic volume of a polypeptide is a unique function of its chain length and, hence, its mass.

SDS-polyacrylamide gel electrophoresis additionally requires that the electrostatic charge on the polypeptide-SDS complex must be proportional to the mass of polypeptide. These conditions are generally obtained for linear polypeptides complexed with SDS in the normal fashion, namely the amount of bound SDS is 1.4 g per g of protein polypeptide (Reynolds & Tanford, 1970a,b). However, glycoproteins have non-polypeptide substance, namely carbohydrate, and behave like branched-chain polymer. Anomalous behavior of glycoproteins mentioned above in the empirical methods is the result of difference of structure of glycoprotein from that of simple globular protein. The use of gel chromatography or SDS-polyacrylamide gel electrophoresis for the purpose of molecular weight estimation of glycoprotein should be precluded or employed with great caution.

We have succeeded in application of the GPC/LALLS technique to determination of the molecular weight and subunit composition of membrane protein solubilized by surfactants as shown in chapters 2 and 3. The technique has been successfully applied to membrane proteins solubilized by surfactant (Kameyama et al., 1982; Imamura et al., 1982) and to the proteins of calf eye lens (Bindles et al., 1982). This technique is not only as accurate as the classical physicochemical methods but also as convenient as the methods depending on the molecular sieving effect, as mentioned previously.

We have tried further to improve the technique to established it as a most reliable and versatile technique for determination of protein molecular weight. The measuring system was made more sophisticated by the addition of a spectrophotometer to the system. In this chapter, we present the standard instrumentation and procedure of the improved technique. As described above the molecular weight of glycoprotein can not be estimated by techniques depending on the molecular sieving effect such as gel chromatography and SDS-polyacrylamide gel electrophoresis. We thought, therefore, that the high performance of our improved measuring system would be most persuasively demonstrated if it could determine the molecular weight of typical examples of such proteins. Human α_1-acid glycoprotein and chicken ovomucoid, both with high carbohydrate content, were selected as the examples. The molecular weight of the glycoproteins thus obtained showed excellent agreement with those determined by the amino acid sequential and the carbohydrate compositional analyses. Monitoring of the elution from a GPC column by a spectrophotometer, a LALLS photometer and a precision differential refractometer is recognized to be a powerful tool for determination of protein molecular weight.

4-2 Experimental procedures

Molecular weights of proteins were measured with a system consisted of a spectorophotometer, a LALLS photometer and a precision differential refractometer each equipped with a flow-through cell. Outline of the instrumentation is shown in Fig.1-6. Specification of the equipments are described in the legend to the figure. Measurements were carried out at room temperature ($25 \pm 2^{\circ}C$).

Both human α_1-acid glycoprotein (Lot 81F-9630) and chicken ovomucoid (Lot 60F-8110) were obtained from Sigma Chemical Co.. Bovine serum albumin (66,267(Reed et al., 1980), 0.670 $ml \cdot mg^{-1} \cdot cm^{-1}$), hen's ovalbumin (42,700 (McReynolds et al., 1978), 0.735), bovine carbonic anhydrase (29,000 (Sciaky et al., 1976), 1.90) and bovine pancreatic ribonuclease A (13,700 (Smyth et al., 1963), 0.706) were best commercially available products, and assumed to have molecular weights and extinction coefficients at 280 nm (Fasman, 1976) mentioned in the parentheses.

4-3 Results

4-3-1 Standard procedure

As has been described in chapter 1, molecular weight of a protein can be determined according to the following equation,

$$M = k' \, (dn/dc)^{-1} \, (Output)_{LS} / (Output)_{RI} \qquad (4\text{-}1)$$

where the notations are molecular weight, constant determined by instrumental and experiemntal conditions, specific refractive index increment, output of the scatteirng photometer and that of the refractometer from left to right. The specific refractive index increment can be estimated according to the following equation,

$$dn/dc = k'' \, E \, (Output)_{RI} / (Output)_{UV} \qquad (4\text{-}2)$$

where the newly appeared notations on the right side are constant, extinction coefficient expressed in terms of weight concentration and output of the spectrophotometer from left to right. Thus, the molecular weight can be expressed as follows,

$$M = k \, E^{-1} \, (Output)_{UV} \, (Output)_{LS} / (Output)_{RI}^{-2} \qquad (4\text{-}3)$$

The constant, k, can be estimated by monitoring the elution of a standard protein with known molecular weight and extinction coefficient. To ensure the accuracy, a mixture of four standard proteins were applied to the

measuring system. Fig.4-1 shows the elution curves thus obtained.

4-3-2 Measurement of molecular weights of glycoproteins

Fig.4-2a and b show the elution curves of human α_1-acid glycoprotein and chicken ovomucoid, respectively, recorded by the three detectors shown in Fig.1-6. Each of the glycoproteins gave a single major peak.

Three elution curves explicitly show the high resolution, sensitivity and stability of the measuring system used in the preset study.

A plot of the values of the right side of Eq.4-3 excepting the constant, k, for standard proteins with known molecular weights and extinctin coefficients gives a universal calibration line with inclination equal to k. Molecular weight of a protein thus can be determined uniquely when its accurate extinction coefficient is available. The line A in Fig.4-3 was obtained using the data shown in Fig.4-1. The peak heights were adopted as the measures of the corresponding outputs. The instrumental constant, k, in Eq.4-3 was fairly stable. It was sufficient to determine the k value once a day in a series of experiments during which the solvent was stationarily flowing through the system. The lines B and C in Fig.4-3 were obtained in different series of experiments carried out to determine molecular weights of α_1-acid glycoprotein and ovomucoid, respectively.

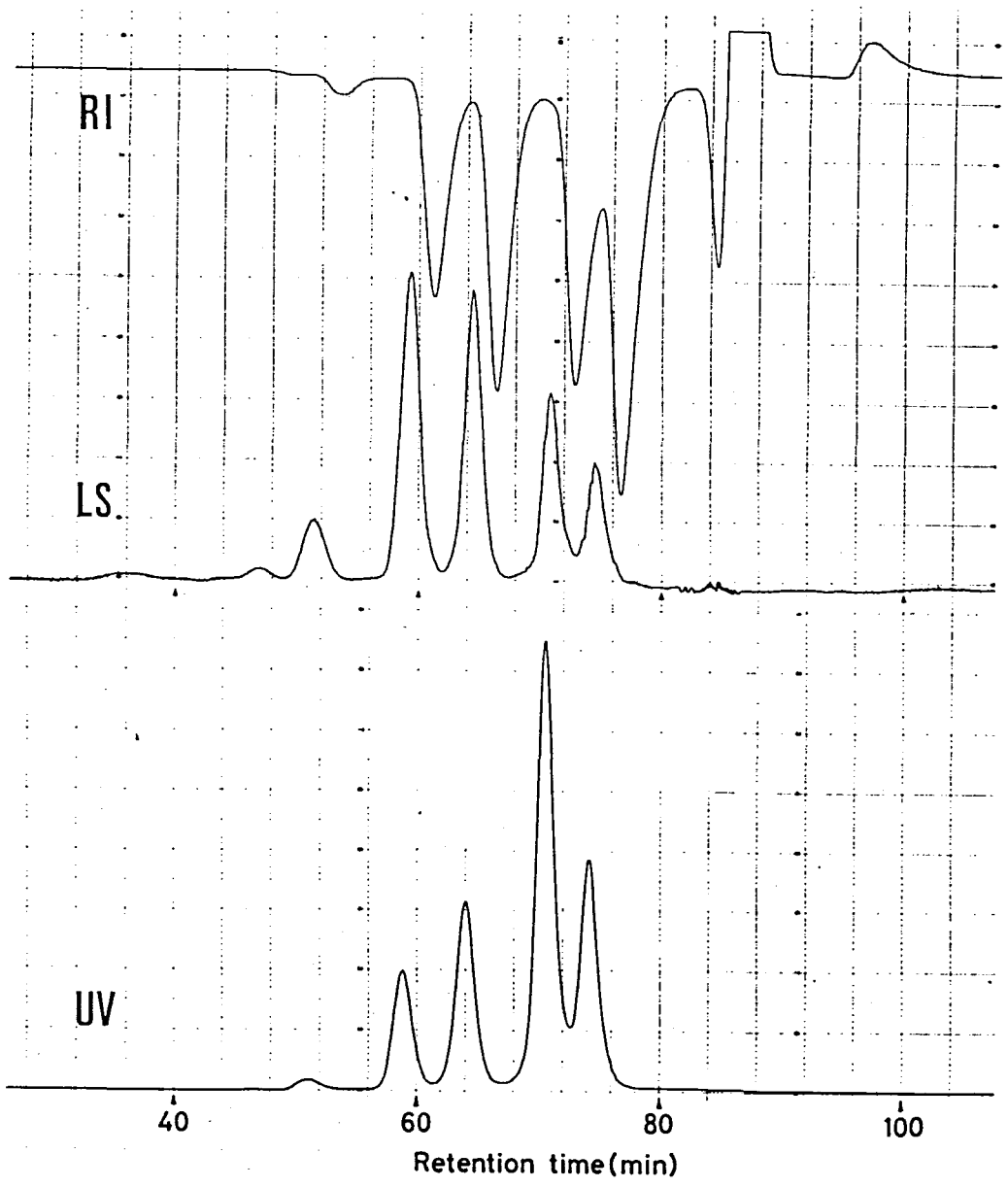


Fig.4-1 Elution patterns of the mixture of four standard proteins obtained by the spectrophotometer at 280 nm (UV), the LALLS photometer (LS) and the differential refractometer (RI). The sample contained bovine serum albumin (330 µg), ovalbumin (440 µg), carbonic anhydrase (550 µg) and ribonucrease A (610 µg) in 100 µl of the elution buffer. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4, 0.2 M sodium chloride and 0.02 % sodium azide, pH 7.1. Gain setting: spectrophotometer, 1.28 fs; LALLS photometer, 32; refractometer, 128. Flow rate: 0.33 ml/min.

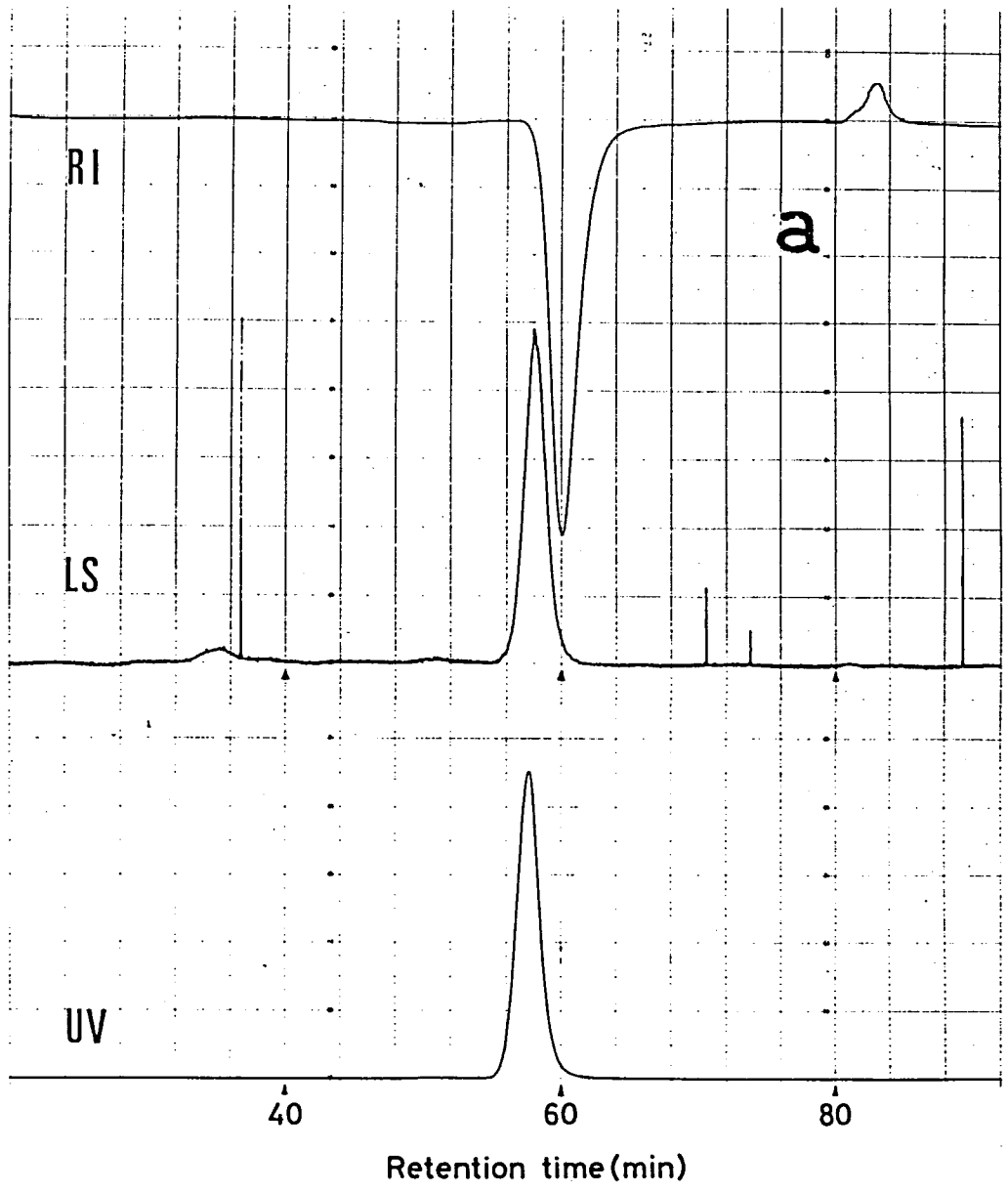


Fig.4-2 Elution patterns of (a)α_1-acid glycoprotein and (b) ovomucoid recorded by the spectrophotometer at 280 nm (UV), the LALLS photometer (LS) and the differential refractometer (RI). The sample contained (a)0.5 mg of α_1-acid glycoprotein and (b)1.0 mg of ovomucoid in 100 µl of the eluant. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4, 0.2 M sodium chloride and 0.02 % sodium azide, pH 7.1. Gain setting: spectrophotometer, 1.28 fs; LALLS photometer, 32; refractometer, 128. Flow rate: (a)0.33 ml/min; (b)0.31 ml/min.

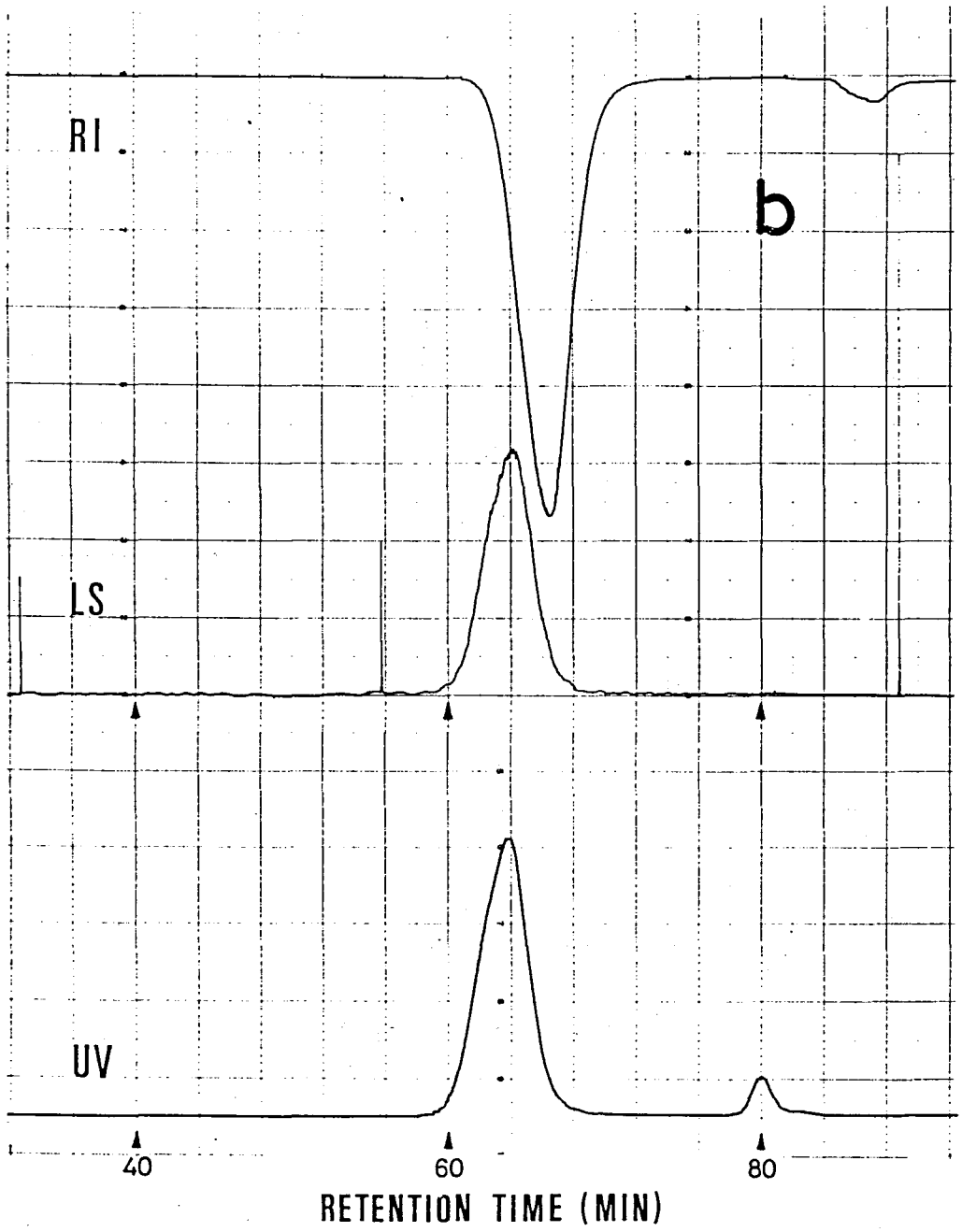


Fig.4-2 (continued)

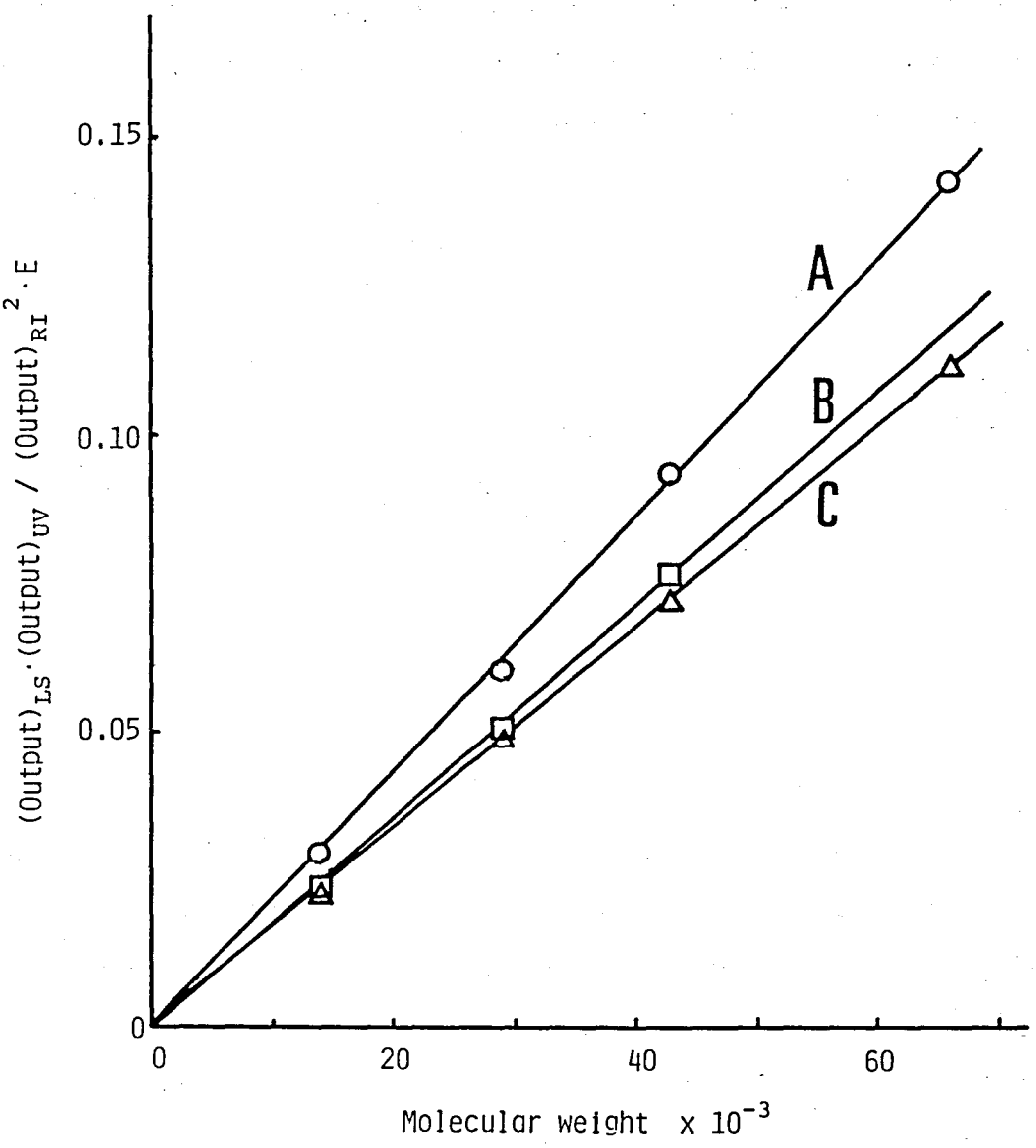


Fig.4-3 Calibration lines for molecular weight determination of proteins. For details, see text.

The extinction coefficients of α_1-acid glycoprotein and ovomucoid were assumed to be 0.881 and 0.406 $ml \cdot mg^{-1} \cdot cm^{-1}$ at 280 nm, respectively, which were calculated using the reported values at 278 nm (Schmid, 1953; Donovan, 1967) and their absorption spectra. The average value of E^{-1} $(Output)_{UV}$ $(Output)_{LS}$ $(Output)_{RI}^{-2}$ in Eq.4-3 was evaluated from experimental data like Fig.4-2. The height of a peak was taken as the output for the each of the detectors. The molecular weights of α_1-acid glycoprotein was thus determined to be 38,300 $\pm$ 400 (n=4) by using the line B in Fig.4-3 as the calibration line. The molecular weight of ovomucoid was also determined to be 25,000 $\pm$ 500 (n=7) by using the line C.

The peaks of ovomucoid in Fig.4b shared a common feature of each having an obscure shoulder in their fronts. Since the light scattering photometer emphasizes the presence of a species with higher molecular weight, the above feature precludes this possibility. In fact, no heterogeneity in molecular weight was detected in the region around the top of the peaks. One possible explanation might be the incomplete resolution of ovomucoid with a nature of acidic protein according to its heterogeneity in charge (Lin & Feeney, 1972) by the TSK-GEL column which is expected to show ionic-exclusion effect for such a protein (Takagi et al., 1981).

4-4 Discussion

Molecular weight of human α_1-aicd glycoprotein was determined to be 38,300. The molecular weight of the protein moiety of the glycoprotein has been determined to be 21,270 by the sequence study (Schmid et al., 1973). The carbohydrate content of the glycoprotein was calculated to be 44.1 % (w/w) (Li et al., 1983) based on the result of the study of the primary structure of the carbohydrate moieties (Fournet et al., 1978). Thus the molecular weight of the glycoprotein can be assumed to be 38,000. The value obtained in the present study is in excellent agreement with the above value.

Various techniques have been applied to the determination of the molecular weight of human α_1-acid glycoprotein. Gel chromatography gave an extremely higher estimate, 70,000, in the absence of denaturant (Kawasaki et al., 1966) and a lower estimate, 31,500, in the presence of 6 M guanidine hydrochloride (Ui, 1981). In the present experiments, the glycoprotein showed elution behavior comparable to that of bovine serum albumin in the phosphate buffer in the absence of denaturant. This is in agreement with the observation of Kawasaki et al. (1966). Gel electrophoresis in the presence of SDS gave values varied from 53,000 to 40,000 with increase of the concentration of polyacrylamide gel (Leach et al., 1980a). Conventional physicochemical techniques using equipments

such as an osmometor, an ultracentrifuge or a light scattering photometer, have frequently been applied to determine the molecular weight of the glycoprotein. The values obtained (complied by Schmid , 1975) were not far from the point, but ranged between 39,000 and 41,000 with a general tendency of overestimation.

Molecular weight of chicken ovomucoid was determined to be 25,000. Molecular weight of the protein moiety of the glycoprotein has been determined to be 20,080 by the sequence study (Kato et al., 1978). The carbohydrate content has been estimated to be between 20 and 25 % (w/w) (Lin & Feeney, 1972). Thus the total molecular weight of the glycoprotein is estimated to be between 25,100 and 26,800 from its composition. This glycoprotein has been demonstrated to behave anomalously in both SDS-polyacrylamide gel electrophoresis and gel chromatography (Leach et al., 1980a,b). The glycoprotein also behaved anomalously in the present experiments to be eluted in a position comparable to that of ovalbumin.

The results described above clearly indicate that the technique, described in this chapter, can be used to determine the molecular weight of proteins to which no technique depending on the molecular sieving effect can be applied. The reported value of molecular weight of α_1-acid glycoprotein next to ours in accuracy is that (39,000) obtained by Kawahara et al. (1973) under extreme care based on their profound experience. In the case of

ovomucoid, the sedimentation equilibrium technique gave molecular weight of ca. 28,000 which is significantly higher estimate.

The present technique also requires experience in the operation and familiarity with the performance of the measuring system shown in Fig.1-6. Once become acquainted with the system, a series of measurements can be finished within several hours. For a protein of which extinction coefficient is unknown, determiantion of the coefficient will require more time than that required for the main experiments. To make the procedure time- and sample-saving, the quantitative amino acid analysis technique (Craig and Kyte, 1980; Peters et al.,1981a,1982) might be the best choice. The practical application of this technique to an unknown sample will be described in the next chapter.

It is not necessary to measure specific refractive index increment in the improverd GPC/LALLS technique. However, this value can be conveniently determined by Eq.4-2). The specific refractive index increments of α_1-aicd glycoprotein and ovomucoid were determined to be 0.188 ± 0.002 and 0.215 ± 0.003 $ml \cdot g^{-1}$, respectively, from the outputs of spectrophotometer and differential refractometer, where bovine serum albumin was adopted as standard protein. The specific refractive index increment and extinction coefficient of bovine serun albumin were assumed to be 0.187 $ml \cdot g^{-1}$ and 0.67 $ml \cdot mg^{-1} \cdot cm^{-1}$,

respectively. Specific refractive index increment must be measured as accurately as possible in identical condition to those where experiments of light scattering were performed. In these senses, we can recognize that the improved GPC/LALLS technique by which molecular weight and specific refractive index increment can be determined simultaneously is very desirable.

The present technique can be applied to any protein applicable to a presently available high performance gel chromatography column. Exceptions are proteins with absorption in the visible region. As has been discussed in chapter 2, the situation will be improved by the change of the light source of the refractometer. Proteins with absorption at or near the wavelength of He-Ne laser will still be outside of the range of the present technique.

5 Application of the GPC/LALLS technique to the characterization of membrane polypeptides solubilized by SDS

Summary

Application of the GPC/LALLS technique to estimation of molecular weights of polypeptides constituting a membrane protein solubilized by a denaturing surfactant, sodium dodecyl sulfate (SDS) was demonstrated. As a test, molecular weights of the α and β subunits of canine kidney (Na^+,K^+)-ATPase were measured by the technique. Extinction coefficient was required for each component for determination of molecular weight in a single run. Quantitative amino acid analysis was found to be most suitable to determine the coefficient using a very small amount of sample protein. It was found that the GPC/LALLS technique is highly promising as a method for determining the molecular weight of subunit of membrane protein. This result indicates that subunit composition of membrane protein can be analyzed using the GPC/LALLS technique firstly by the determination of molecular weight of the assembly in the presence of non-denaturing surfactant and secondly by that of constituent polypeptides in the presence of SDS.

5-1 Introduction

Most membrane proteins consist of two or more subunits and the interaction between them plays important roles in their functions. Therefore, elucidation of the subunit composition is very important in order to understand the function of a membrane protein. In order to clarify the subunit composition of a membrane protein in native state, it is necessary to determine molecular weight for both of the molecular assembly in the native state and the subunits constituting it. Fractionation of the subunits in high resolution is prerequisite to the subunit study. We have demonstrated in chapter 2 that the molecular weight of membrane protein solubilized by a non-denaturing surfactant, $C_{12}E_8$, could be precisely determined by the GPC/LALLS technique. The solubilized membrane protein could be presumed to retain the native properties. Moreover, in chapter 3, we could successfully estimate the subunits composition of canine kidney (Na^+,K^+)-ATPase solubilized by $C_{12}E_8$ with some assumptions.

On the other hand, the GPC/LALLS technique (monitoring of the elution from a GPC column by a LALLS photometer and a differential refractometer) in the presence of denaturing surfactant, SDS, has been applied to porin, the outer membrane protein of E.coli, to confirm the subunit composition (Kameyama et al., 1982). The study

is to be noted as the first application of the GPC/LALLS technique in the presence of SDS to the estimation of molecular weight of a membrane protein. Since porin is, however, consisted of one kind of subunit and is exceptionally stable, the study described above could not provide general strategy for application of the technique to membrane proteins.

The purpose of the present study is to establish the GPC/LALLS technique (monitoring of the elution from a GPC column by three kinds to detector, namely a spectrophotometer, a LALLS photometer and a differential refractometer) in the presence of SDS as a general method to determine molecular weights of polypeptides constituting a membrane protein. Canine kidney (Na^+,K^+)-ATPase was taken up as an example of typical intrinsic membrane proteins with subunit structure. The enzyme consists of two kinds of subunits, namely the α and β subunits. The enzyme has been outlined in chapter 3.

The GPC/LALLS technique has the advantage that molecular weight of a solubilized membrane protein can be determined in a very small amount of the protein. To make the best use of this technique, it is necessary to determine weight concentration of the protein also using a minute amount to estimate its extinction coefficient. In the present study, quantitative amino acid analysis was chosen as the method complying the above demand.

SDS is a powerful solubilizing reagent for membrane

proteins and widely used in the field of biochemistry. Assessment of the use of the GPC/LALLS technique in the presence of SDS, therefore, seems to be very important.

5-2 Experimental procedures

5-2-1 The GPC/LALLS technique in the presence of SDS

The measuring system used in the present study was almost the same as that described in chapter 4. Sequence of detectors was modified and was as follows: a spectrophotometer, a differential refractometer and a LALLS photometer. Buffer solution used had following composition; 21.5 mM NaH_2PO_4, 28.5 mM Na_2HPO_4, 2.0-7.6 mM SDS and 0.02 % sodium azide. SDS was purchased from Bio-Rad Co. and used without further purification.

5-2-2 Solubilization of membrane-bound (Na^+,K^+)-ATPase by SDS

Membrane bound (Na^+,K^+)-ATPase suspended in 1 mM EDTA neutralized with imidazole to pH 7.2 was solubilized by SDS to the following final composition; 0.2-2 mg/ml protein, 0.1 % SDS, 1 mM EDTA, 4 mM dithiothreitol (DTT) and 0.1 M phosphate buffer solution. The centrifugation was started 30 min after the incubation at 37°C for 1 hr, and carried out for 15 min at 40,000 rpm and 23°C. The supernatant was collected as ATPase solubilized by SDS.

5-2-3 Isolation of the α and β subunits of (Na^+,K^+)-ATPase

ATPase solubilized by SDS was subjected to Sepharose

CL-6B column equilibrated with 0.02 M Tris-HCl buffer containing 0.2 % SDS, 2 mM EDTA and 2 mM dithioerythritol (DTE) (pH 8.0). Fractions corresponding to the α or β subunit were collected and concentrated to a concentration of about 2 mg/ml of protein.

5-2-4 Quantitative amino acid analysis

Quantitative amino acid analysis for the α and β subunits of (Na^+,K^+)-ATPase was carried out with a Hitachi 835-S amino acid analyzer. Norleucine was used as an internal standard to measure a recovery of amino acids applied to the analyzer. Sample solution containing norleucine was dried up under reduced pressure. Protein hydrolysis was performed by heating a solution of 6N twice distilluted HCl containing 0.2 % phenol, 20-30 μg protein and 5-6 nmol norleucine in a vacuum-sealed glass tube at 110°C for 24 hrs. Sample solution was dried up and 0.2 M citrate buffer (pH 2.25) was added to it. Volume of sample solution actually analyzed was determined from the weight of sample solution applied to the amino acid analyzer.

Contents of appropriate amino acids contained in a constant volume of sample protein solution could be estimated from this amino acid analysis. On the other hand, contents of the amino acids containing in one mole of the α or β subunit have been reported by Kyte (1972). Accordingly, concentration of sample protein solution

could be determined by taking these two data into consideration. Amino acids used in this estimation were selected as follows: 1) amino acid analysis of bovine pancreatic ribonuclease A was carried out, 2) the results obtanied were compared with the established value reported by Smyth et al. (1963), and 3) amino acids representing the correct contents under this experimental condition were selected.

5-2-5 Measurement of circular dichroism

Circular dichroic (CD) spectra were measured with a JASCO J-500 recording spectropolarimeter equipped with a data processor (model DP-501). The spectra were obtained at protein concetration of 0.3-0.5 mg/ml in 18 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-6.3 mM Tris buffer using a cell with 1 mm light path length. The data were expressed as molar residue ellipticity, $[\theta]$, in $deg \cdot cm^2/dmol$. CD intensity was calibrated with a dioxane solution of androsterone having molar ellipticity of 11,170 $deg \cdot cm^2/dmol$ at 303 nm.

Secondary structure of (Na^+,K^+)-ATPase was estimated according to the method described by Chang et al. (1978). Contents of typical ordered structures were calculated by the simulation of an observed CD spectrum by the liner combination of CD spectra presumed for the respective structure based on X-ray crystallographic data. That is,

the mean residue ellipticity, $[\theta]$, at any wavelength, λ, of a protein in an aqueous solution can be expressed as

$$[\theta]_\lambda = f_H[\theta]^\infty(1-k/\bar{n}) + f_\beta[\theta]_\beta + f_t[\theta]_t + f_R[\theta]_R \quad (5\text{-}1)$$

with two constraints; $0 \leq f_j \leq 1$ and $f_j = 1$.

The subscripts H, β, t, and R refer to helix, β-form, β-turn and unordered form, respectively. The $[\theta]_j$'S are the reference values for β-form(β), β-turn (t) and unordered form (R), and $[\theta]^\infty$ is that for helix of infinite length. The f_j's are corresponding fractions of the structural elements. The constants, k and $\bar{n}$ are dependent on wavelength (Chen et al, 1974) and the average number of amino acid residues per helical segment, respectively. The $[\theta]_j$'s at each wavelength were determined from CD spectra of proteins of known three-dimensional structure. Assigning a suitable value as initial value to $\bar{n}$ in Eq.5-1, computer-simulated pattern best fitted to measured CD spectrum was obtained. The contents of secondary structure were estimated from the simulated pattern.

5-3 Results

5-3-1 Elution patterns of (Na^+,K^+)-ATPase

Fig.5-1 shows elution patterns obtained when a solution of (Na^+,K^+)-ATPase solubilized by SDS was applied to the measuring system shown in Fig.1-6. Details of the measurements were described in the legend. The broad peaks appeared at retention time of about 43 min were assigned to dust particles, air bubbles in the sample solution and highly aggregates of the α and/or β subunits (mention of retention time in this chapter refers to that observed for the tracing of the LALLS photometer). Since such particles act as strong scatterers, they give big output in the tracing of the LALLS photometer even exist in very small amounts. Clearly these particles are contained only in very small amounts in the sample solution as judged from small deflection in the corresponding positions in the tracings of the refractometer and spectrophotometer. Major peaks at retention times of 47 and 59 min can be unambiguously assigned to the α and β subunits, respectively. The big peak appeared at 76 min can be safely assigned to mixed micelles of SDS and phospholipids.

The elution pattern shown in Fig.5-1 is not adequate for molecular weight determination of the α and β subunits. It is because the peak of the α subunit is

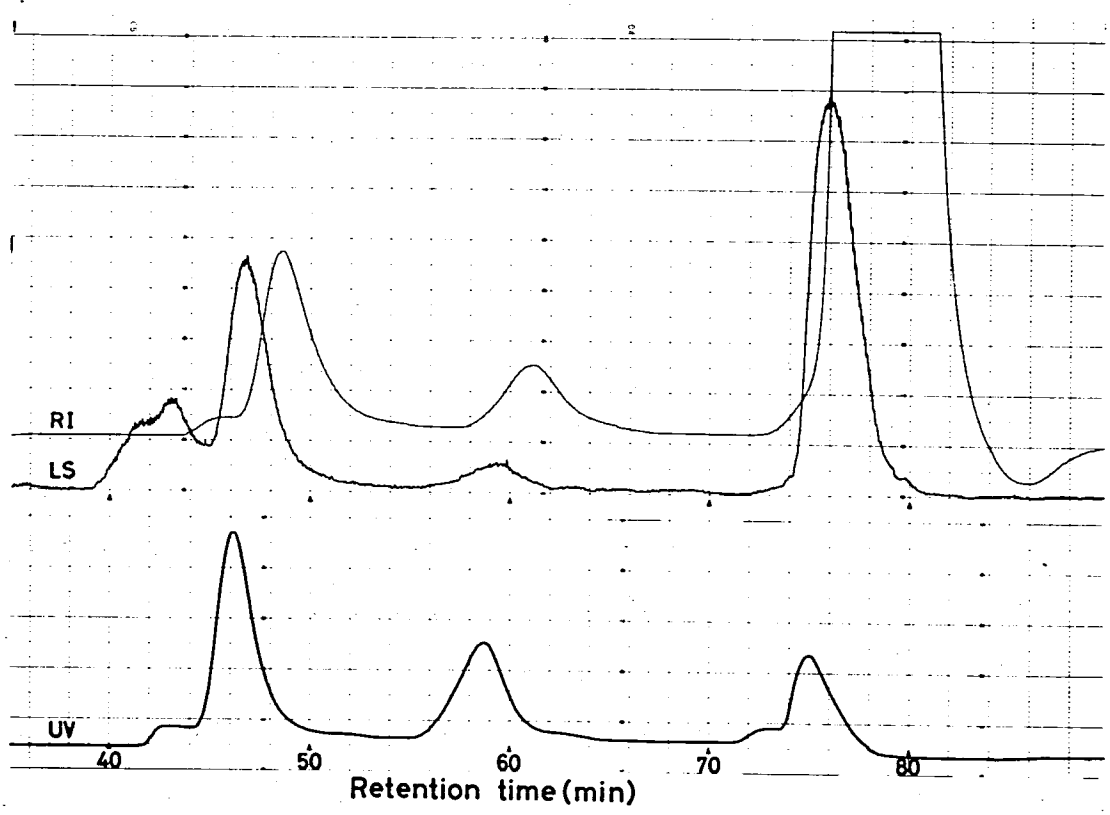


Fig.5-1 Elution patterns of (Na^+,K^+)-ATPase solubilized by SDS using measuring system shown in Fig.1-6. The three curves are the tracings obtained by the spectrophotometer (UV), the LALLS photometer (LS) and the differential refractometer (RI), respectively. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4 (pH 7) containing 3.5 mM SDS and 3 mM sodium azide. Flow rate: 0.22 ml/min. Gain setting: spectrophotometer, 0.64 fs; LALLS photometer, 16; refractometer, 64.

overlapped with the trailing part of the peak assigned to the unwelcomed strongly scattering particles, and the peak of the β subunit is uncomparably small compared to that of the α subunit. We decided, therefore, to determine molecular weights of the α and β subunits separately using their purified samples.

The α and β subunits were separated by Sepharose CL-6B gel chromatography in the presence of SDS, and the fraction of each subunit was concentrated and applied to the measuring system. Figs.5-2 and 5-3 show the elution patterns of the α and β subunits thus obtained, respectively. Although Figs.5-2 and 5-3 differ from Fig.5-1 in flow rate, the author wishes to express the results on the basis of the retention time. As shown in Fig.5-2, the α subunits were eluted at retention time of about 35 min. Although the small peak assigned to aggregates of the α and/or β subunits appeared only in the tracing of the LALLS photometer at a retention time of about 32 min, this peak did not substantially effect the estimation of output of the α subunit detected by the LALLS photometer. The peak observed at 57 min can be assignable to mixed micelles of SDS and phospholipids. As shown in Fig.5-3, the β subunit was eluted at a retention time of 42 min. The peak assigned to mixed micelles was eluted at 56 min. The peaks observed at 31 and 35 min may be assigned to aggregate of the α and/or β subunits and residual α subunit, respectively. Both in Figs.5-2 and

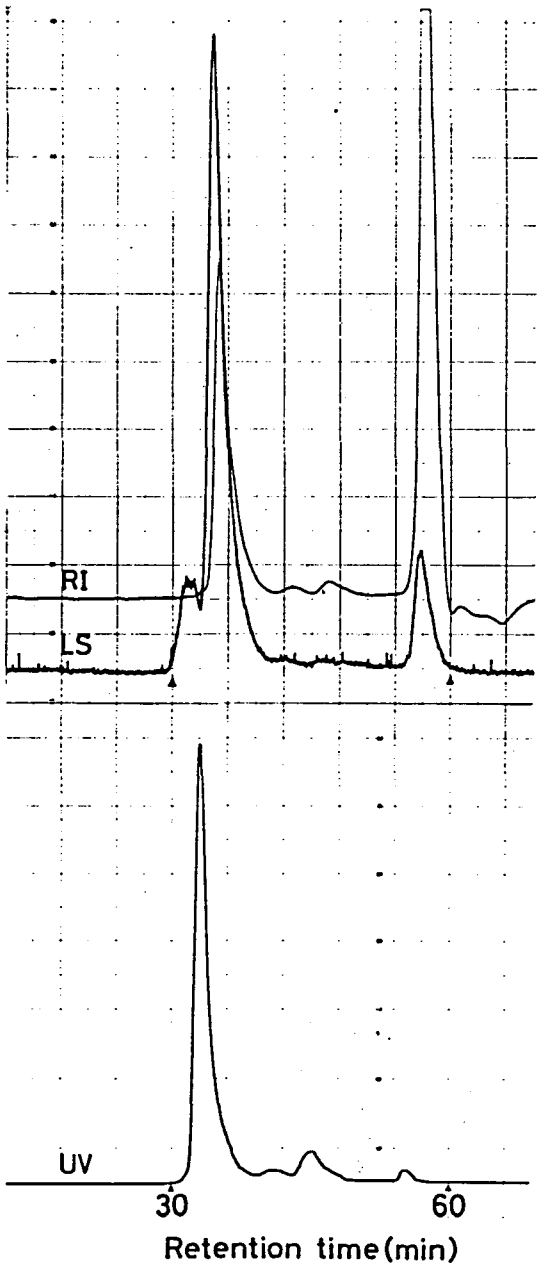


Fig.5-2 Elution patterns of the α subunit of (Na^+,K^+)-ATPase. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4 (pH 7) containing 3.5 mM SDS and 3 mM sodium azide. Flow rate: 0.30 ml/min. Gain setting: spectrophotometer, 0.16 fs; LALLS photometer, 32; refractometer, 32.

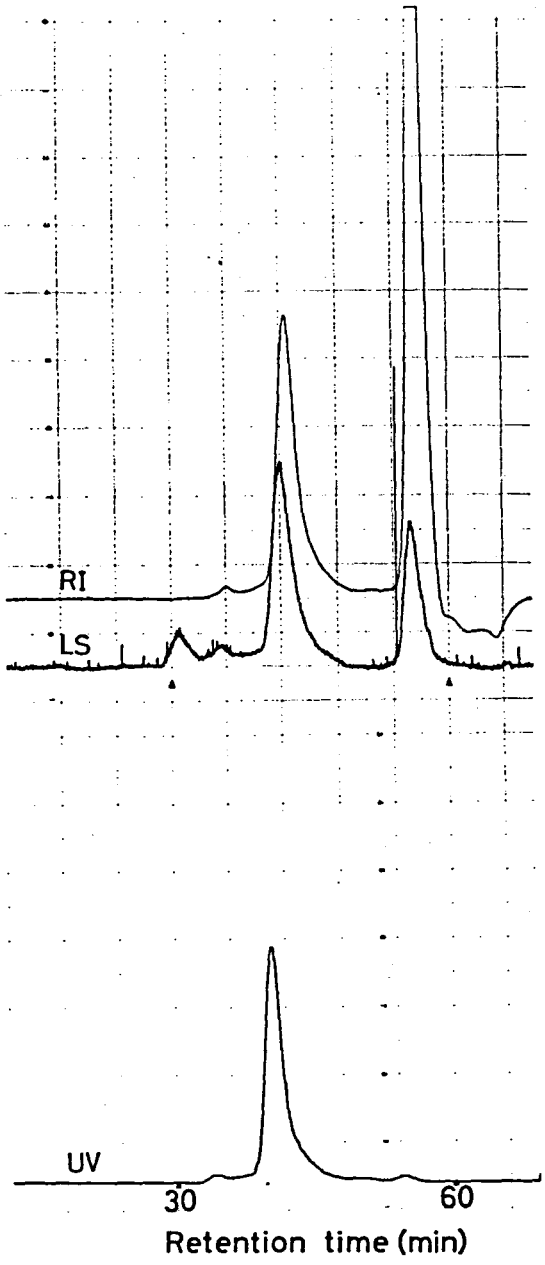


Fig.5-3 Elution patterns of the β subunit of (Na^+,K^+)-ATPase. Eluant: 0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4 (pH 7) containing 3.5 mM SDS and 3 mM sodium azide. Flow rate: 0.30 ml/min. Gain setting: spectrophotometer, 0.32 fs; LALLS photometer, 32; refractometer, 64.

5-3, the differential refractometer gave negative deflection bihind the peak of SDS micelles. It may be attributable to the perturbation of solvent composition described in chapter 2.

Outputs of the three detectors were expressed in terms of the corresponding peak heights.

5-3-2 Determination of extinction coefficients of the α and β subunits

In the GPC/LALLS technique, it is important to have knowledge in advance about extinction coefficient of a sample for accurate estimation of molecular weight. Quantitative amino acid analysis seems to be excellent technique for concentration determination of a protein with known amino acid composition (Craig and Kyte, 1980; Peters et al., 1981, 1982).

Fig.5-4 shows the relationship between the number of amino acids contained in ribonuclease A (Smyth et al., 1963) and the amounts of amino acid estimated by the present amino acid analysis. As shown in Fig.5-4, points for the following amino acids gave a linear plot: leucine, glycine, phenylalanine, histidine, arginine, valine and alanine. It can be concluded that these seven amino acids were analyzed with sufficient accuracy in the present amino acid analysis. Weight of protein in a sample was calculated by the multiplication of the weight of each

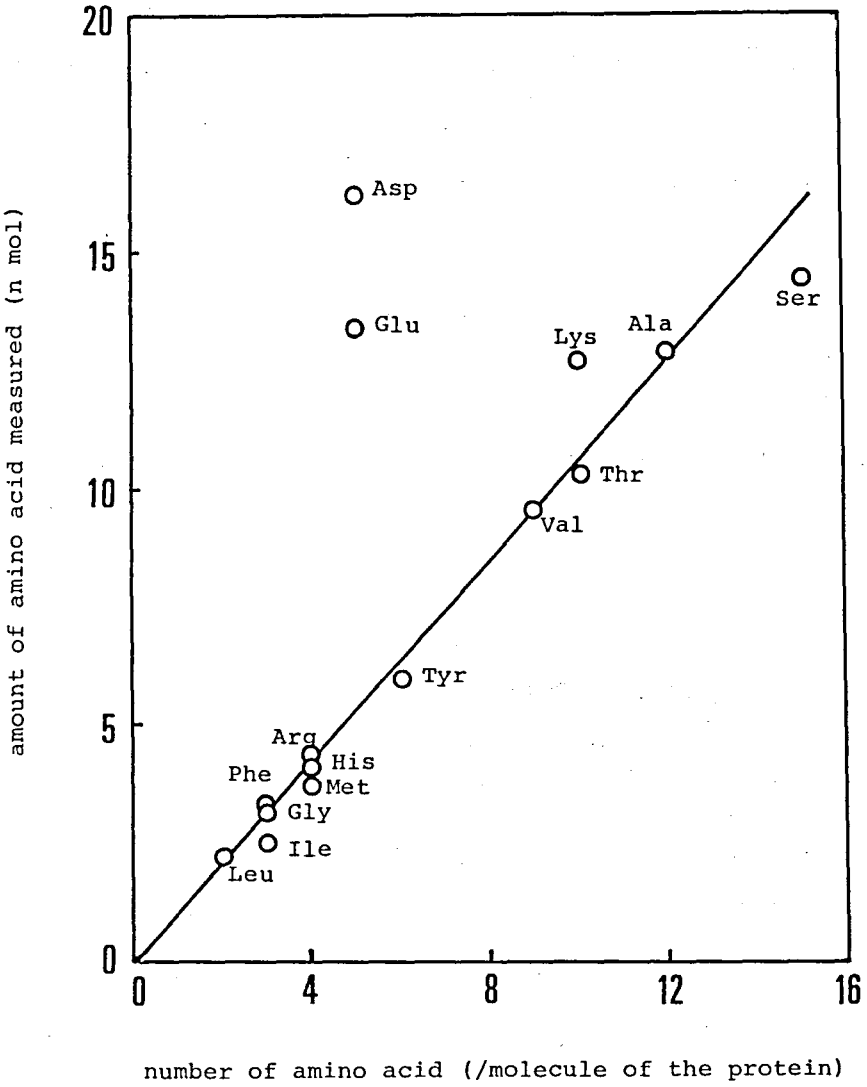


Fig.5-4 Plots of the amounts of amino acid estimated by amino acid analysis for ribonuclease A versus the number of amino acids contained in a molecule of the protein. The line is drawn for the following seven amino acids by a least square method; leucine (Leu), glycine (Gly), phenylalanine (phe), histidine (His), arginine (Arg), valine (Val) and alanine (Ala).

amino acid by corresponding conversion factor. Values obtained for the seven amino acids were averaged.

Extinction coefficients at 280 nm of the α and β subunits were determined to be 0.931 and 1.41 ml mg^{-1} cm^{-1}, respectively. We adopted the amino acid compositions of the α and β subunits reported by Kyte (1972) on calculation of the coefficients.

5-3-3 Determination of molecular weights of the α and β subunits

Molecular weights of the α and β subunits were calculated from the following equation which has been used in section 1-3-3.

$$M_p = K \frac{(\text{Output})_{LS} \; (\text{Output})_{UV}}{(\text{Output})_{RI}^{2} \; E} \qquad (5\text{-}2)$$

Instrument constant, K, in the above equation must have been determined from experiments for standard proteins with known molecular weights and extinction coefficients. In this study, following three standard proteins were used in order to make a calibration line necessary for molecular weight determination: bovine serum albumin (66,267, 0.670 ml mg^{-1} cm^{-1}), hen's ovalbumin (42,700, 0.735) and bovine carbonic anhydrase (29,000, 1.90). These were applied to the column separately. Molecular weights and extinction coefficients at 280 nm for each protein

were given in the parentheses. Extinction coefficients were assumed not to change in the presence of SDS. Fig.5-5 shows the calibration line thus obtained. Molecular weight of the α and β subunits could be determined to be 117,900 ± 3,000 (n=6) and 39,400 ± 900 (n=4), respectively, according to the above equation. Extinction coefficients of the α and β subunits were assumed to be 0.931 and 1.41 ml mg^{-1} cm^{-1}, respectively.

5-3-4 Estimation of the amounts of SDS bound to the protein

The values of dn_c/dc_p of the α and β subunits were estimated to be 0.420 ± 0.008 (n=6), and 0.495 ± 0.005 (n=4), respectively according to the following equation;

$$\frac{dn_c}{dc_p} = k\ E\ \frac{(Output)_{RI}}{(Output)_{UV}} \qquad (5\text{-}3)$$

Instrument constant, k, in the above equation was calculated from the experiment for bovine serum albumin. The value of dn_c/dc_p for the albumin was assumed to be 0.376 ml g^{-1} which was determined using a batch-type differential refractometer (Kameyama et al., 1982). Kameyama et al. (1982) showed that the relation between the amounts of SDS bound to protein, δ, and dn_c/dc_p can be expressed as follows;

$$dn_c/dc_p = 0.140 * \delta + 0.188 \qquad (5\text{-}4)$$

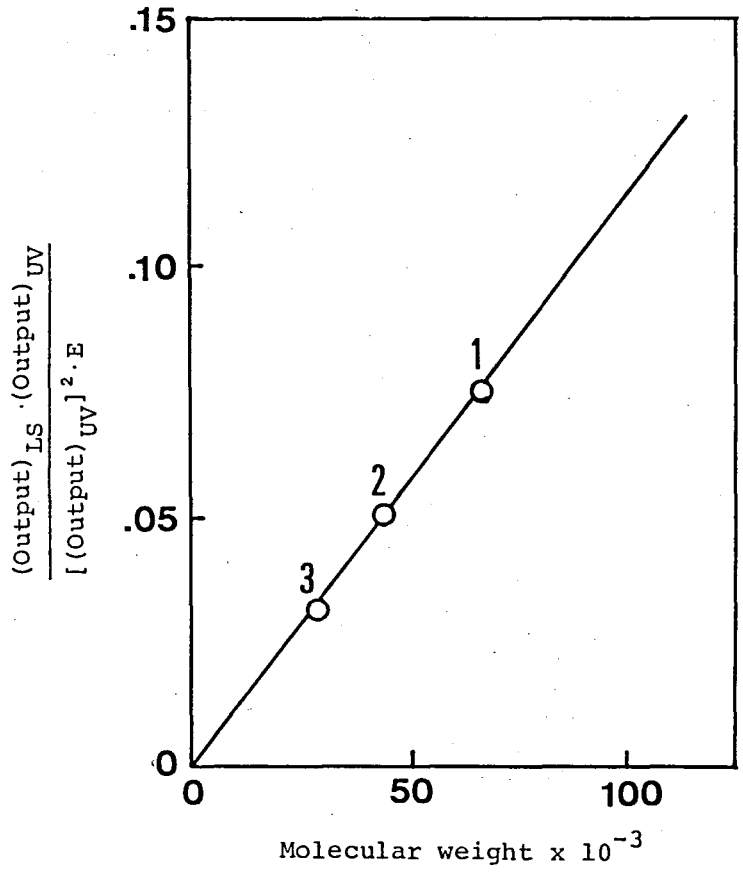


Fig.5-5 Calibration line for molecular weight determination of proteins. Following standard proteins were used; bovine serum albumin(1), ovalbimin(2) and carbonic anhydrase(3).

where 0.140 and 0.188 were equal to dn/dc of SDS and water-soluble globular proteins, respectively. Using above equation the amounts of SDS bound to the α and β subunits were estimated to be 1.66 and 2.19 g per g of protein, respectively.

5-3-5 CD spectra of the α and β subunits

Figs.5-6 and 5-7 show CD spectra in the wavelength region between 200 and 240 nm of the α and β subunits, respectively. Dotted lines show computer-simulated curves. Contents of secondary structure were estiamted from the curve fitting. CD spectrum for the α subunit represents typical α-helical pattern. The content of α-helix were estiamted to be 23 % and that of β-structure to be 17.6 %. On the other hand, the β subunit was estimated to contain 16.5 % of α-helix and 35.2 % of β-structure.

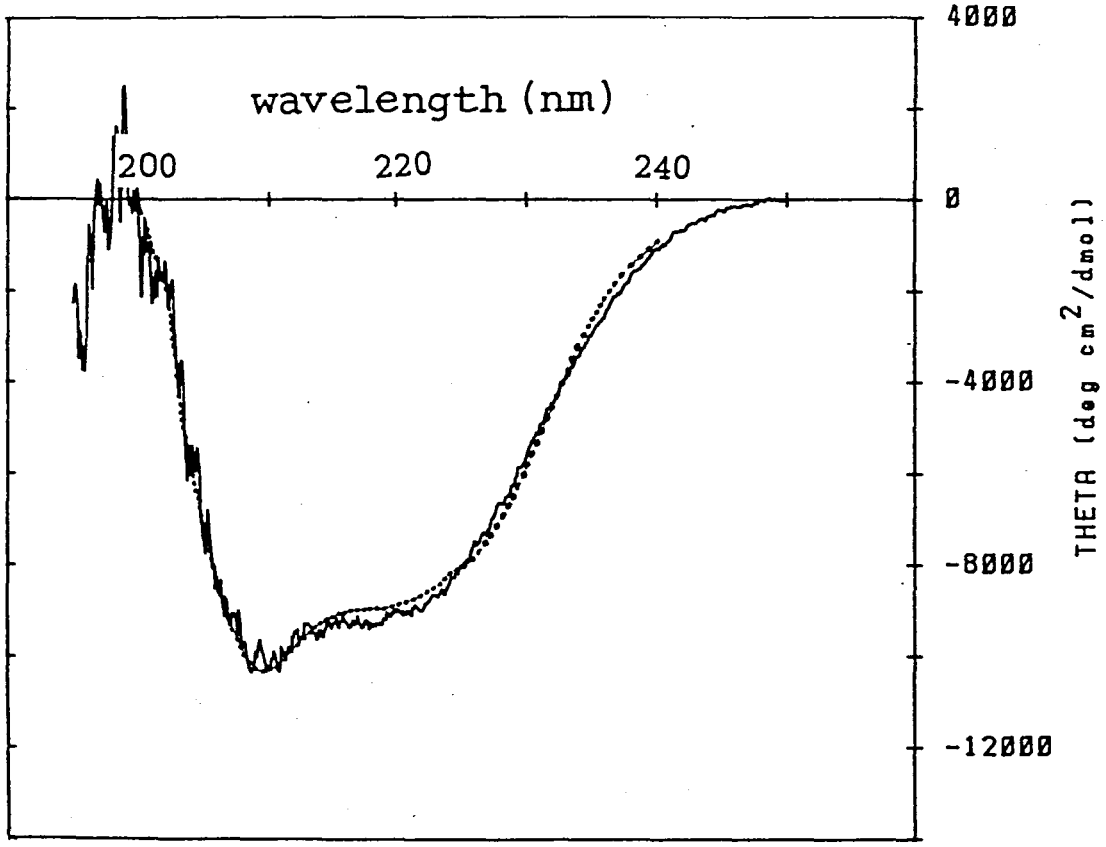


Fig.5-6 CD spectra of the α subunit of (Na^+,K^+)-ATPase. Dotted line shows computer-simulated curve.

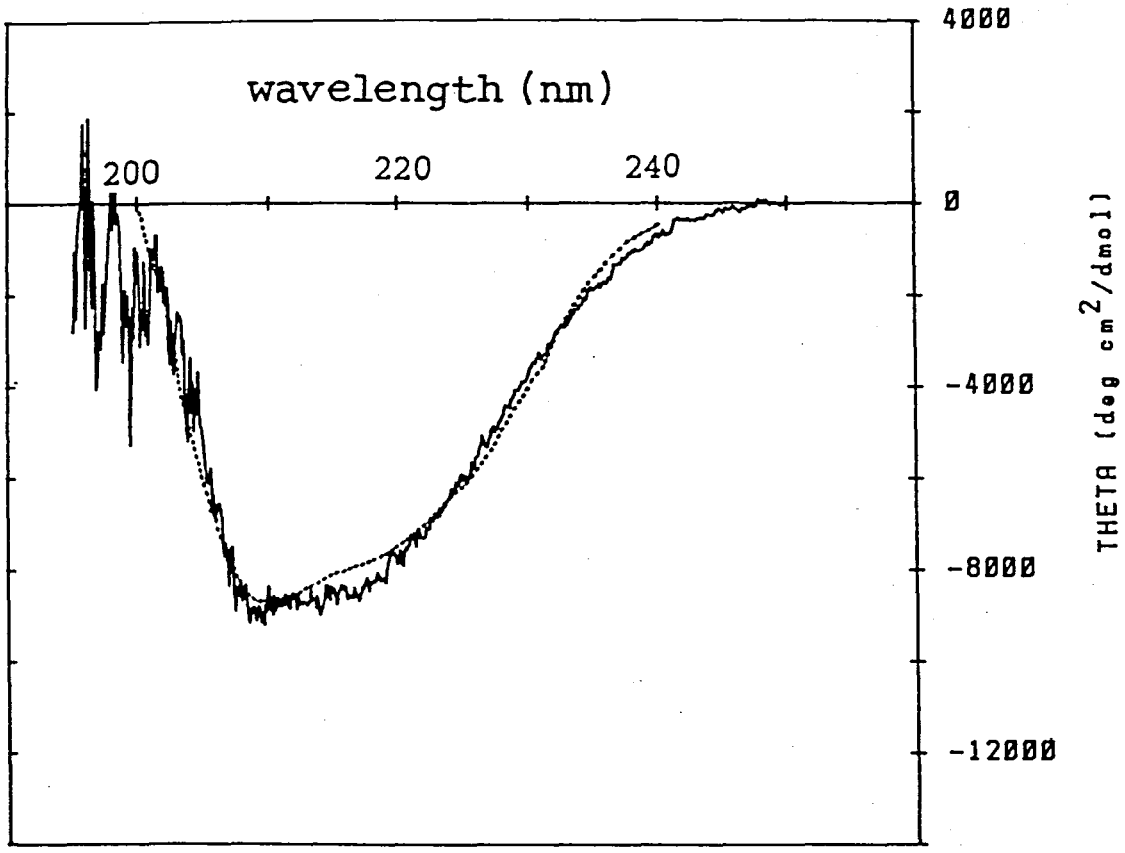


Fig.5-7 CD spectra of the β subunit of (Na^+,K^+)-ATPase. Dotted line shows computer-simulated curve.

5-4 Discussion

Molecular weights of the α and β subunits were estimated to be 117,900 and 39,400, respectively. Molecular weights of the α and β subunits have been reported for (Na^+,K^+)-ATPase from several sources. Molecular weights of the α and β subunits of (Na^+,K^+)-ATPase from shark rectal gland were estimated to be 106,000 and 39,700, respectively, by the sedimentation equilibrium technique in the presence of SDS (Esmann et al., 1980). Peterson and Hokin (1981) estimated the molecular weights of the α and β subunits of the enzyme from canine kidney to be 97,000 and 40,200, respectively, by SDS-polyacrylamide gel electrophoresis. On the other hand, Freytag and Reynolds (1981) estimated the molecular weights of the α and β subunit from pig kidney to be 93,000 and 32,300, respectively, by the sedimentation equilibrium technique in the presence of SDS or guanidine hydrochloride. The value obtained in this study were in agreement with those reported by Esmann et al. (1980). However, according to the present result, molecular weight of the αβ-protomer can be calculated to be 157,300. This value is lager than that of 123,000 reported for the αβ-protomer in chapter 3. In chapter 3, the concentrations of species eluted from the column were calculated assuming that extinction coefficients for all protein species were 1.22 ml $mg^{-1}cm^{-1}$, which was average value of two

extinction coefficients estimated in SDS solution and $C_{12}E_8$ solution, depending on the Bradford method (1976). Therefore, the molecular weight estimated for protein species in chapter 3 may have included error in estimation of the extinction coefficient in addition to experimental errors. However, the origin of difference between molecular weight of αβ-protomer reported in this chapter and that in chapter 3 is not clear.

SDS is one of the most popular denaturing surfactants used in the field of biochemistry. Characteristics of SDS is fully utilized in polyacrylamide gel electrophoresis in its presence. This technique depends on the phenomenon that SDS binds to most proteins to give a uniform complex concerning the binding ratio of SDS to the protein. Relative electrophoretic mobility of the SDS-protein complex is thus a unique funciton of only polypeptide molecular weight. When one wants to investigate the properties of SDS-protein complex, it is indispensable to know the amount of bound SDS to the protein. General method to measure the amount of bound SDS to protein is colorimetry of methyleneblue-SDS complex extracted with chloroform. This method is tedious although sensitive.

In the present study, the amount of bound SDS was conveniently estimated from the specific refractive index increment of SDS-protein complex expressed in terms of the weight concentration of protein moiety, dn_c/dc_p, according to the method reported by Kameyama et al. (1982). The

amount of bound SDS to the α and β subunits of (Na^+,K^+)-ATPase could be readily calculated from the dn_c/dc_p measured by the simplified procedure described in chapter 2. The validity of this procedure was confirmed by measurement of the amount of SDS bound to ovalbumin. The value obtanied was 1.92 g/g of protein, and coincided well with the value reported previously, 1.88 g/g. (Kameyama et al., 1982)

Thus the GPC/LALLS technique applied to characterization of SDS-protein complex gives the information of amount of bound SDS to protein in addition to the molecular weight of protein moiety of the complex. This suggests the possibility of application of this technique in future to study of interaction between protein and surfactant and, to the protein-ligand interaction.

CD spectra of the α and β subunits of the enzyme are shown in Figs.5-6 and 5-7. Tes-Tris buffer solution was used as solvent of sample solution for CD measurements because HEPES-imidazole buffer solution has CD bands in the region. The α and β subunits were separated by the high performance gel chromatography in the presence of SDS. Even if the sample solution thus obtained was dialyzed against buffer solution containing no SDS, the sample solution remained clear and incomplete removal of SDS or formation of protein micelles (Simons et al., 1978) were unlikely. The CD spectra for the α and β subunits

indicate that both subunits retain ordered structure in the given condition.

It is taken for granted that a membrane protein can be solubilized by SDS when its equilibrium concentration is above the critical micelle concentration of the surfactant. It is common practice, however, to keep the equilibrium concentration of SDS appreciably higher than the critical micelle concentration. SDS existing in a solution in exess over the critical concentration present in the solution forming micelles. A micelle of SDS is a scatterer far stronger than free SDS present in the same number as constituting a micelle. Therefore, the higher the concentration of SDS in an equilibrium buffer solution, the lower is the S/N ratio in the record of the LALLS photometer. On the other hand, the micelles present in excess might distrub molecular weight determination. This is presumed from the consideration of the principle of light scattering phenomenon. Light scattering from a protein solution is the outcome of the flactuation of protein concentration. Things are the same for the micelles of SDS. If there were interaction between protein-SDS complex and SDS micelles from the point of thermodynamics, an error would be introduced due to the perturbation of concentration fluctuation of the complex by SDS micells. This possible hazardous effect of coexisting micelles has been pointed out by Jones et al. (private communication). Based on their experimental

results, they are dubious of molecular weight determination of proteins in the presence of excess amount of SDS. Jones et al. assessed the issue making batch-type experiments where each sample must have been dialyzed against the solvent. Due to the difficulties pertinent to such experimental procedures, we are dubious of their pessimistic conclusion. It would be disgusting if the equilibrium concentration of SDS were confined in the region just above the critical micelle concentraiton. Thus, we decided to assess the reliability of the GPC/LALLS experiments when carried out in the presence of exess amount of SDS.

Firstly, elution behavior of the following standard proteins were examined: bovine serum albumin, hen's ovalbumin and bovine carbonic anhydrase, in following concentrations of SDS: 2.0, 3.2, 5.0 and 7,6 mg/ml. The range covers concentration range between the critical micelle concentration and four times of it. The change of SDS concentration had no appreciable effect on the elution behavior of the three proteins. Moreover, experimental data obtained in these experiments gave plots with good linearity when calibration lines like that in Fig.5-5 were drawn. No significant change was observed in inclination among the lines.

The molecular weight of the α subunit was estiamted under the conditions described above. The values obtained agree well with those obtained previously within

experimental error. This observation suggests that the GPC/LALLS technique gives molecular weight of membrane protein accurately independent of concentration of SDS in elution buffer.

The results obtained indicate that the possible effect proposed by Jones et al. and unfavorable for molecular weight determination of proteins in the presence of SDS, is not significant, if present, in the concentration range of SDS mentioned above. Jones et al. insisted that the effect is significant even in the above concentration range of SDS. The inconsistence might be due to the difference in experimental conditions. In our experiemtns, the complexes between protein and SDS were made in equilibrium with solvent by gel chromatography which secures the attainment of equilibrium conveniently and promptly. GPC/LALLS experiments in the presence of SDS higher than the present maximum concentration must be carried out with due care after suitable assessment study.

Concluding remarks

The present study was planned to develop and establish a technique for determination of molecular composition of membrane proteins. There was no technique suitable for the purpose at the beginning of the present study. We have noticed that the optimum one is the GPC/LALLS technique in which elution from a GPC column is monitored by a spectrophotometer, a low-angle laser light scattering photometer and a precision differential refractometer connected in series. The technique was first utilized in the research field of synthetic polymers where organic solvents are mostly used. Recent development of high performance GPC column for use of aqueous solvents had opened a way to the application of the technique to biological macromolecules. Due to experimental difficulties pertinent to the use of aqueous solvents, the technique had not been successfully used for characterization of biological macromolecules. The present study is a part of a series of studies for the introdcution of the technique for characterization of proteins.

Membrane proteins were solubilized by a suitable surfactant, applied to a GPC column equilibrated with an aqueous solvent containing the surfactant and then detected by the measuring system described above. We have overcome several difficulties pertinent to the application

to membrane proteins. Molecular composition of a membrane protein was made clear according to the following procedures: 1) molecular weight of the total assembly of the membrane protein was determined; and 2) subunit composition of the assembly was made clear and molecular weight of each of them was determined. The first could be carried out by the GPC/LALLS technique using a solvent containing a non-denaturing surfactant. In the present study, octaethyleneglycol n-dodecyl ether ($C_{12}E_8$) was used as the surfactant. The second could be carried out using the same measuring system as above and a denaturing surfactant, sodium dodecyl sulfate (SDS). It was shown that the GPC/LALLS technique is quite effective to reveal molecular composition of a membrane protein. Membrane proteins used as test materials were porin and λ-receptor protein of E.coli outer membrane and (Na^+,K^+)-ATPase of canine kidney. Especially in the case of the ATPase, the compositions of major molecular assemblies were found to be stoichiometric and 2:2 and 1:1 of the α and β subunits. The application of the GPC/LALLS technique to the ATPase opened a way to the identification of functional molecular species of this biologically very important enzyme, which could not be revealed by conventional techniques.

The two surfactants showed good performance for the present purpose. Other suitable surfactants should also be used for solubilization in a non-denaturing condition depending on the nature of sample protein. The

non-denaturing surfactant should satisfy following conditions: 1) a membrane protein must be solubilized without giving rise of non-specific aggregates and still keeping intrinsic molecular assembly; 2) the surfactant must be transparent in the near UV region not to interfer with the monitoring by a spectrophotometer; 3) the peak of micelle derived from the excess amount of the surfactant in a sample solution should not overlap with the peak of any proteins to be measured. Use of such a surfactant should not be limited to $C_{12}E_8$ used in the present study. Surfactants suitable for the GPC/LALLS technique for characterization of membrane proteins should be further sought. To be free from the overlap with a protein peak, a set of surfactants with various micelle sizes should be provided. Sodium dodecyl sulfate (SDS) seems to be the best choice both from the viewpoints of performance and availability.

The GPC/LALLS technique was found to be applicable to glycoproteins from the results of α_1-acid glycoprotein and ovomucoid used as test materials. These are not membrane proteins. Membrane proteins are, however, often glycoproteins, and, therefore, the know-how obtanied are indispensable in the application of the GPC/LALLS technique to membrane proteins.

In the application of the GPC/LALLS technique to membrane proteins solubilized by a surfactant, elution must be monitored in principle with respect to weight

concentration of the proteins. In the present study, the concentration was monitored indirectly by the measurement at 280 nm by the spectophotometer. This requires accurate estimation of extinction coefficient at the wavelength for each protein. For determiantion of the coefficient, we again need weight concentration of the sample protein. We could introduce the quantitative amino acid analysis for the purpose.

The assessment phase of the application of the GPC/LALLS technique to the characterization of membrane proteins is believed to have come to a conclusion. The results obtained indicate that the technique is quite efficient and promising for the purpose. The technique is actually attracting interest of people working in the field of membrane biochemistry, and will find a wide range of application in near future.

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