Studies on the Structure of Filamentous Bacteriophage fd.
I. Physicochemical Properties of Phage
fd and its Components

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Detailed structural information of phage fd has been obtained by using physicochemical techniques. Spectrophotometric results indicate that the DNA in the virion exhibits a hypochromicity about 60% as large as that of double-stranded native DNA, and the major native coat protein contains about 90% α-helical conformation. From the electrophoretic mobility of the phage protein in SDS polyacrylamide gel, it was elucidated that the A-protein, which is considered to bind with the tip of the virion, exists as an aggregate consisting of four molecules. The molecular weights of the various constituents of the virus were determined as: DNA, 1.85×10^6 daltons; A-protein, 7.4×10^4 daltons; A-protein aggregate, 30×10^4 daltons; B-protein, smaller than 1×10^4 daltons.

INTRODUCTION

The phage fd is an *E. coli*[†] male-specific bacteriophage and similar in physical and biological properties to M13, f1, ZJ/2 or else. This phage is shaped like a flexible rod about 8500 Å long by 50 Å in diameter, and it contains a single-stranded circular DNA and at least two kinds of coat proteins (for a review see Marvin and Hohn[1]).

This phage is one of the smallest DNA virus known and is released without lysis from the bacterial cells. The major coat protein usually called as the B-protein are bound directly to the single-stranded DNA,[2] and there is a species specificity of coat protein–DNA association in vivo.[3] Furthermore, the assembly of the virion may take place on the host cell membrane.[4] These properties of the filamentous phage are regarded suitable for studying the molecular interactions within the nucleoprotein complexes, and, up to the present, many workers have investigated the physical properties of this virus.[2,5~13]

In the earlier studies, however, intrinsic viscosity and sedimentation coefficient have been determined without careful consideration of its morphology. Intrinsic viscosity, for instance, has been measured in the range of fairly high shear stress and sedimentation coefficients have been determined at high solute concentrations.[6] The secondary structures of the coat protein and DNA in the virion have been obtained with less direct

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† The following abbreviations are used in the text: *E. coli, Escherichia coli*; ORD, optical rotatory dispersion; CD, circular dichroism; SDS, sodium dodecyl sulfate; Tris, tris hydroxymethyl aminomethane; Gu-HCl, guanidine hydrochloride; BPES buffer, $0.006M$-Na_2HPO_4, $0.002M$-NaH_2PO_4, $0.001M$-disodium-EDTA, $0.179M$-NaCl.

methods than those reported here. Namely, the α-helix content was obtained from the measurements of ORD[7] and hyperchromic effect in the far ultraviolet region,[8] not from the CD measurements. In addition, the content in the virion of the A-protein, the minor coat protein, has been obtained with a phage preparation labelled with ^{14}C amino acid mixture.[13]

In this paper, we report a detailed structural information of phage fd obtained with physicochemical techniques which were modified in these points.

EXPERIMENTAL

Preparation of Phages

E. coli K38 was used as the host cell for the preparation of fd according to the method essentially similar to that described by Marvin and Hoffman-Berling.[6] Occasionally, the final purification procedure of the CsCl density gradient centrifugation was replaced by repeated differential centrifugations. Concentrations were determined spectrophotometrically using a specific absorption coefficient of $A_{1cm}^{1\%}=39$ at 269 nm.[5]

The phage virion labelled with ^{3}H-histidine was prepared as follows. *E. coli* KCI101, a his⁻ derivative of strain K38, was grown with shaking at 37°C in M9 minimal medium supplemented with 2 μg/ml cold histidine to 5×10^7 cells/ml. Radioactive histidine was then added at a concentration of 1 μCi/ml, followed by the addition of CaCl₂ at a concentration of 0.1 mM and inoculation of fd at a multiplicity of 5 phages per cell. After the growth for more 5 hr, the bacteria were separated by centrifugation. The viruses were precipitated from the supernatant by the addition of acetic acid and ethanol. The precipitate collected by centrifugation was solubilized in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.6, and further purified by means of Sephadex G100 column chromatography and CsCl equilibrium centrifugation.

Strains *E. coli* K38 and fd were kindly supplied by Professor M. Takanami.

Preparation of fd DNA and Coat Proteins

Phage DNA and proteins were extracted and purified with phenol according to the procedure of Knippers and Hoffman–Berling.[11] In short, a phage solution was added to an equal volume of redistilled phenol having been saturated in advance with the same buffer. The mixture was vigorously shaken and the aqueous and phenolic phases were separated by centrifugation. After re-extracting each phase with either phenol or the buffer, DNA and the coat protein were obtained from the aqueous and phenolic phases, respectively. The solutions were used for the measurements described below after extensive dialysis.

Ultraviolet Absorption

The ultraviolet absorption spectrum was measured in a Carl Zeiss PQ2 spectrophotometer at the room temperature. The UV absorption change of phage with temperature was measured at 260 nm in Gu-HCl solutions in a Hitachi automatic spectrophotometer, Model 124, as the temperature was raised at a rate of 0.5°C/min.

Circular Dichroism and Optical Rotatory Dispersion

CD and ORD measurements were performed with a Jasco automatic recording

polarimeter, Model ORD/UV6 and ORD/UV5 in Shionogi Research Laboratory. The CD spectrum, as well as the ORD spectrum, was measured in the wavelength region of 205 nm to 320 nm.

Sedimentation Velocity

All the sedimentation velocity measurements were made at 25°C in a Beckmann—Spinco Model E ultracentrifuge using an An-E type rotor and UV optics. Sedimentation coefficients of the phage were measured in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.6 and in 0.14 M NaCl, 0.02 M Tris-HCl, pH 7.6 by boundary sedimentation. Sedimentation constants at infinite dilution were estimated from plots of 1/S versus concentration, and the results obtained were converted to that for the standard condition of water at 20°C by multiplying by $(\eta_{solvent}/\eta_{H_2O})$ and by $(1-\bar{v}\rho_w)/(1-\bar{v}\rho_{solvent})$.

Sedimentation coefficients of the viral DNA were measured in neutral and alkaline solutions. Sedimentation coefficients in alkaline solutions pH 12.1 were obtained by band sedimentation as described by Studier.[14] The concentration of DNA in the sample well was 22 μg/ml. In this method the extrapolation to zero concentration was unnecessary, because the concentration of DNA sedimenting as a band through the bulk solvent was sufficiently low. At times, DNA dissociated from phage in an alkaline solution has been used for the measurement. That is, the concentrated phage stock solution was diluted to make an alkaline solution pH 12.1 at a concentration of 0.19 mg/ml. After keeping the solution for 1 hr at 28°C, a sedimentation experiment was performed on it without any further treatment. The contaminated protein does not interfere the measurement.

Polyacrylamide Gel Electrophoresis

Proteins of the virion were analysed by polyacrylamide gel electrophoresis on 10% or 7.5% gels according to the method of Weber and Osborn.[16] Phages dissociated with SDS were applied on the gel column after the addition of glycerol and bromophenol blue. The gel electrophoresis was run at a current of 5 mA/gel for 5 hr. The gels were then removed from the tubes, stained with Coomassie brilliant blue, and finally destained by diffusion in a mixture containing 9% methanol and 9% acetic acid. Gels containing radioactive protein were sliced into 1 mm thick disks. After each disk was dissolved completely in 30% H_2O_2 at 60°C for 20 hr, radioactivity was counted in a Beckman liquid scintillation spectrometer, Model LS-100.

RESULTS AND DISCUSSION

UV Absorption, ODR and CD Spectra of Phage

The spectrophotometric properties of phage fd were measured in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.6) in order to obtain the conformational information about DNA and the major coat protein in the virion. From the optical density ratio, 270 nm/245 nm= 1.36 and the DNA content, 12.2%,[5] it can be deduced that the single–stranded DNA in the virion is in a highly hypochromic conformation in agreement with the results reported by several investigators.[5,8]

The ORD and CD spectra of phage are shown in Fig. 1. The ORD spectrum reveals a strong Cotton effect with a minimum at 233 nm and a maximum at 199 nm,
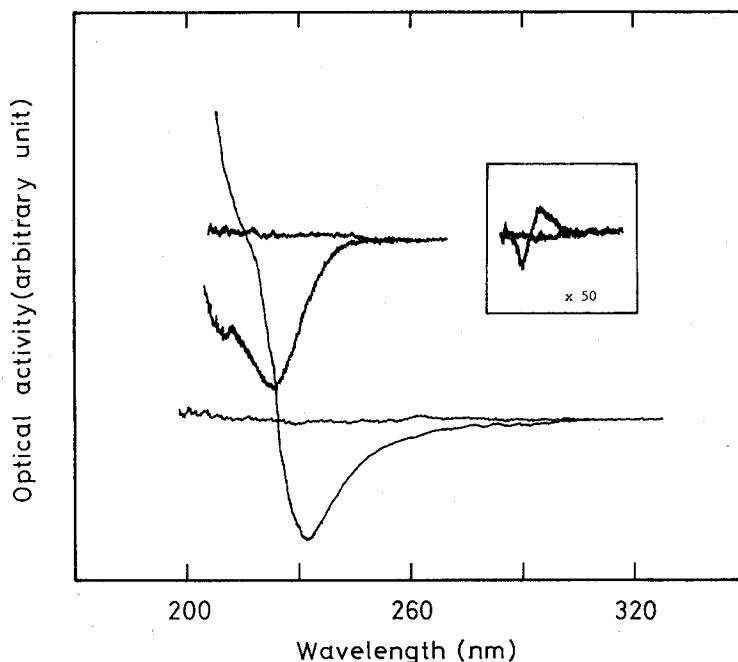
Fig. 1. ORD(the lower curve) and CD(the upper curve) spectra of intact phage fd in 0.14M-NaCl, 0.1M-Tris-HCl at pH 7.6.

which is characteristic of right-handed α-helix in polypeptides or proteins. Some weak Cotton effects can also be seen in a longer wavelength region. In Fig. 1 is also shown the CD spectrum because it yields better resolution of the Cotton effects. In fact, the weak Cotton effects are seen to be caused by the negative Cotton band at 289 nm and the positive one at 294 nm. The right-handed α-helical conformation detected above from the ORD measurements reveals itself in the CD spectrum with peaks at 210 nm and 222 nm.

The results obtained above indicate that the major coat protein in the virion is largely α-helical and DNA in the virion assumes a highly base-stacked conformation.

Sedimentation Coefficient and Intrinsic Viscosity of Phage

Since the molecular weight of fd is about 1.5×10^7 daltons and the length of the virion is about 8500 Å, it is expected that the sedimentation coefficient should become highly concentration dependent. Therefore, the measurements were made at very small solute concentrations with an An-E type rotor and UV optics. The sedimentation coefficients in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.6) and in 0.14 M NaCl, 0.02 M Tris-HCl (pH 7.6) are shown in Fig. 2. The plotted points were extrapolated to zero solute concentration and the ordinate intercepts yield $s^0_{20,w}$ values of 40.1 and 40.5, respectively.

Experimental results of viscosity measurements are shown in Fig. 3 for the intact phage fd in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.6) at 25°C. The shear stress used in the measurements was as small as 0.17 dynes/cm². The plotted points were extrapolated at infinite dilution so as to obtain a common intercept and the same value of Huggins' constant in the two types of plot. The intrinsic viscosity value of 8.68 dl/g was obtained within the experimental error of $\pm 5\%$. This value is only slightly greater than the value of 8.5 dl/g reported by Marvin and Hoffmann–Berling[6] which was determined by
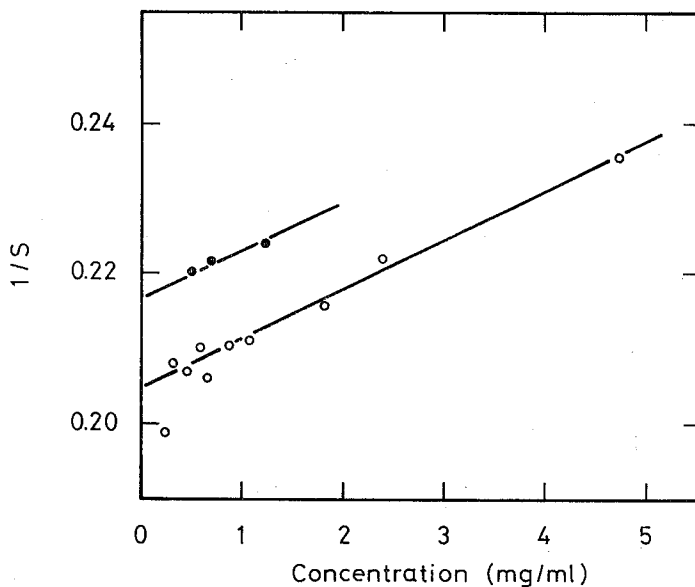
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Fig. 2. Sedimentation velocity data for intact phage fd in 0.14M-NaCl, 0.1M-Tris-HCl, pH 7.6(filled circles) and in 0.14M-NaCl, 0.02M-Tris-HCl, pH 7.6(open circles), at 25°C as plots of 1/S versus c.
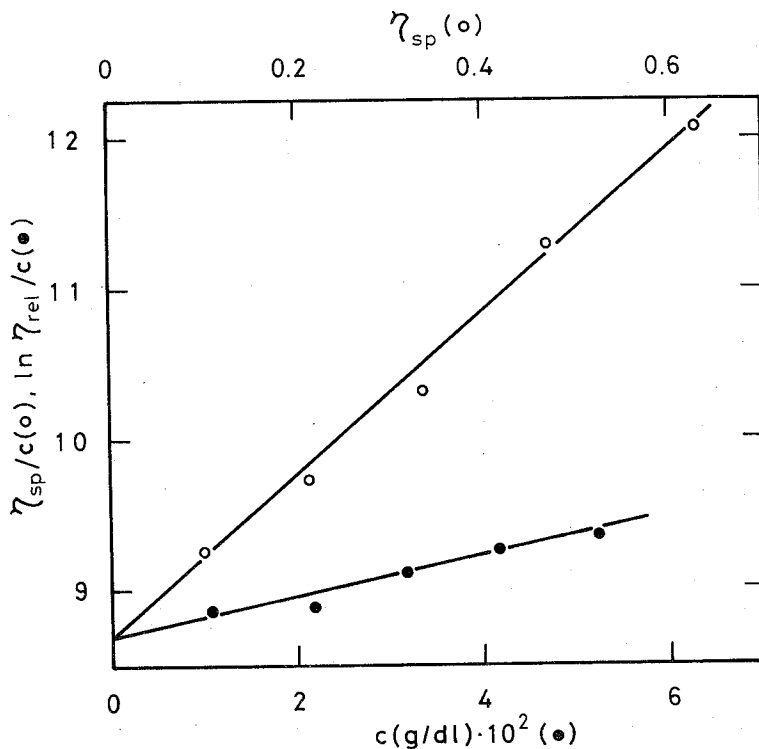

Fig. 3. Viscosity data for intact phage fd in 0.14M-NaCl, 0.1M-Tris-HCl, pH 7.6 at 25°C as plots of η_{sp}/c versus η_{sp} (open circles) and of $\ln \eta_{rel}/c$ versus c. (closed circles).

measurements with a usual capillary viscometer. This suggests that the phage fd is flexible enough to make the correction of shear rate effect essentially negligible. An attempt was made to estimate the persistence length as a quantitative measure of the flexibility, but the sedimentation and viscosity data did not yield a consistent result. The lateral aggregation of phages may account for the discrepancy, and light-scattering investigations are now in progress to clarify this point.

Sedimentation Coefficient and Molecular Weight of DNA

Although the techniques for measuring molecular weights have made much progress, there still exist some difficulties in the case of DNA, mainly due to its high molecular weight. One convenient procedure is the determination of molecular weight from the sedimentation coefficient with use of the molecular weight-sedimentation coefficient relationship. In 1965, Studier reported three equations relating $S^0_{20,w}$ and molecular weight of DNA in different conformations:[14]

$$S^0_{20,w} = 0.0105 \ M^{0.549} \tag{1}$$

$$S^0_{20,w} = 0.0528 \ M^{0.400} \tag{2}$$

$$S^0_{20,w} = 0.0882 \ M^{0.346} \tag{3}$$

Equation (1) represents the relation for denatured single-stranded DNA in 1 M NaCl, 0.01 M Tris-HCl (pH 8), equation (2) for alkaline denatured DNA in 0.9 M NaCl, 0.1 M NaOH (pH 12.1), and equation (3) for native double-stranded DNA in 1 M NaCl, 0.01 M Tris-HCl (pH 8). Among these, equation (2) is the most useful to obtain information of the primary structure and molecular weight of single-stranded fd DNA, because all the configurations specific to the base sequence are melted out in the medium.

The analytical band sedimentation experiments on phenol extracted fd DNA were performed in alkaline solutions according to the procedure described by Studier.[14] Some samples yielded two bands migrating with the sedimentation coefficients of 19.3S and 17.0S. It may be suggested that the faster sedimenting material is the circular DNA and the slower one the linear molecule having a single chain scission. The theoretical calculation on flexible macromolecules predicts that the ring: linear ratio of the sedimentation coefficients is 1.18 in the limit of large hydrodynamic interaction.[17] Experimental results above in fact agree with this theoretical prediction. As an further support to this view, a band sedimentation experiment was performed with the same bulk solution on a phage solution denatured for one hour at pH 12.1. The sedimentation pattern showed two bands in the ultracentrifuge with the sedimentation coefficient of 19.2S and 3.7S. As a conclusion, therefore, the 19S and 17S components may be identified as the intact circular DNA and the singly nicked linear DNA, respectively, and the 4S component the major coat protein dissociated in the alkaline solution. Using in eq. (2) the sedimentation coefficient of linear DNA so obtained in alkaline solution, its molecular weight is calculated as 1.85×10^6 daltons. These results are consistent with those reported previously.[8]

The sedimentation coefficient of fd DNA in BPES buffer at a concentration of 22 μg/ml was $S_{20,w} = 23.1$.

Degree of Base Stacking of DNA in the Virion

As mentioned earlier, fd DNA in the virion is probably taking a highly hypochromic conformation. Generally speaking, the conformation of DNA in the nucleoprotein

complex cannot be investigated in a straightforward manner. But the UV absorption change with temperature measured at 260 nm may yield valuable information, because the protein denaturation proceeds with negligible absorption change at this wavelength.

Absorbance-temperature profiles of phage measured at 260 nm in Gu-HCl solutions are shown in Fig. 4. We added Gu-HCl, because otherwise the absorption increased more slowly and the solution became turbid above 80°C due to the aggregation of the dissociated coat proteins. In the presence of the denaturant the heated solutions remained all the way clear. It is seen that the absorbance at 260 nm increases with the transition of DNA from the native conformation in the virion into random coil. The transition temperature defined as the temperature of half transition decreases as the concentration of Gu-HCl increases, but the hyperchromicity is kept almost constant around a value of 1.23 except the measurement in 6 M Gu-HCl. From the result obtained above, the degree of the base stacking of fd DNA in the virion is calculated as 63% as large as that of native DNA, using the DNA content of fd (12.2%) and the value of the hyperchromicity of double-stranded native DNA (1.42). This value is considerably smaller than the value of about 90% reported by L. A. Day. The reason for this discrepancy is yet unknown.

Molecular Weights and Structure of A- and B-proteins

Since Shapiro *et al.* originally reported that the mobility of proteins in SDS-poly-acrylamide gel electrophoresis is simply related to the molecular weight,[18] this technique has become one of the most convenient methods for the determination of molecular weight and composition of proteins. In this method, however, many well characterized proteins are required as the reference material for obtaining the reliable relation between the electrophoretic mobility and molecular weight. We have found recently that the lysozyme aggregates of various size are formed in SDS solutions, which can be used as the internal standard (see Appendix for detailed preparation of the reference line with lysozyme
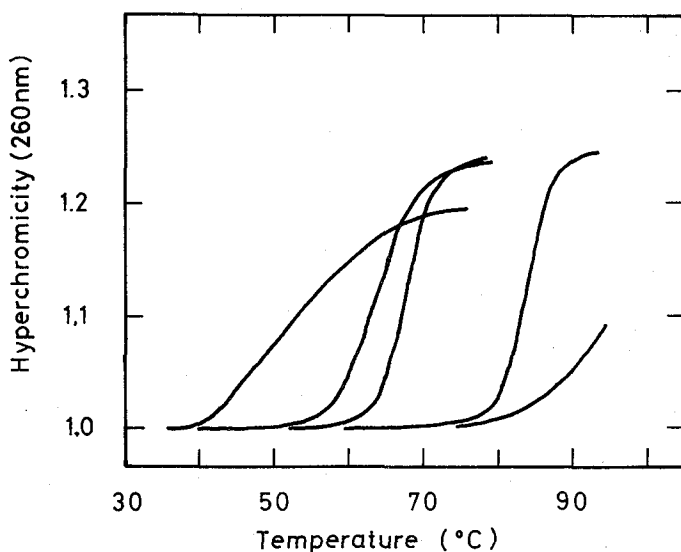

Fig. 4. Absorbance-temperature profiles of intact phage fd in 0.14M NaCl, 0.1M-Tris-HCl, pH 7.6 at the concentration of 0M, 2M, 4M, 4.8M, and 6M of Gu-HCl (from right to left).
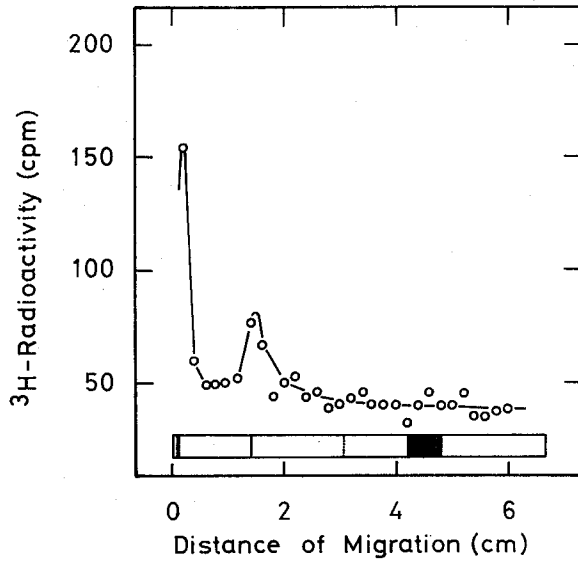
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Fig. 5. SDS-polyacrylamide gel electrophoresis on 10% gel of fd labelled with ^{3}H-Histidine and dissociated in 0.1% SDS solution. The anode is taken to the right. The insert at the bottom represents a rough sketch of the staining. The bands indicate, from left to right, A-protein aggregate, A-protein monomer, presumably contaminated cell protein, and the major component of B-protein.
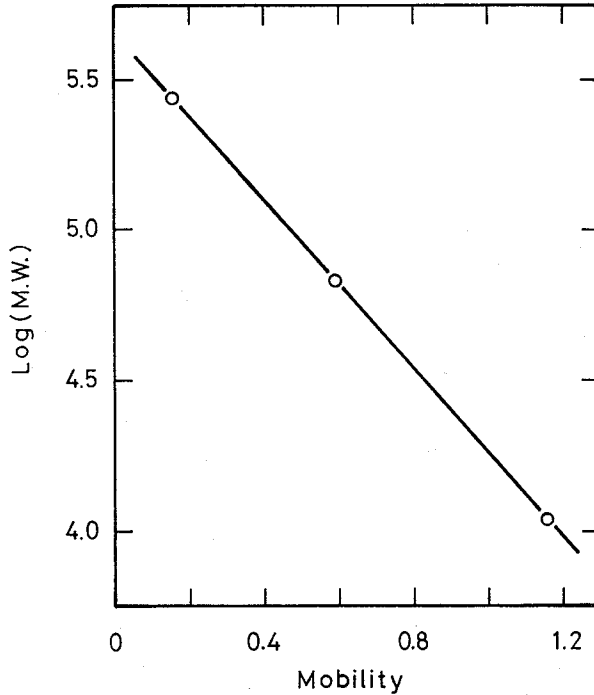

Fig. 6. Determination of the molecular weights of fd coat proteins using lysozyme aggregates as the internal standards (see Appendix). Open circles indicate, from left to right, A-protein aggregate, A-protein monomer, and B-protein.

(147)

aggregates). Using this method, the composition of fd coat proteins was analyzed and the molecular weight of each component was determined. It was convenient that viral coat proteins could be made molecularly dispersed in a SDS solution. The results obtained are shown in Fig. 5 and 6. It is seen in Fig. 5 that fd dissociated in 0.1% SDS gives two bands of A-protein which was preferentially labelled with ^{3}H-histidine. When fd was dissociated in a SDS solution of 1.0%, some amount of the A-protein in the band of the slow migration was observed to be transferred to that of the faster migration. Therefore, it may be suggested that A-protein exists in the virion as an aggregated form.

By comparing the data with the reference line of log of molecular weight versus mobility, the molecular weights of A-protein monomer, A-protein aggregate and of B–protein were determined as shown in Fig. 6, and the numerical data are summarized in Table I. The ratio of molecular weight of A-protein agregate to that of the monomer

Table I. Numerical Results of Physicochemical Measurements of Intact Phage. DNA and Proteins of *E. coli* Phage fd

Intact Phage fd

		This work	Previous works
UV	E_{270}/E_{245}	1. 36	1. 39 [8]
Sedimentation coefficient	$S^0_{20,w}$	40. 3	40 [6]
Intrinsic vicsocity	$[\eta]$	8. 68	8. 5 [6]

fd DNA

		This work	Previous works
Sedimentation coefficient	$S^0_{20,w}$ alkaline	19. 3 (17. 0)	—
	$S^0_{20,w}$ neutral	23. 1	14. 7* [6] 13. 4*
M.W.		1.85×10^6	1.7×10^4 [8]
base stacking		63%	90% [8]

* measurements at low ionic strength

fd coat protein

		This work	Previous works
ORD**	$[\alpha]_{233}$	-1.4×10^4	-1.5×10^4 [7]
CD**	$[\theta]_{210}$	-2.5×10^4	—
	$[\theta]_{222}$	-3.7×10^4	—
	$[\theta]_{289}$	-1.2×10^2	—
	$[\theta]_{294}$	1.2×10^2	—
M.W.	A-protein aggregate	30×10^4	—
	A-protein monomer	7.4×10^4	7×10^4 [3]
	B-protein	$<1 \times 10^4$	5169 [1]
helix content		93%	80-90% [8]

** values estimated from results with phage fd

is about 4. This implies that A-protein in the virion exists in the form of a tetramer. This result can be compared with that of Marvin *et al.*[13] They have suggested by using proteins labelled with ^{14}C amino acid mixture that there are three or four molecules of A-protein in a fd virion. No protein other than A- and B-protein was found.[19]

Content of α-Helix in the Major Coat Protein

As shown in Fig. 1 ORD and CD spectra for the intact phage reveal patterns characteristic of α-helix of polypeptides or proteins. The α-helical content was obtained from the CD spectrum, because closely located Cotton bands can be better resolved. We have calculated the content of α-helix in B-protein in the native state from the large negative ellipticity at 222 nm, because the contribution of DNA to the ellipticity in this region can be neglected and the content of the other coat protein is also negligibly small. The content of α-helix was calculated according to the relation

$$\% \ \alpha\text{-helix} = \frac{[\theta]_{222}}{-3.98 \times 10^4} \tag{4}$$

where $[\theta]_{222}$ represents the ellipticity at 222 nm. The value of the α-helix content obtained as 93% indicates that there is contained a very large amount of α-helix in the native form of this protein. This value is in good agreement with the results obtained by other investigators.[2,7,8]

All the experimental data obtained in the present investigation and numerical results calculated therefrom are summarized in Table I, and compared with those reported so far.

<div align="center">APPENDIX</div>

Use of Lysozyme Aggregates as the Internal Standards for Molecular Weight Estimation of Proteins from Polyacrylamide Gel Electrophoresis

Kenji IKEHARA and Hiroyasu UTIYAMA

Although SDS-polyacrylamide gel electrophoresis has become one of the most convenient method of molecular weight determination of proteins or polypeptides, many well characterized proteins are required for obtaining the reliable relation between electrophoretic mobility and molecular weight.[16,18] We have found recently that lysozyme at a concentration of 5 mg/ml in SDS solutions forms aggregates of various sizes due to the strong tendency of SDS-lysozyme complexes to associate and that these aggregates could be made use of for internal standards of different molecular weights for polyacrylamide gel electrophoresis. In an earlier study the similar internal standards have been provided by DANS-derivatives of a protein,[20] but the procedure of preparation is more laborious and time consuming. This appendix describes the detailed procedure of preparing the internal standard line with lysozyme aggregates for molecular weight estimation from SDS-polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis of Lysozyme Aggregates

Egg white lysozyme (Sigma, three times crystallized) was solubilized at a concentration of 5 mg/ml in 0.1% SDS solution, pH 7.6 at the room temperature. SDS-gel electrophoresis was carried out according to the method of Weber and Osborn[16] in glass tubes, 5 mm in diameter by 80 mm long, containing polyacrylamide gel of 10% or 7.5%. The electrode buffer contained 4.4 g $NaH_2PO_4·2H_2O$, 25.8 g $Na_2HPO_4·12H_2O$ and 1 g SDS (pH 7.2) in 1 liter of solution. Electrophoresis was performed at a current of 6 mA per tube at the room temperature for 6 hr. Gels were stained with 0.05% Coomassie brilliant blue in methanol-acetic acid-water (5 : 1 : 5) for 30 min at 60°C and destained by diffusion in a mixture containing 9% methanol and 9% acetic acid for 20 hr.

There appeared in the gel column as many as five lightly stained bands in addition to the thick monomer band. These minor components are considered to be the products of successive aggregation of lysozyme (*i.e.,* dimer, trimer, tetramer, pentamer, and hexamer). As a matter of fact the log of molecular weight of the aggregate calculated on this assumption follows a straight line when plotted against the relative migration as shown in Fig. 7.

Preparation of the Internal Standard Line with Lysozyme Aggregates

The straight line obtained above was compared with that obtained with the four well-characterised proteins, hemoglobin, chymotrypsinogen A, ovalbumin, and bovine serum albumin. The latter results agree well with those of Weber and Osborn.[16] Figure 8 shows that lysozyme aggregates as well as lysozyme itself migrate more slowly in the
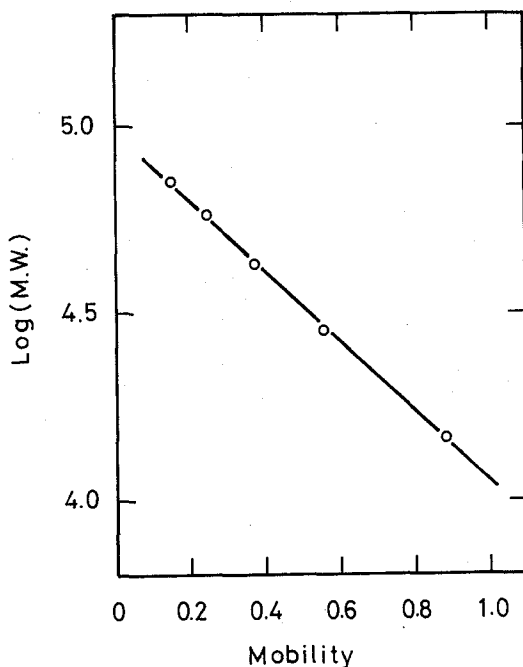

Fig. 7. Relationship between mobilities of lysozyme aggregates and logarithm of their molecular weights. Open circles from right to left indicate monomer, dimer, trimer, tetramer, and pentamer.

acrylamide gel than the other proteins. The reason for this difference is now unknown, but from the practical point of view we may obtain the calibration line provided that some small correction be applied on the data on aggregates. For this purpose we determined the effective molecular weight of each aggregate of lysozyme from the comparison of two lines. The molecular weights thus obtained are listed in Table II, which are identical for both 10% and 7.5% gels. Thus, lysozyme aggregates can be used as the internal standards by plotting the effective molecular weights against their mobilities.

The egg white lysozyme aggregates in SDS solutions seem stable complexes judged from the well separated bands. These bands did not disappear even if lysozyme was reduced with 1% mercaptoethanol. Not only lysozyme but all the proteins examined such as hemoglobin and chymotrypsinogen A, were found to form several oligomers
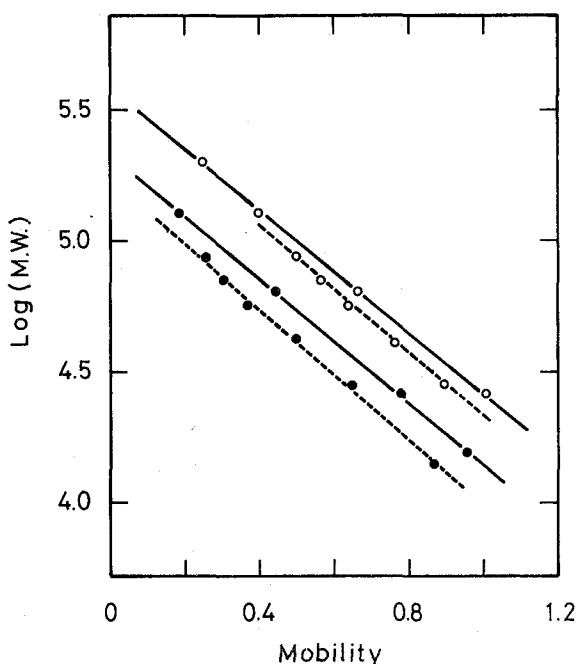
Fig. 8. Log of molecular weight versus electrophoretic mobility for well-characterized proteins (straight lines) and lysozyme aggregates (dashed lines) in 7.5% (open circles) and 10% (closed circles) gels.

Table II. Effective Molecular Weights of Lysozyme Aggregates in SDS-polyacrylamide Gel Electrophoresis Applicable to Both 10% and 7.5% Gels.

lysozyme aggregate	effective molecular weight ($\times 10^{-4}$)
monomer	2.0
dimer	3.5
trimer	5.5
tetramer	7.6
pentamer	8.9
hexamer	9.8

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in SDS solutions. The electrophoretic mobilities of lysozyme aggregates seem somewhat smaller in comparison with those of the other aggregates. From these observations it may be suggested that SDS-protein complexes have a general tendency to make aggregates, and proteins other than lysozyme could be used for the similar purpose.

ACKNOWLEDGMENTS

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