Molecular Weight of the Coat Protein of Alfalfa Mosaic Virus*

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ABSTRACT: Degradation of alfalfa mosaic virus in 0.5 M $MgCl_2$ gives rise to an RNA precipitate and a soluble protein. Experiments with the protein in the analytical ultracentrifuge yielded $s^0_{20°,w} = 2.93$ S and $D^0_{20°,w} = 5.30 \times 10^{-7}$ cm²/sec. From these figures a molecular weight of 51.6×10^3 was calculated, in agreement with the results of sedimentation equilibrium measurements which gave a molecular weight of 50.9×10^3. Carboxymethylated protein was solubilized with sodium dodecyl sulfate (SDS). Sedimentation equilibrium measurements, after correction for SDS bound by the protein, yielded a molecular weight of 25.2×10^3. On basis of the cysteine, tryptophan, and tyrosine content of the protein molecular weights of 23.3×10^3, 23.2×10^3, and 23.3×10^3 were calculated. This is in agreement with the minimum value obtained from total amino acid composition (24.8×10^3). As a mean from these results a value of 24,500 was accepted for the subunit molecular weight. Electrophoresis in SDS polyacrylamide gel revealed no difference in molecular weight for protein of eight different strains of the virus. The molecular weight obtained with this technique agreed within 12% with the value of 24,500. This value is considerably lower than those found by others ($34–38 \times 10^3$, Kelley, J. J., and Kaesberg, P. (1962), *Biochim. Biophys. Acta 55*, 236; and 32.6×10^3, Hull, R., Rees, M. W., and Short, M. N. (1969), *Virology 37*, 404). The product obtained by degrading the virus in $MgCl_2$ is a stable dimer of the subunit.

Preparations of alfalfa mosaic virus contain at least five components (Figure 1), which have been designated bottom component, middle component, top component b, top component a and top component o (Bancroft and Kaesberg, 1960; Kelley and Kaesberg, 1962b; Jaspars and Moed, 1966). As far as known, all components are nucleoproteins and have the same coat protein (Kelley and Kaesberg, 1962a; Moed and Veldstra, 1968). In the last years insight has been obtained concerning the biological role of these components (Van Vloten-Doting and Jaspars, 1967; Van Vloten-Doting *et al.*, 1968, 1970). In these studies evidence is presented that one of the top components contains the genetic information for the coat protein, while the middle and bottom components do not contain this information. In order to establish whether, *e.g.*, the RNA of the top component b contains only the genetic information for the coat protein or is large enough to code for other proteins as well (*i.e.*, whether it is a monocistronic or a polycistronic messenger), it is necessary to know the molecular weights of the subunit of the coat protein and of the RNA of the top component b, respectively. The investigations described in this paper were aimed at a good estimate of the molecular weight of the protein subunit. Physical as well as chemical methods were used and yielded values in good agreement with each other, however, being much lower than earlier estimates (Kelley and Kaesberg, 1962a; Hull *et al.*, 1969a).

Materials and Methods

Viruses. Unless stated otherwise, the alfalfa mosaic virus used in this work was derived from isolate 425 of Hagedorn and Hanson (1963). The virus was grown in *Nicotiana tabacum* L. var. "Samsun NN" and isolated according to Van Vloten-Doting and Jaspars (1967), unless stated otherwise. Crude virus (a preparation containing all AMV[1] components) was fractionated by use of Mg^{2+} ions to a top fraction (containing the smaller components) and a bottom fraction (containing the heavier components) as described by Van Vloten-Doting and Jaspars (1967). Purified bottom component was obtained by zonal gradient centrifugation (Van Vloten-Doting *et al.*, 1970).

TMV (U1 strain) and phage MS2 were gifts from Drs. A. van Kammen and H. O. Voorma, respectively. TYMV was isolated from chinese cabbage (*Brassica chinensis* L. var. Wong Bok) by the procedure of Dunn and Hitchborn (1965).

Reference Proteins. Proteins used as references were obtained from Sigma: serum albumin, carboxypeptidase A, and lysozyme; from Boehringer Mannheim: pyruvate kinase, glutamate dehydrogenase, fumerase, and cytochrome *c;* from Worthington: aldolase; from Nutritional Biochemicals Corp.: ovalbumin, trypsin, and bovine pancreatic ribonuclease; from Koch-Light: carbonic anhydrase; from Mann: myoglobin; from Calbiochem: lysozyme.

Preparation of Viral Proteins. AMV protein was prepared from AMV bottom fraction or purified bottom component (unless stated otherwise) in the following way (Moed, 1966). A virus solution at a concentration of 25 mg/ml in 0.01 M sodium phosphate buffer (pH 7.0) was slowly and under vigorous shaking added at 4° to an equal volume of 1 M $MgCl_2$, containing 5% 2-mercaptoethanol. The mixture became turbid as the RNA from the dissociated virus precipitated. After 15 min the precipitate was removed by centrifugation at 6500*g*, during 15 min. The supernatant which contained the protein was subsequently dialyzed against buffer or distilled water. After dialysis the preparation was once more centrifugated to remove aggregated material.

The protein as prepared in this way contained only traces

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); HNB, 2-hydroxy-5-nitrobenzyl; SDS, sodium dodecyl sulfate; AMV, alfalfa mosaic virus; TYMV, turnip yellow mosaic virus.

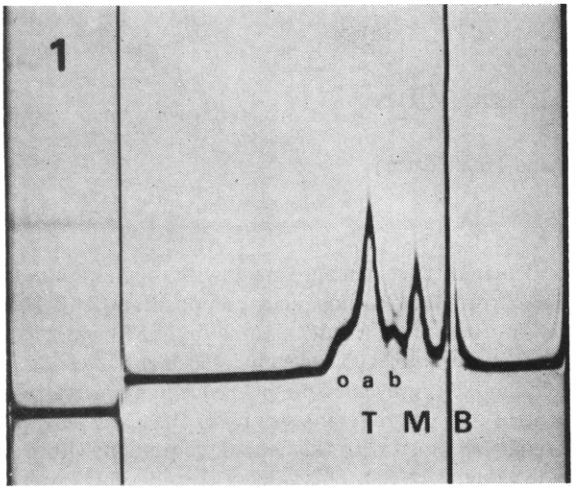

FIGURE 1: Schlieren diagram of AMV. Sedimentation from left to right; 20°; 0.01 M sodium phosphate buffer (pH 7.0); schlieren angle 70 deg; concentration 10 mg/ml. Photograph taken 12 min after the rotor reached a speed of 42,040 rpm. T (o, a, b), M and B, top (o, a, b), middle, and bottom components.

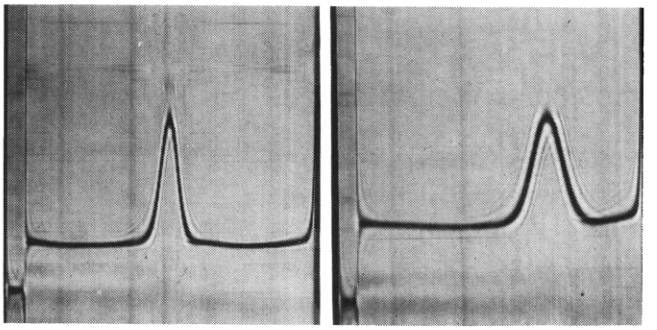

FIGURE 2: Sedimentation of AMV protein, as obtained by schlieren optics. Sedimentation from left to right; 5°; 0.01 M magnesium acetate pH 5.5; schlieren angles 70 and 60 deg, respectively; concentration 6 mg/ml. Photographs taken 16 and 88 min, respectively, after the rotor reached a speed of 59,780 rpm. An artificial boundary cell (valve type) was used in this experiment.

of impurities as judged by sedimentation (Figure 2) and SDS polyacrylamide gel electrophoresis (Figure 12B).

TMV and TYMV proteins were prepared by the acetic acid method of Fraenkel-Conrat (1957). MS2 protein was isolated according to Ling *et al.* (1969).

Reagents. DTNB was obtained from Aldrich Chemical Corp., HNB bromide from the British Drug Houses and Rosaniline from George T. Gurr Ltd.

Guanidine hydrochloride (Aldrich Chemical Corp.) was crystallized from methanol, SDS (Serva), and urea (British Drug Houses) from ethanol.

2-Mercaptoethanol (Puriss. p. a.) was a product of Fluka AG.

S-Carboxymethylated Protein. Carboxymethylation of salt-free lyophilized protein was performed as described previously (Kruseman, 1969). Analysis of the CM-protein revealed that more than 98% of the cysteine residues were carboxymethylated and that less than 1% of other amino acid residues were changed.

Oxidized Protein. Performic acid oxidation was carried out according to the method of Hirs (1956).

Sedimentation Measurements. SEDIMENTATION VELOCITIES were measured with schlieren optics in a Spinco Model E analytical ultracentrifuge using a valve-type artificial boundary cell. Sedimentation coefficients were converted into water basis with the use of measured values of the density and viscosity of the buffer.

SEDIMENTATION EQUILIBRIUM experiments were made in double-sector cells with 3-mm solution columns (Van Holde and Baldwin, 1958) at relatively high speed, according to Yphantis (1964), and at constant temperatures between 15 and 18°. Equilibrium distribution of the protein was measured with a split-beam photoelectric scanner, using monochromatic light of 280-nm wavelength (Schachman and Edelstein, 1966).

The method was checked by measuring bovine pancreatic ribonuclease in 0.1 M NaCl–0.01 M sodium phosphate buffer (pH 6.4). Using a partial specific volume of 0.695 (Harrington and Schellman, 1956) a molecular weight of 13.3×10^3 was calculated, as compared to the theoretical value of 13,683 (Scheraga and Rupley, 1962).

Diffusion. Diffusion experiments were performed in a double-sector snythetic boundary cell (capillary type) in a Spinco Model E analytical ultracentrifuge, using the split-beam photoelectric scanner and with the rotor operating at 3397 rpm. The experiments and calculations were performed according to Markham (1967). As a check on the procedure we measured the diffusion coefficient of turnip yellow mosaic virus, at a concentration of 0.1 mg/ml in 0.05 M sodium acetate buffer (pH 6.4). We obtained $D_{20°,w} = 1.48 \times 10^{-7}$ cm²/sec, in agreement with the literature data (1.55×10^{-7} cm²/sec, Markham, 1951; and 1.48×10^{-7} cm²/sec, Stols, 1964).

Gel Electrophoresis. Electrophoresis in SDS polyacrylamide gel was performed exactly as described by Weber and Osborn (1969). Gels of 10% acrylamide with a length of 7 cm and a diameter of 6.6 mm were used.

Dry Weight and Ash Content Determinations of Protein. Dry weight and ash were determined as reported previously (Kruseman, 1969).

Protein Determinations. The nitrogen content of the samples was determined by direct Nesslerization of Kjeldahl digest, using the procedure of Minari and Zilversmit (1963), and converted into protein on the basis of the amino acid composition (17.27% N). AMV protein from a batch, on which a Dumas nitrogen analysis had been performed, was used for the calibration line. Concentrations of lysozyme and bovine pancreatic ribonuclease were measured spectrophotometrically at 280 nm taking 2.65 and 0.695, respectively, as the extinction coefficients of a 1-mg/ml solution (Edelhoch, 1967).

Phosphorus Determination. Phosphorus was determined with the method of Knight and Woody (1958).

SDS Determination. SDS was determined with the procedure of Karush and Sonenberg (1950).

Cysteine Determinations. The number of cysteine residues was determined with DTNB (Ellman, 1959) in the following way. Salt free protein was incubated in a concentration of 0.73–1.15 mg/ml at 40° in 8 M urea, 10^{-3} M EDTA, and 10 mg/ml of sodium borohydride. At intervals 0.4-g samples were transferred to tubes containing 2.5 ml of 8 M urea, 0.7 N HCl, and 10^{-3} M EDTA. When all samples had been taken, 0.19 ml of 0.8 M Tris was added to them. The pH rose to pH 8.0. Directly afterward 0.18 ml of 0.01 M DTNB in 0.05 M sodium phosphate (pH 7.0) was added and the optical density at 512 nm was read immediately. After correction of the appropriate blanks optical density values were plotted against time. From the optical density in the plateau, and the protein concentration the equivalent weight per SH group was calcu-

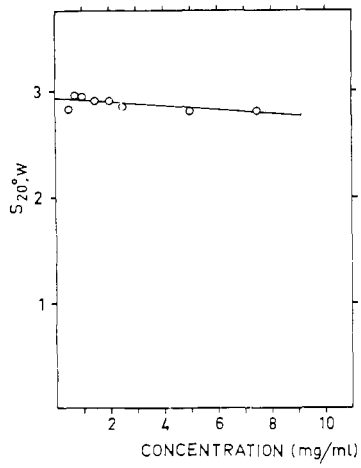

FIGURE 3: Sedimentation coefficients of AMV protein as a function of initial concentration, in 0.01 M magnesium acetate (pH 5.5); 20° and 52,640 rpm.

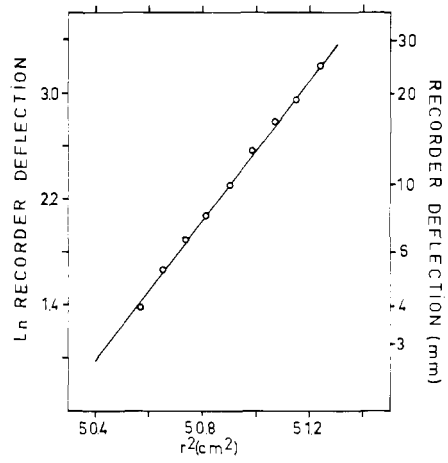

FIGURE 4: Molecular weight determination of AMV protein from a sedimentation equilibrium experiment, in 0.05 M sodium acetate–0.1 M NaCl (pH 4.5). Rotor speed was 29,500 rpm. A straight line was fitted by least squares. A molecular weight of 50.9×10^3 was calculated from this experiment.

lated. The method was checked with 2-mercaptoethanol, lysozyme, and bovine pancreatic ribonuclease.

Spectrophotometric Determination of Tryptophan and Tyrosine. Tryptophan and tyrosine contents of the protein were determined spectrophotometrically according to Edelhoch (1967) after correction for light scattering (Englander and Epstein, 1957) and RNA content. Protein preparations containing more than 0.25% RNA were not used. The method was checked with bovine pancreatic ribonuclease and lysozyme.

Chemical Modification and Estimation of Tryptophan Residues. The protein was treated with 2-hydroxy-5-nitrobenzyl bromide in varying amounts according to the standard procedure of Barman and Koshland (1967). Preceding the reaction the protein, at a concentration of 8 mg/ml, was incubated in 8 M urea and 10 mg/ml of sodium borohydride for 30 min at 40°. At the end of this period the pH was adjusted to 2.7 by adding 6 N HCl and the incubation was continued for 16–24 hr at 37°. After the reaction with HNB bromide the modified protein remained in solution and could be eluted from the Sephadex column in 0.18 M acetic acid. Precipitation and washing procedures were followed by drying the protein in a desiccator over phosphorus pentoxide. The dried protein was dissolved in 0.1 N NaOH. This solution was used both for nitrogen determination and for the spectrophotometric assay of HNB groups.

As Barman and Koshland point out that a new ingredient (in this case sodium borohydride) may change the chemistry of the reaction, and as cysteine reacts slowly with HNB bromide, our unfolding procedure was checked by treating lysozyme at a concentration of 5 mg/ml in the same way.

Amino Acid Analysis. About 6 mg of protein was dissolved in 1.2 ml of 50% acetic acid, which contained 1.0 μmole of norleucine as an internal standard. The protein solution was divided among four hydrolysis tubes, and desiccated under vacuum. Subsequently each tube received 0.5 ml of 6 N HCl; when tryptophan was to be determined, also 4% thioglycolic acid was present, following the method of Matsubara and Sasaki (1969). Oxygen was removed then by a stream of nitrogen, the tubes were frozen in liquid nitrogen, evacuated, and sealed. Hydrolysis was performed at 110° in the dark for 12, 24, 48, and 72 hr, respectively. The hydrolysates were taken rapidly to dryness in a rotary evaporator and immediately analyzed on a Beckman Unichrom amino acid analyzer according to the method of Spackman *et al.* (1958).

Computer Calculations of the Best-Fitting Molecular Weight. From the percentages of amino acids, as they were found in the amino acid analysis, the best-fitting molecular weight was determined following the method used by Black and Hogness (1969). For an extended series of molecular weights, increasing gradually with intervals of 50 units, the corresponding amino acid residue numbers were calculated on base of their relative contents. As a rule the residue values found will not be integral numbers as should theoretically be expected for a complete polypeptide chain. Indicating the difference between found value and nearest integer I_i with ΔI_i, the relative deviation from theoretical values is $\Delta I_i/I_i$. As the maximum relative deviation for each amino acid is $0.5/I_i$, we can define $f = \Sigma(\Delta I_i/I_i)/0.5\Sigma(1/I_i)$ as the fraction of maximum deviation from the ideally fitting amino acid composition for a given molecular weight. So the best-fitting molecular weight for a protein is to be found at the lowest minimum for f. Calculations were made with the aid of a program written in Algol 60 on a IBM 360 computer.

Results

Physical Studies on the Native AMV Protein

Sedimentation Velocity. The sedimentation velocity of the native protein was measured at 20° in 0.01 M magnesium acetate buffer (pH 5.5). The protein, when freshly prepared, sedimented with a single symmetrical boundary (Figure 2). There appeared to be a slight concentration dependence over the range of protein concentrations (8–0.5 mg/ml) explored (Figure 3). Extrapolation of the $s^0_{20°,w}$ *vs.* c plot to $c = 0$ gave $s^0_{20°,w} = 2.93$ S.

Diffusion and Molecular Weight by Sedimentation Velocity and Diffusion. Diffusion coefficients were measured at a concentration of 1 mg/ml in 0.05 M magnesium acetate (pH 5.5). As the protein concentration was low enough, no extrapolation to zero concentration was needed. Our measurements yielded a value of $D_{20°,w} = 5.30 \times 10^{-7}$ cm²/sec (mean of 5.34 and 5.26). This, together with the sedimentation coefficient and a partial specific volume of 0.735 (calculated from the

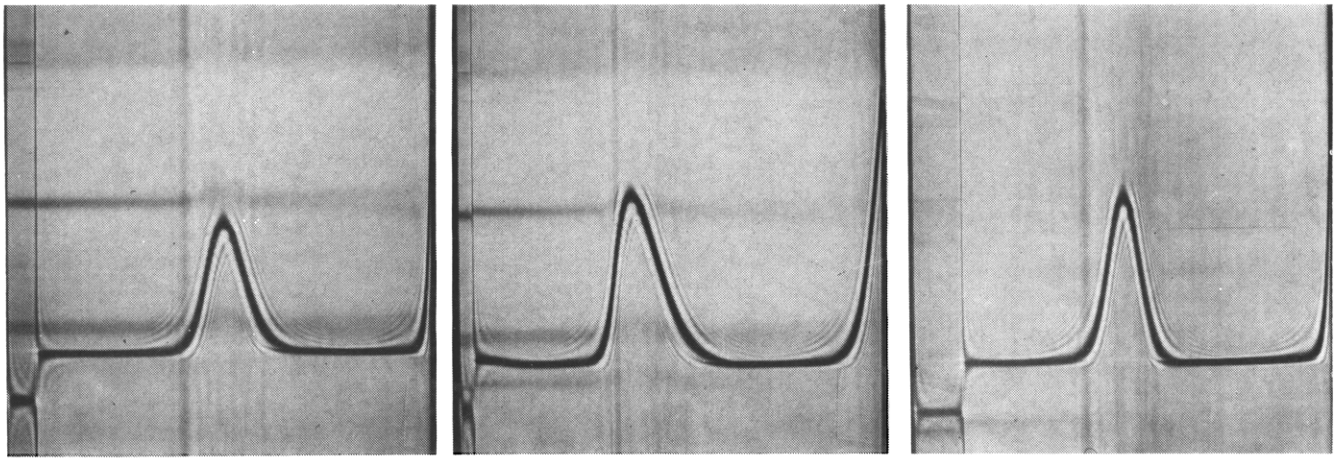

FIGURE 5: Effect of SDS and carboxymethylation on the sedimentation behavior of AMV protein. Sedimentation from left to right; 20°; schlieren angle 70 deg; concentration ±7 mg/ml. Photographs taken 20 min after the rotor reached a speed of 52,640 rpm. Artificial boundary cells (valve type) were used in these experiments. (A, left) AMV protein in distilled water; (B, middle) the same preparation as in A dialyzed against 0.01 M SDS in distilled water; and (C, right) AMV CM-protein in 0.01 M sodium phosphate buffer, 0.1 M NaCl, 10^{-3} M SDS, and 10^{-3} M NaN_3 (pH 7.0).

amino acid composition) gives a molecular weight of 51.6 × 10^3.

Sedimentation Equilibrium and Molecular Weight. Confirmation of the above value for the molecular weight was obtained by sedimentation equilibrium experiments with the rotor operating at 29,500 rpm. A typical plot of ln c *vs.* r^2 is represented in Figure 4. In Table I the molecular weights obtained by sedimentation equilibrium under different conditions of ionic strength and pH are summarized. A mean value of 50.9 × 10^3 was calculated from these experiments.

Physical Studies on the Protein in the Presence of SDS

Stubbs and Kaesberg (1964) found that when bromegrass mosaic virus is dissociated into protein and nucleic acid by means of a high concentration of $CaCl_2$, the resulting protein is present in the form of a dimer of the true chemical subunit. In order to investigate the possibility that the protein of AMV prepared with $MgCl_2$ behaves in a similar way, we measured the molecular weight of the protein in the presence of SDS. However, when the protein was dialyzed directly against an SDS solution, the resulting product appeared to be heterogeneous in the analytical ultracentrifuge (Figure 5B) as was also experienced by Kelley and Kaesberg (1962a) with their preparations of AMV protein in the presence of SDS. We have found that this heterogeneity does not occur when the SH groups of the protein are carboxymethylated prior to the addition of SDS. This is illustrated in Figure 5C.

Sedimentation Equilibrium and Molecular Weight. The molecular weight of the CM-protein was measured by sedimentation equilibrium analysis at 33,450 rpm after equilibrium dialysis against two buffers, *viz.*, 0.01 M sodium acetate–0.1 M NaCl–10^{-3} M NaN_3–10^{-3} M SDS (pH 5.0) and 0.01 M sodium phosphate–0.1 M NaCl–10^{-3} M NaN_3–10^{-3} M SDS (pH 7.0). From the difference in SDS concentration inside and outside the dialysis bag the amount of SDS bound by the protein was calculated. A typical plot of ln c *vs.* r^2 is shown in Figure 6. In Table II the molecular weights of the protein–SDS complexes, the composition of the complexes and the molecular weights of the CM-protein, are summarized. Partial specific volumes of the protein–SDS complexes were calculated from their composition, using a value of 0.885 as the partial specific volume of SDS (Granath, 1953). To check the whole procedure, we determined the molecular weight of bovine pancreatic ribonuclease using a buffer containing 0.1 M NaCl, 0.01 M sodium phosphate, 10^{-3} M SDS, and 10^{-3} M NaN_3 (pH 6.4). We found a protein weight fraction in the complex of 0.547 and a molecular weight of the protein of 13.3 × 10^3.

TABLE I: Molecular Weight of Native AMV Protein.

Expt	Buffer	pH	Concn[c] (mg/ml)	Mol Wt × 10^{-3}
1	Magnesium acetate[a]	5.5	0.28	46.1
2	Magnesium acetate[a]	5.5	0.30	54.8
3	Magnesium acetate[a]	5.5	0.32	51.0
4	Sodium acetate[b]	4.5	0.26	50.9
5	Sodium acetate[b]	5.0	0.29	50.6
6	Sodium acetate[b]	5.0	0.31	52.2

[a] 0.05 M magnesium acetate. [b] 0.05 M sodium acetate–0.1 M NaCl. [c] Initial protein concentration.

TABLE II: Determination of Molecular Weight of AMV CM-Protein in the Presence of SDS.

Expt	Buffer	Concn[c] (mg/ml)	Wt Fraction Protein in the Complex	Mol Wt of the Complex × 10^{-3}	Mol Wt of the Protein × 10^{-3}
1	Acetate[a]	0.43	0.425	58.3	24.8
2	Phosphate[b]	0.36	0.555	48.2	26.8
3	Phosphate[b]	0.54	0.555	45.0	25.0
4	Phosphate[b]	0.33	0.586	41.3	24.2

[a] 0.01 M sodium acetate–0.1 M NaCl–10^{-3} M NaN_3–10^{-3} M SDS (pH 5.0). [b] 0.01 M sodium phosphate–0.1 M NaCl–10^{-3} M NaN_3–10^{-3} M SDS (pH 7.0). [c] Initial protein concentration.

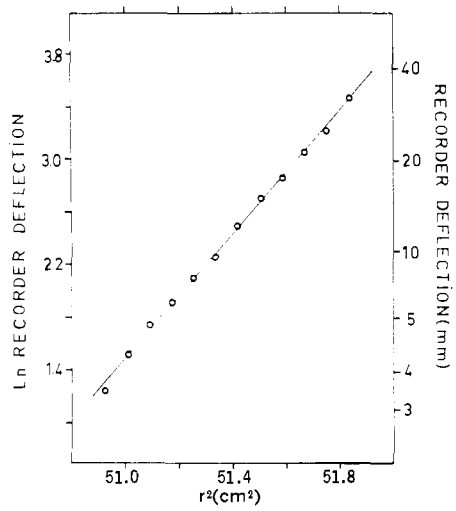

FIGURE 6: Molecular weight determination of AMV CM-protein in 0.01 M sodium phosphate, 0.1 M NaCl, 10^{-3} M SDS, and 10^{-3} M NaN_3 (pH 7.0). Rotor speed was 33,450 rpm. A straight line was fitted by least squares. A molecular weight of 48.2×10^3 was calculated for the protein–SDS complex from this experiment.

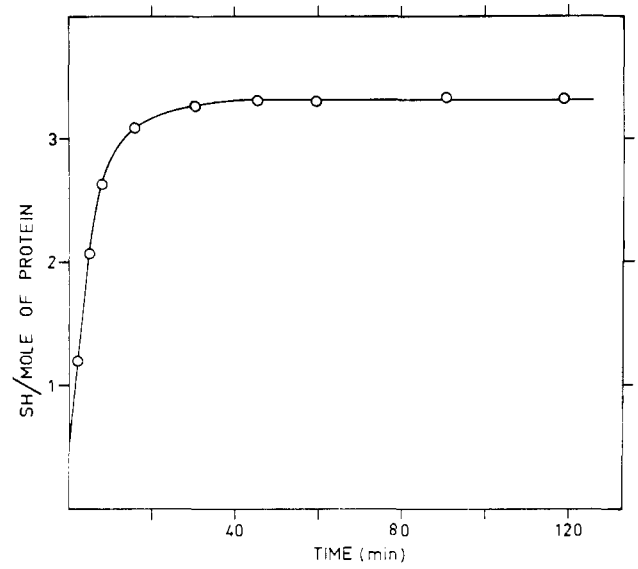

FIGURE 7: Course of the reduction of AMV protein (0.73 mg/ml) by 1% sodium borohydride in 8 M urea–10^{-3} EDTA at 40°. At intervals samples were taken for the determination of SH content with DTNB. The molecular weight of the protein was taken as 24,500.

A mean value of 25.2×10^3 is found for the molecular weight of the CM-protein of AMV. From this it is concluded that the protein prepared with $MgCl_2$ is a dimer of the true chemical subunit.

Chemical Studies on the Protein

The purity of the four protein preparations, used in the chemical studies, was tested by comparing nitrogen content with dry weight, and by determining ash and phosphorus content. The results are summarized in Table III. The possibility that the discrepancy between the nitrogen content (agreeing within 2% with the amino acid analysis) and the dry weight determination would be due to salt or RNA is ruled out, by the relevant analyses. Possibly we have to do with plant material strongly absorbed by or maybe even covalently bound to the protein. In view of the variable amounts of impurities we have made our calculations on the basis of nitrogen content.

In Table IV the results of the determinations of cysteine, tryptophan, and tyrosine content of the protein are summarized. The results obtained with 2-mercaptoethanol, lysozyme, and bovine pancreatic ribonuclease are included and show a good agreement with the values from the literature. On the basis of three cysteine residues an average value of 23.3×10^3 was found for the molecular weight of the protein. In Figure 7 is shown the course of the reduction for one of the protein preparations. Apparently in this case the protein before treatment was almost completely oxidized. However, this is not the native situation as in several other preparations the treatment hardly introduced any new SH groups. For four tyrosine and two tryptophan residues the values of the molecular weight were 23.3×10^3 and 23.2×10^3, respectively.

Figure 8 shows the results obtained when reduced AMV protein and reduced lysozyme were labeled with increasing amounts of HNB bromide. It is clear that two and six HNB groups per molecule, respectively, are incorporated in the first phase of the labeling process. This corresponds with the

TABLE III: Purity of AMV Protein Preparations.

Prepn No.	Protein Content as Percentage of Dry Wt[a]	Percentage Ash	Percentage RNA[b]
I	100.1	<0.2	0.11
II	94.6	<0.2	0.14
III	89.8	*c*	0.25
IV	94.3	0.3	0.65

[a] From N content. [b] From P content. [c] Not determined.

TABLE IV: Determination of Molecular Weight on Basis of Cysteine, Tryptophan, and Tyrosine Content.

Prepn	Mol Wt $\times 10^{-3}$ on Basis of Content of: Cys[a]	Trp[b]	Tyr[c]	Lit. Value $\times 10^{-3}$
I	24.2	24.0	22.5	
II	23.0	22.8	24.0	
III	22.2	22.9	23.2	
IV	23.7	*d*	23.4	
2-Mercaptoethanol	0.0781			0.0781
Lysozyme	13.9	14.3	14.2	14.307[e]
Ribonuclease	13.9		*d*	13.683[f]

[a] On basis of three SH groups per molecule for AMV protein, one for 2-mercaptoethanol, eight for lysozyme, and eight for ribonuclease, respectively. [b] On basis of two tryptophan residues per molecule for AMV protein, and six for lysozyme. [c] On basis of four tyrosine residues per molecule for AMV protein and three for lysozyme. [d] Not determined. [e] Canfield (1963). [f] Scheraga and Rupley (1962).

TABLE V: Amino Acid Composition.[a]

	AMV Protein						AMV CM-Protein					
	Time of Hydrolysis (hr)				Value Taken	Nearest Integer	Time of Hydrolysis (hr)				Value Taken	Nearest Integer
	12	24	48	72			12	24	48	72		
Aspartic acid	21.3	21.1	20.8	29.0	21.0	21	21.3	21.3	21.6	21.4	21.4	21
Threonine	13.1	12.9	12.6	11.5	13.2	13	12.9	12.9	12.4	11.8	13.0	13
Serine	14.0	13.5	11.9	10.6	14.7	15	14.1	13.3	12.0	10.7	14.8	15
Glutamic acid	19.7	20.3	19.9	19.7	19.9	20	20.3	20.0	20.1	20.2	20.1	20
Proline	17.7	16.9	17.4	18.0	17.5	17	17.1	17.2	17.1	17.2	17.1	17
Glycine	16.8	17.0	16.9	16.9	16.9	17	17.1	16.8	17.0	16.9	17.0	17
Alanine	20.0	20.3	20.2	20.2	20.2	20	20.4	20.0	20.2	20.4	20.2	20
CM-cysteine							3.1	3.5	3.3	3.1	3.2	3
Valine	12.4	13.1	13.2	13.3	13.3	13	12.2	12.8	13.1	13.2	13.2	13
Methionine	3.0	3.0	3.0	2.9	3.0	3	3.0	2.7	3.0	2.9	2.9	3
Isoleucine	4.8	4.9	4.9	4.9	4.9	5	4.8	5.0	5.0	5.0	5.0	5
Leucine	20.5	20.8	20.7	20.7	20.7	21	20.7	20.7	20.7	21.0	20.8	21
Tyrosine	3.7	3.9	3.9	3.8	3.8	4	3.8	3.8	3.9	3.8	3.8	4
Phenylalanine	17.4	17.6	17.5	17.7	17.6	18	17.5	17.4	17.6	17.7	17.6	18
Tryptophan	1.9	1.9	1.7	1.6	1.9	2						
Lysine	14.2	13.8	13.2	13.0	13.6	14	14.1	14.3	14.1	14.2	14.2	14
Histidine	7.2	7.0	6.8	7.0	7.0	7	7.3	7.4	7.4	7.4	7.4	7
Ammonia	19.0	19.9	21.3	22.0	18.1	18						
Arginine	11.2	11.1	11.0	11.0	11.1	11	11.3	11.6	11.6	11.5	11.5	11

[a] Data are expressed as amino acid residues per molecule, based on the best-fitting molecular weight of 24.8×10^3 (see also Figure 9). Values of different hydrolysis times are all normalized to the same amount of norluecine, added as an internal standard before hydrolysis. Ammonia, threonine, serine, and tryptophan values are extrapolated to zero time. Isoleucine and valine data are taken from 72-hr hydrolysis time.

number of tryptophan residues which are supposed to be present in both proteins.

The results of the amino acid analyses of AMV protein and CM-protein are shown in Table V. From these data the best-fitting molecular weight was calculated. This is illustrated for AMV protein in Figure 9. The lowest minimum emerges at mol wt 24,450. Adding to this value the molecular weight of three cysteine residues (determined as CM-cysteine in CM-protein) we obtain 24.8×10^3 as the most probable molecular weight, which corresponds with 224 amino acids.

Molecular Weight from Electrophoresis on SDS Polyacrylamide Gel. The values for the molecular weight of AMV protein obtained thus far in this study are considerably lower than those reported previously in the literature ($34–38 \times 10^3$, Kelley and Kaesberg, 1962a; 32.6×10^3, Hull *et al.*, 1969a).

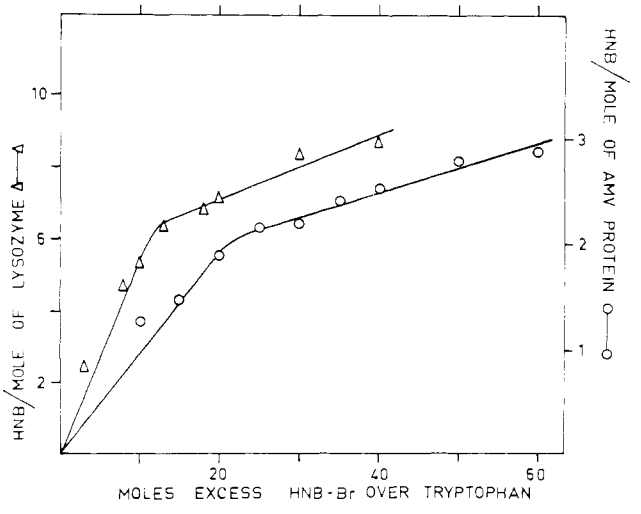

FIGURE 8: The titration of the tryptophans of reduced lysozyme and reduced AMV protein with HNB bromide. The proteins in a concentration of 5 and 8 mg per ml, respectively, reacted in 8 M urea (pH 2.7), with increasing amounts of HNB bromide. The HNB content was determined as described under Materials and Methods. The molecular weights of the proteins were taken as 14,300 and 24,500, respectively.

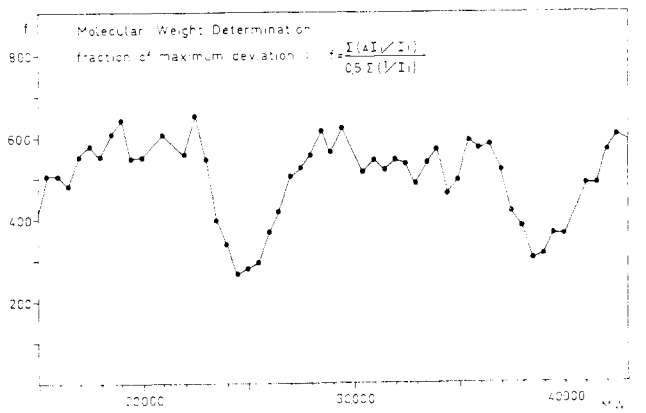

FIGURE 9: Molecular weight determination from amino acid analysis data for AMV protein.

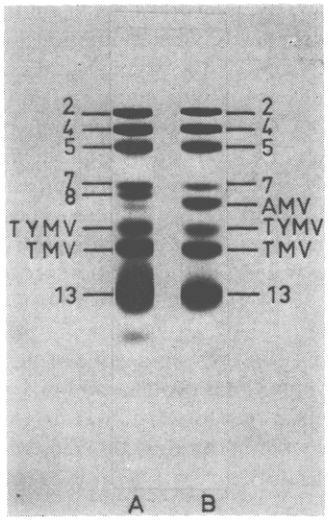

FIGURE 10: Electrophoretogram illustrating the position of the polypeptide chains of AMV, TYMV, and TMV between a set of marker proteins. The anode is at the bottom. The markers are identified by their numbers in the legend of Figure 11. Gel B differs from A in that carbonic anhydrase (8) is replaced by AMV protein.

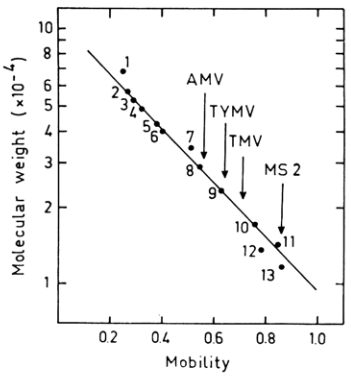

FIGURE 11: Relationship between the logarithm of the molecular weight and the electrophoretic mobility of a number of marker proteins: (1) serum albumin (68,000), (2) pyruvate kinase (57,000), (3) glutamate dehydrogenase (53,000), (4) fumarase (49,000), (5) ovalbumin (43,000), (6) aldolase (40,000), (7) carboxypeptidase A (34,600), (8) carbonic anhydrase (29,000), (9) trypsin (23,300), (10) myoglobin (17,200), (11) lysozyme (14,300), (12) ribonuclease (13,700), and (13) cytochrome *c* (11,700). For referenc esof molecular weights, see Weber and Osborn (1969). The mobilities of four viral proteins are indicated by arrows.

This discrepancy can be caused by differences in experimental procedures as well as by the fact that in the separate investigations different strains of AMV are used and the virus is isolated in different ways. The work of Hull *et al.* (1969a), which is the most extensive, was done with strain 15/64, while their virus was isolated by a milder treatment with butanol–chloroform (1:1, v/v) than used in our procedure. That such differences may influence the molecular weight of the protein subunit by about 5000 has recently been shown for potato virus X (Koenig *et al.*, 1970). Electrophoresis on SDS polyacrylamide gel is a simple method to detect small differences in protein molecular weights (*e.g.*, Shapiro *et al.*, 1967; Weber and Osborn, 1969). Therefore we decided to compare the electrophoretic mobilities of protein from a number of AMV strains and from virus isolated in different ways. Running them in combination with a set of marker proteins of known molecular weight also allowed an estimation of their molecular weight by this technique.

Eight strains of AMV were compared: 425 (the strain used in this study), 15/64 (Hull, 1969), ATCC-106 (Bancroft *et al.*, 1960), vein necrosis virus and yellow spot mosaic virus (Zaumeyer, 1959, 1963), ta 59 and lu 64s (Verhoyen, 1961, 1966), and the California isolate of AMV (Oswald *et al.*, 1954). Protein was prepared from crude virus as well as from top or bottom fractions. Electrophoresis on SDS polyacrylamide gels revealed that protein of all AMV strains had the same electrophoretic mobility. Between a set of marker proteins AMV protein was found just below carbonic anhydrase (Figure 10). When the mobilities of the marker proteins were plotted against their molecular weight, a straight line was obtained. From this a molecular weight of 27,500 ± 500 was extrapolated for the protein of the investigated AMV strains (Figure 11). Treatments which might influence the state of unfoldedness in SDS solution, like carboxymethylation and performic acid oxidation, did not alter the electrophoretic mobility of AMV protein. Strain 15/64 was also isolated according to the method of Hull *et al.* (1969a) from "White Burley" tobacco (the host plant used by Hull *et al.*). Electrophoresis of protein prepared from this virus yielded a molecular weight of 27,500 as well.

To test the reliability of the molecular weight determination in SDS gel we subjected protein of the well characterized viruses: TMV, TYMV, and MS2 to electrophoresis (Figure 11). Molecular weights of 19,500, 22,500, and 13,500 were extrapolated, respectively, deviating up to 12% from the values reported in the literature (TMV: 17,530, Caspar, 1963; TYMV: 20,000, Harris and Hindley, 1965; MS2: 14,700, Lin *et al.*, 1967).

A further possible explanation for the discrepancy between our results and those of Hull *et al.* (1969a) might be a difference in purity between the protein preparations used. Hull *et al.* prepared their protein from crude virus, while in our study gradient purified virus was used as the starting material. Protein prepared from crude AMV 425 contained some impurities (Figure 12A). However, when gradient purified virus was used only a minor contamination with material of a mo-

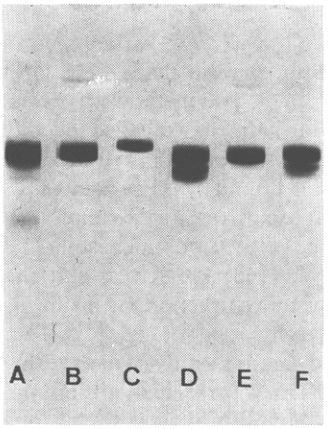

FIGURE 12: Purity of different AMV–protein preparations. Protein prepared from: (A) crude AMV 425; (B) AMV 425 purified by gradient centrifugation, protein carboxymethylated; (C) the same preparations as in B; (D) crude AMV 15/64; (E) top fraction of AMV 15/64; and (F) crude AMV 15/64 isolated from "White Burley" tobacco by the method of Hull *et al.* (1969a). Gel C is loaded with 10 μg of protein. To reveal minor impurities the other gels are loaded with 50 μg of protein. Anode is at the bottom.

TABLE VI: Summary of Molecular Weight Determinations.

Method	Mol Wt of the Chemical Subunit ($\times 10^{-3}$)
Sedimentation equilibrium	25.2
Cysteine content	23.3
Tyrosine content	23.3
Tryptophan content	23.2
Amino acid analysis	24.8
Sedimentation equilibrium of dimer ($\times$ 1/2)	25.5
Sedimentation diffusion of dimer ($\times$ 1/2)	25.8
SDS polyacrylamide gel electrophoresis	27.5

lecular weight of 53,000 was present (Figure 12B,C). Possibly this is an aggregate of AMV protein. On the other hand, protein prepared from crude AMV 15/64 showed considerably more heterogeneity (Figure 12D). In addition to material with a molecular weight of 27,500 two other bands were visible with molecular weights of about 24,000 and 22,000. This heterogeneity disappeared on further purification of the virus. When protein was prepared from an AMV 15/64 top or bottom fraction only protein with a molcular weight of 27,500 was found (Figure 12E). Protein prepared from crude AMV 15/64 isolated by the method of Hull *et al.* (1969a) showed a comparable heterogeneity (Figure 12F).

Discussion

In Table VI the results of the molecular weight determinations of AMV protein are summarized. As a mean from these results a value of 24,500 was accepted for the subunit molecular weight. The molecular weight obtained by electrophoresis in SDS polyacrylamide gel differed about 12% from this value, but a comparable deviation was found for TMV- and TYMV-protein with this technique. A point in need of comment is the difference between our results and those found by others. Kelley and Kaesberg (1962a) found 34–38 $\times 10^3$ and Hull *et al.* (1969a) 32.6 $\times 10^3$. The measurements by Kelley and Kaesberg were made on a protein preparation solubilized with SDS. The method was the approach to sedimentation equilibrium, the so-called Archibald principle, as described by Ehrenberg (1957). The fact that no correction was made for SDS bound by the protein (J. J. Kelley, personal communication) seems to offer a simple explanation for the discrepancy as it is clear from our results (Table II) and those of others (Boeyé, 1965; Pitt-Rivers and Impiombato, 1968) that omission of this correction results in too high values for the molecular weight.

As Kelley and Kaesberg did not dialyze their protein against SDS till equilibrium was reached, it is not possible to say how much SDS was bound in their case, but one would expect a lower molecular weight for such a complex than for the complex obtained after equilibrium dialysis. This expectation is fulfilled as we found still higher values for the protein–SDS complex (Table II).

Hull (Hull *et al.*, 1969a) found a subunit molecular weight of 32.6 $\times 10^3$. This was determined from amino acid analyses and specific staining for certain amino acids on peptide maps from tryptic and chymotryptic digests of the protein. The result was complemented by determinations of the C- and N-terminal residues and of the tryptophan content, although the end-group determinations show rather large differences (39,000 and 23,400 for N and C terminal, respectively). In view of the different experimental approach it is difficult to give a reasonable explanation for the difference between his results and ours. Our gel electrophoresis experiments revealed that the discrepancy did not originate from differences between AMV strains or from the isolation procedures, but that the cause might be a difference in purity between the protein preparations used.

As a consequence of the lower value of the subunit molecular weight, the number of protein subunits present in each of the components, as put forward by Hull (Hull *et al.*, 1969b) will have to be revised in our opinion. This will be the subject of a separate paper.

Addendum

During the preparation of this manuscript we learned from Dr. A. J. Gibbs that in his laboratory (Rothamsted Experimental Station, Harpenden, Herts, England) Dr. J. Carpenter determined a molecular weight for AMV protein (strain 15/64) of 27,250 by electrophoresis in SDS polyacrylamide gel. Also Dr. Stéhelin informed us that Dr. Collot (Institut de Botanique, Strasbourg, France) has found a molecular weight of AMV protein of 23–24 $\times 10^3$ using chemical methods.

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Subunit Structure of Azoferredoxin from *Clostridium pasteurianum* W5*

George Nakos and Leonard Mortenson†

ABSTRACT: Azoferredoxin from *Clostridium pasteurianum* W5 has a molecular weight of 55,000 ± 5000 as determined by gel filtration. Treatment of azoferredoxin with sodium dodecyl sulfate results in the dissociation of the "native" enzyme into two subunits of mol wt 27,500 ± 1350. The subunits are identical in size and they appear to be chemically similar. Each dimer contains 4 iron atoms and about 4 acid-labile sulfide groups.

Azoferredoxin is one of the two proteins required by *Clostridium pasteurianum* to catalyze nitrogen fixation (Mortenson *et al.*, 1967). Moustafa and Mortenson (1969) reported that azoferredoxin of 90–95% purity had an estimated molecular weight of 40,000 and 2 iron and 2 acid-labile sulfide atoms per molecule.

This paper presents evidence that azoferredoxin exists in solution as a dimer of two polypeptide chains of identical molecular weights and that the molecular weight of the dimer is 55,000 and contains 4 iron and 4 acid-labile sulfide atoms per molecule.

Materials and Methods

Chemicals. The chemicals used were obtained: from Eastman Organic Chemicals, acrylamide and *N,N'*-methylbisacrylamide; from Sargent Co., *N,N,N',N'*-tetramethylethylenediamide; from Fisher Scientific Co., sodium dodecyl sulfate and bromophenol blue; from Mann Research Laboratories, comassie brilliant blue R-250, from Pharmacia, Sephadex G-200 and Blue Dextran 2000. All other reagents were commercially obtained and of the best available grade.

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