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Concerning the Tertiary Structure of the Soluble Surface Proteins of Paramecium*

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ABSTRACT: Six of the water-soluble globular proteins found on the surface of *Paramecium aurelia* (serotypic or immobilization antigens) are compared with respect to their amino acid compositions. They contain a remarkably high percentage of cystine (>10%) and hydroxylamino acids (24–29%), particularly threonine (12–16%).

The complement of hydrophobic amino acids is low, their average hydrophobicities, $H\phi_{av}$, ranging from 818 to 892 cal per mole of residue. The sedimentation coefficients of these molecules show marked concentration dependence and although hydrodynamic data indicate that the molecules are effective hydrodynamic prolate ellipsoids with axial ratios of about 11:1, the validity of this finding as regards their true shape is not supported by electron microscopy which presents a rough circular image about 175 Å in diameter. Analyses of the amino acid composition using Jennings' equation, predict that the oblate ellipsoidal dimensions should be about 200 × 15 Å while the prolate ellipsoid would measure about 1000 × 24 Å. We suggest that these molecules are in fact disk like. Optical rotary dispersion measurements indicate that little helical structure is present. A model for these molecules is presented in which it is suggested that they might resemble double bimolecular leaflets and may act as surface protectants.

Coating the surface of the ciliated protozoan, *Paramecium*, are a family of proteins termed the serotypic or immobilization antigens. Any one animal may have the genetic potential for expressing over a dozen members of the family but normally only one of the group is detectable at a given time. In short, a system of mutual exclusion operates (see Beale, 1954, Beale and Wilkinson, 1961, and Preer, 1968, for reviews).

Preer (1959) demonstrated the proteinaceous character of these molecules and estimated that one of them (51A from *Paramecium aurelia* syngen 4) has a molecular weight of about one-quarter million (±20%). Later studies by Jones (1965) and Steers (1965) have extended Preer's findings, so that amino acid analyses are available for antigens 90D, 178D, and 90G (syngen 1) and 51A. In addition Steers determined the molecular weight of 51A to be 310,000 g/mole while Jones estimated the syngen 1 molecules to have molecular weights of 250,000 g/mole.

Comparative analyses of these data together with our findings on 51A, 51B, and 51D show that the serotypic anti-

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gens are a hitherto unrecognized type of globular protein closely related to one another and with a remarkably low ratio of nonpolar to polar amino acids. The α-helical content is low or nonexistent. They contain an extraordinary amount of hydroxylamino acids and cystine. The evidence presented suggests that these molecules are in the form of highly asymmetrical oblate spheroids which act as a surface protectant, are bound to the pellicular surface electrostatically rather than hydrophobically, and which may resemble double bimolecular leaflets.

Materials and Methods

Protein Purification. Antigens 51A, 51B, and 51D were obtained and purified by methods previously described (Macindoe and Reisner, 1967; Reisner and Rowe, 1969). The initial ammonium sulfate precipitation of 51D was at 67% saturation while reprecipitations were done at 50% saturation.

Physical Determinations. Molecular weights were determined by meniscus depletion equilibrium (Yphantis, 1964) in a Spinco Model E ultracentrifuge and measurements of the photographic plates were made using a Nikon Model 6C comparator equipped with 2-μ/division Mitutoyo micrometers. The partial specific volume, $\bar{v}$, of 51A was determined using the H_2O–D_2O system in the ultracentrifuge (Edelstein and Schachman, 1967). Sedimentation coefficients were determined using two separate preparations of each protein and extrapolating to infinite dilution by the method of least squares. Both $s^0_{20,w}$ and $s^0_{25,w}$ were computed. Tris buffer was used in all these determinations (0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.1 M $MgCl_2$). In addition $\bar{v}$ was calculated from the amino acid compositions for all antigens analyzed (Cohn and Edsall, 1943).

Optical rotary dispersion measurements were done in a Perkin-Elmer Model 141 photoelectric polarimeter at wavelengths of 365, 436, 546, and 578 (Hg lamp) and 589 mμ (Na lamp) at 18° using a 1-dm cell. The solvent was 0.01 M phosphate (pH 7.0). The Drude dispersion parameter, λ_c, and the constants a_0 and b_0 of the Moffitt equation were obtained by the methods described by Fasman (1963); λ_0 was taken as 212 mμ.

Isoelectric points were determined by isoelectric focusing (Svensson, 1961, 1962a,b) in a 33 density-step 110-ml column using 1% Ampholine pH 3–5 buffer (LKB, Stockholm). Columns were run at 800 V for 65–72 hr at either 1 or 20° and column temperature controlled to ±0.2°. Positions of the antigens were determined by pumping the contents of the column through a flow analyzer and recording the absorbance at 280 mμ. Fractions of 2 ml were collected and analyzed for antigenicity using adsorption titration (Macindoe and Reisner, 1967) and the pH of the fractions was determined using a Beckman expanded scale pH meter.

Kinematic viscosities were measured using Cannon-Manning semimicro viscometers with flow times for water of 175–200 sec. The temperature was maintained at 25.00 ± 0.005° using a precision water bath and flow times were measured electronically to 0.01 sec. The proteins were dissolved in the Tris buffer described above and passed through 0.45-μ Millipore filters immediately before measuring. Water and buffer flow times were redetermined before each series of measurements. Values for β (Scheraga and Mandelkern, 1953; Scheraga, 1961) were determined using the relationship, $\beta = Ns[\eta]^{1/3}\eta_0/M^{2/3}(1 - \bar{v}\rho)$, where N is Avogadro's number, s is $s^0_{25,w}$, η_0 is the viscosity of the solvent, ρ is the density of the solvent, and $[\eta]$, M, and $\bar{v}$ are the intrinsic viscosity, molecular weight, and partial specific volume, respectively, of the protein.

Protein concentrations were determined interferometrically as described previously (Reisner and Rowe, 1969).

Amino Acid Analysis. Analyses of the amino acid content of 51A, 51B, and 51D were made using a Beckman 120C amino acid analyzer. Freeze-dried samples of the antigen (2.0 mg) were hydrolyzed in evacuated sealed tubes with 2 ml of redistilled constant-boiling HCl for 20 and 70 hr at 110°. The hydrolysates were dried in a rotary evaporator and then 0.5 ml of water and 0.5 ml of 0.5 M phosphate buffer (pH 6.8) were added. The solutions were allowed to stand overnight to permit complete air oxidation of cysteine to cystine (Moore and Stein, 1963). The hydrolysates were then transferred quantitatively to 10-ml volumetric flasks with portions of citrate buffer (pH 2). DL-Norleucine and L-α-amino-β-guanidopropionic acid were added for use as internal standards. Additional samples of the antigen (2.0 mg) were oxidized with performic acid. The samples were dissolved completely in 0.1 ml of formic acid at 0° and 0.2 ml of freshly prepared performic acid (0.5 ml of 30% H_2O_2 + 9.5 ml of 99% formic acid incubated 90 min at room temperature) added to the chilled tubes (Hirs, 1956). After incubating at 0° for 90 min, 5 ml of cold water was added and the samples were freeze dried. These samples were then hydrolyzed for 20 hr and prepared for the amino acid analyzer as described above. Tryptophan was determined spectrophotometrically and the values obtained for tyrosine from the analyzer were checked spectrophotometrically (Edelhoch, 1967) using the relationships

$$N = \frac{(A_1B) - 70C}{3103} - \frac{(A_2B) - 120C}{10{,}318}$$

and

$$M = \frac{(A_2B) - 120C}{825} - \frac{(A_1B) - 70C}{698}$$

where N and M are the number of moles of tryptophan and tyrosine, respectively, per mole of protein, A_1 and A_2 are the absorbences through a 1-cm path at 288 and 280 mμ, respectively, B is the molecular weight of the protein (grams per mole) divided by the concentration analyzed (grams per liter), and C is the number of moles of cystine (whole) per mole of protein.

The amino acid analyses for 90G, 90D, and 178D were obtained from Jones (1965), the published residue values being converted into moles per cent for comparative purposes.

Values Derived from Amino Acid Contents. Difference indices (DI) (Metzger *et al.*, 1968) were computed from the relationship, $DI = 0.5\Sigma[r_1 - r_2]$, where r_1 and r_2 are the moles per cent for a given amino acid in proteins 1 and 2. Brackets indicate the absolute value. Values for the frequency of nonpolar side chains (NPS) were determined as: NPS = 0.01(Trp + Ile + Tyr + Phe + Pro + Leu + Val), where the values of the amino acids are expressed as moles per cent (Waugh, 1954). The ratio of polar to nonpolar residue volume,

TABLE I: Amino Acid Composition of Six Serotypic Antigens from *P. aurelia*.[a]

Amino Acid	Steers' 51A (Moles %)	Serotypic Antigen: 51A Moles %	51A Residues	51B Moles %	51B Residues	51D Moles %	51D Residues	90D Moles %	90D Residues	178D Moles %	178D Residues	90G Moles %	90G Residues
Lysine	5.19	4.64	136	4.28	108	7.64	197	8.55	207	8.17	191	5.74	138
Histidine	0.71	0.60	18	0.69	17	0.35	9	0.45	11	0.38	9	0.87	21
Arginine	1.34	1.21	35	1.66	42	1.26	33	1.45	35	1.67	39	1.45	35
Aspartic acid	12.01	11.80	345	11.74	296	12.94	334	11.94	289	12.40	290	11.26	271
Threonine	15.31	16.42	481	16.53	417	13.94	360	12.27	297	13.39	313	15.34	369
Serine	8.61	9.94	291	9.99	252	7.46	193	7.64	185	7.53	176	7.02	169
Glutamic acid	6.27	6.12	179	5.33	134	6.03	156	6.36	154	6.89	161	5.74	138
Proline	2.42	1.87	55	2.19	55	2.72	70	2.48	60	2.14	50	1.91	46
Glycine	7.50	6.87	201	6.89	174	7.96	206	10.17	246	9.58	224	9.60	231
Alanine	12.57	12.11	354	12.73	321	11.33	293	9.79	237	9.07	212	12.72	306
½ Cystine	9.54	11.19	328	10.98	277	10.85	280	10.41	252	10.44	244	10.52	253
Valine	5.09	4.61	135	4.27	108	4.16	108	3.84	93	3.51	82	4.36	105
Methionine	0.42	0.50	15	0.30	8	0.23	6	0.29	7	0.38	9	0.50	12
Isoleucine	2.88	2.48	73	2.64	67	2.55	66	2.44	59	2.57	60	2.41	58
Leucine	4.09	3.86	113	3.98	100	3.59	93	4.30	104	4.49	105	4.78	115
Tyrosine	2.87	2.72	80	2.47	62	3.51	91	4.05	98	4.19	98	3.16	76
Phenylalanine	1.68	1.55	45	2.00	50	2.47	64	2.89	70	2.52	59	1.54	37
Tryptophan	1.50	1.49	44	1.32	33	1.02	26	0.66	16	0.68	16	1.08	26
Amide		(55.2)[b]	(306)	(53.2)[b]	(229)	(38.3)[b]	(188)	(31.6)[b]	(140)	(28.4)[b]	(128)	(35.7)[b]	(146)
Total	100.00	99.98	2928	99.99	2521	100.01	2585	99.98	2420	100.00	2338	100.00	2406

[a] Values for "Steers" 51A obtained from Steers (1965); for 90D, 178D, and 90G from Jones (1965) (moles per cent calculated from published residue data). The values for 51A, 51B, and 51D are the means of duplicate 20- and 70-hr hydrolysates. Values for serine, threonine, and ammonia were obtained by extrapolating to zero time. Methionine was determined as methione sulfone and cystine both as cysteic acid and cystine while tryptophan values were obtained using Edelhoch's (1967) method. The residue values are based on molecular weights of 301,500 (51A), 271,000 (51D), 259,000 (51B), and 250,000 (90D, 178D, and 90G). [b] Amide as a percentage of aspartic acid plus glutamic acid.

p, was obtained from the expression, $p = \Sigma V_p r / \Sigma V_{np} r$, where V_p and V_{np} are the volumes of the polar and nonpolar amino acid residues, respectively, while p_s is p as calculated for a sphere (Fisher, 1964). The volumes (in Å^3) of the amino acid residues were obtained by converting the molal volumes given by Cohn and Edsall (1943). The average hydrophobicity of the antigens $H\phi_{av}$ is computed using Tanford's free energies of transfer from organic to aqueous solvents by the relationship, $H\phi_{av} = 0.01\Sigma(H\phi)r$, where $H\phi$ is the hydrophobicity in calories per mole of a given amino acid residue (Bigelow, 1967). Molecular dimensions were obtained by solving for b the quartic expression developed by Jennings (1968), $db^4 - 2d^2b^3 - b^2(\theta - d^3 - \theta/(1 + p) + 2d\theta b - \theta d^2 = 0$, where d, the polar shell thickness, is assumed to be 4 Å, b is the semiaxis of "nonrotation," p is defined above, and θ is derived from the interior volume, V_{np} (nonpolar residues), and external volume, V_p (polar residues), by the expression $\theta = (3/4\pi)(V_p + V_{np})$. The semiaxis of rotation, a, is given by the relationship $a = \theta/b^2$.

Results

Amino Acid Composition of Serotypic Antigens. The amino acid compositions of six of the serotypic antigens are given in Table I. Referring to the three proteins analyzed by us (51A, 51B, and 51D) no significant differences were noted in the determinations of cysteic acid from the performic acid oxidized samples compared with the cystine values obtained from the unoxidized antigens. However, the values for methionine sulfone were substantially higher than those for methionine. It is immediately apparent from the data that this group of proteins is remarkable for its high content of cystine (11.19 moles %, 51A to 10.41 moles %, 90D), and threonine plus serine (26.36 moles %, 51A to 19.91 moles %, 90D). Note must be made that the tryptophan content given in Table I for "Steers" 51A has been altered from the published value of 3.50 moles % to 1.50 moles % and the amounts of the other amino acids adjusted accordingly. This was done because of the large discrepancy between the value Steers used (obtained from Preer, 1959) compared with our value for 51A and the values for the other five proteins examined. Finally the values for tyrosine obtained from the analyzer were checked spectrophotometrically and agreement between the two methods was within 3%.

Molecular Weights and Partial Specific Volumes. Determination of partial specific volumes of the six antigen mole-

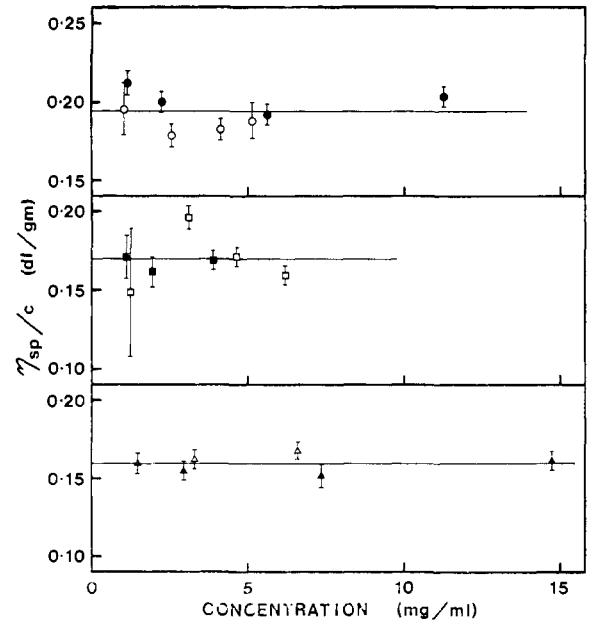

FIGURE 1: Reduced viscosities of 51A (O—O), 51B (□—□), and 51D (△—△). Open and closed symbols indicate different preparations. The standard error for each value is indicated by I.

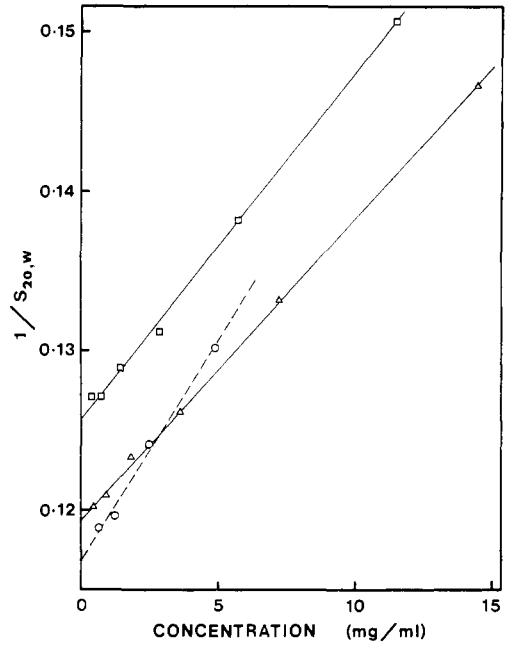

FIGURE 2: Sedimentation velocity data for 51A (O—O), 51B (□—□), and 51D (△—△). Each line represents one of two preparations examined (see text).

cules based on their amino acid content yielded the following values: 51A, 0.702 cc/g; 51B, 0.703 cc/g; 51D, 0.706 cc/g; 90D, 0.708 cc/g; 178D, 0.706 cc/g; and 90G, 0.707 cc/g. The experimentally determined values for 51A gave $\bar{v} = 0.708 \pm 0.013$ cc/g in agreement with Preer's pycnometric determination of 0.705 cc/g (Preer, 1959). For molecular weight determinations $\bar{v} = 0.705$ cc/g was used in all cases. The molecular weight of 51A was found to be 301,500 ± 4,500 g/mole based on 25 determinations at starting protein concentrations of 1.0 to 0.1 mg per ml. The values for 51B and 51D were 259,000 ± 8,500 g/mole (six determinations) and 271,000 ± 8,400 g/mole (eight determinations), respectively, using starting concentrations of 1.0, 0.6, 0.3, and 0.1 mg per ml. In all cases log C *vs.* x^2 plots gave straight lines. Thus there is a significant difference in the molecular weights of 51B and 51D, on the one hand, and 51A, on the other hand. The molecular weight of 250,000 g/mole for 90D, 178D, and 90G used in the analyses described below is taken from Jones (1965).

Hydrodynamic Measurements. Intrinsic viscosities for 51A, 51B, and 51D were determined to be 0.195 ± 0.003, 0.168 ± 0.003, and 0.159 ± 0.002 dl per g (pooled standard error), respectively, in contrast to Preer's (1959) finding of 0.11 dl/g for 51A. Figure 1 shows that the reduced viscosity of none of the antigens was concentration dependent. $s^0_{20,w}$ values for the antigens were 8.50 and 8.55 (51A), 7.92 and 7.95 (51B), and 8.37 and 8.40 S (51D). The sedimentation coefficients for all three antigens were concentration dependent (Figure 2). Table II summarizes the physical parameters of the three proteins. The values for β show that the effective hydrodynamic ellipsoids of revolution are prolate with axial ratios of about 11:1, while the values for V_e, $V_{\bar{v}M}$, and V_{aM} indicate the extent of hydration of the molecule. Using the relationship developed by Bull and Breese (1968) the degree of hydration of these three proteins is estimated at about 30%.

Optical Rotary Dispersion of 51A. Both the Drude and the Moffitt plots gave straight lines. From the Drude equation, λ_c was found equal to 222.2 mμ, a value similar to that found for denatured proteins. The Moffitt equation yielded values of $a_0 = -306$ and $b_0 = -42$. Calculating the helical content using the expression % helix $= -b_0/6.30$ the value of 6.7% is obtained, a figure which is probably exaggerated because of the large cystine content. In sum the data suggest that there is little helical content in this molecule.

Isoelectric Points of 51A, 51B, and 51D. Steers (1961) determined the isoelectric points of 51A, 51B, and 51D using moving boundary electrophoresis in buffers of ionic strength equal to 0.1. He obtained values of 3.9 (51B), 4.0 (51A), and 4.3 (51D). Using isoelectric focusing we found the order of increasing isoelectric points to be the same but our values were consistently lower for all three antigens. Three determinations were made for each antigen and the values obtained (including the range of variation) were 3.48 ± 0.05 (51B), 3.64 ± 0.03 (51A), and 3.95 ± 0.06 (51D). No significant differences were noted when the columns were run at 1° as compared with running them at 20°.

Average Hydrophobicity, $H\phi_{av}$, Nonpolar Side-Chain Frequency, NPS, and Polar Volume Ratio, p, of the Serotypic Antigens. Table III shows the values for $H\phi_{av}$, *NPS*, *p*, and p/p_s determined for the six antigens. As was found by Bigelow (1967) in analyzing about 150 proteins the correlation between $H\phi_{av}$ and *NPS* is excellent. The values for p/p_s (Fisher, 1964) all fall between 3.3 and 3.9 thus predicting a highly aspherical shape for these proteins (see the analysis using the Jennings equation, below). Comparing $H\phi_{av}$ and *NPS* with the selection of proteins analyzed by Bigelow we find that the serotypic antigens all fall below the seventh percentile and only fibrion, squid tropomysin, salmine, and erythrocuprein have $H\phi_{av}$ values below 51A. In short the serotypic antigens have a

TABLE II: Summary of Physical Parameters of Serotypic Antigens 51A, 51B, and 51D.

	Protein		
	51A	51B	51D
$s^0_{20,w}$ (S)	8.53 ± 0.025	7.94 ± 0.015	8.38 ± 0.015
$s^0_{25,w}$ (S)	9.62 ± 0.028	8.95 ± 0.017	9.45 ± 0.017
$[\eta]$ (dl/g)	0.195 ± 0.003	0.168 ± 0.003	0.159 ± 0.002
$\bar{v}$ (cc/g)[a]	0.702	0.703	0.706
M (g/mole)	301,500 ± 4,500	259,000 ± 8,500	271,000 ± 8,400
β ($\times 10^{-6}$)[k]	2.46 ± 0.12	2.43 ± 0.12	2.46 ± 0.12
$(1/p)$[b]	11.7	10.6	11.7
$(1/F)$[c]	1.63	1.58	1.63
(ν)[d]	16.90	14.80	16.90
(V_e)[e] (Å^3)	575,000	488,000	423,000
$(V_{\bar{v}M})$[f] (Å^3)	353,000	303,000	317,000
(V_{aM})[g] (Å^3)	356,000	305,000	320,000
V_e/V_{aM}	1.62	1.60	1.32
pK[h]	3.64	3.48	3.95

[a] $\bar{v}$ = determined from amino acid composition. [b] $1/p$ = axial ratio of the effective hydrodynamic prolate ellipsoid of revolution. [c] $1/F$ = frictional coefficient of the effective hydrodynamic prolate ellipsoid. [d] ν = shape factor. [e] V_e = effective volume (Å^3) of the prolate ellipsoid as determined from the relationship $V_e = (M[\eta]/N\nu) \times 10^{26}$. [f] $V_{\bar{v}M}$ = volume of the molecule as calculated from $\bar{v}$ and its molecular weight. [g] V_{aM} = volume of the molecule as calculated from its molecular weight and the anhydrous values of its constituent amino acids. [h] Isoelectric point as determined by isoelectric focusing. [k] The determination of the standard error of β assumes a standard error of $\bar{v}$ of ±0.013.

TABLE III: Relationships of Polar to Nonpolar Amino Acids in Six Serotypic Antigens of *P. aurelia*.[a]

	Serotypic Antigen					
	51A	51B	51D	90D	178D	90G
$H\phi_{av}$ (cal/mole residue)	818	826	882	892	878	846
NPS	0.186	0.189	0.200	0.207	0.201	0.192
p	1.254	1.212	1.299	1.313	1.404	1.187
p/p_s	3.82	3.47	3.74	3.66	3.88	3.35

[a] The values for $H\phi_{av}$, *NPS*, and p were obtained as described in Materials and Methods. The amino acid analyses published by Jones (1965) were used for the calculations involving 90D, 178D, and 90G.

relatively small hydrophobic core; indeed over 85% of the proteins analyzed by Bigelow have $H\phi_{av}$ above 1000 cal/mole of residue.

Difference Indices of the Serotypic Antigens. The difference indices were calculated for the 15 possible combinations of the antigens, comparing the antigens as a whole, their polar residues and their nonpolar residues. In these computations the convention was followed (Metzger *et al.*, 1968) not to distinguish between aspartic acid and asparagine or between glutamic acid and glutamine. The difference indices are given in Table IV and range from 2.4 (51A–51B) to 11.9 (51B–90D) when comparing whole molecules. Although the evaluation of these data from a microevolutionary viewpoint is difficult because of making comparisons across syngens, the difference indices suggest the order of decreasing similarity is A → B → G → D and it is interesting that this correlates with the $H\phi_{av}$ of these molecules. Comparing the difference index values of polar to nonpolar residues (Table IV) we find that the two groups show about equal variability, *i.e.*, within this group of six molecules there is no indication of preferential conservation of either polar or nonpolar residues. In addition the difference index ratio of polar to nonpolar residues shows that relatively large differences in DI_p are not necessarily reflected in DI_{np} or *vice versa.*

Dimensions of the Serotypic Antigens Using Amino Acid Composition. Solving the Jennings equation yields four solutions for the semiaxis of "nonrevolution." Two of these roots while real are less than 4 Å and therefore physically meaningless. The remaining two roots are also real and they are the semiaxes of nonrotation for a prolate and oblate ellipsoid of revolution in which the nonpolar core is completely surrounded by a monolayer of polar amino acids.

TABLE IV: Difference Indices for Six Serotypic Antigens.[a]

	Serotypic Antigens Compared														
	51A/ 51B	90D/ 178D	51D/ 178D	51D/ 90D	51A/ 90G	51B/ 51D	51B/ 90G	51D/ 90G	178D/ 90G	51A/ 51D	90D/ 90G	51B/ 178D	51A/ 178D	51A/ 90D	51B/ 90D
DI_w	2.4	2.9	5.3	5.7	6.3	6.5	6.6	6.9	7.8	7.9	8.2	11.1	11.5	11.6	11.9
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
DI_p	1.1	1.5	1.8	2.5	3.5	4.2	3.8	3.4	4.5	5.1	4.6	6.3	6.3	6.2	7.1
	(1)	(2)	(3)	(4)	(6)	(8)	(7)	(5)	(9)	(11)	(10)	(14)	(13)	(12)	(15)
DI_{np}	1.3	1.4	3.5	3.2	2.8	2.3	2.7	3.5	3.4	2.8	3.6	4.8	5.2	5.3	4.8
	(1)	(2)	(10)	(7)	(6)	(3)	(4)	(9)	(8)	(5)	(11)	(12)	(14)	(15)	(13)
DI_p/DI_{np}	0.84	1.07	0.51	0.77	1.22	1.86	1.40	0.97	1.32	1.86	1.26	1.33	1.19	1.17	1.47

[a] Difference index values rounded to the nearest tenth. DI_w = difference indices comparing whole antigens, DI_p = difference indices comparing the polar residues, and DI_{np} = difference indices comparing nonpolar residues. Figures in parentheses indicate the relative position of the difference index in the set.

Table V lists the ellipsoidal dimensions calculated from the Jennings relationship and shows that the prolate ellipsoids predicted are highly asymmetrical and of very different dimensions from those predicted from hydrodynamic data.

Electronmicroscopy. Negatively stained preparations of 51A protein (in water) were examined in the electron microscope. Figure 3 shows that the only structured material observed in these preparations projects as rough disks of about 175-Å diameter or aggregates of these disks (caused presumably by the potassium phosphotungstate). Figure 3f shows an individual molecule. Although variations in image intensity are seen, they appear no different from the variations seen in the background. A careful examination of original plates gave no indication of subunit structure. Since sedimentation velocity, sedimentation equilibrium, isoelectric focusing, and gel electrophoretic analyses showed no heterogeneity in the preparation we conclude that these disks are in fact the serotypic antigen. Because of the concentration dependence of the sedimentation coefficients, we suggest that these proteins are *oblate* ellipsoids despite the fact that their β values are considerably larger than 2.14×10^6.

Discussion

Previous studies on the serotypic antigens have left doubt concerning the mass of these molecules. Steers (1965) reported a value of 310,000 ± 17,000 daltons for 51A while Preer (1959) and Jones (1965) obtained values of about 250,000 daltons (51A and 145D, respectively). The measurements reported here on 51A, 51B, and 51D support Steers' finding for 51A but in addition they demonstrate that 51B and 51D have masses significantly lower than 51A. Recent evidence (Reisner and Rowe, 1969; Reisner *et al.*, 1969) suggests strongly that these three molecules, at least, are composed of single polypeptide chains despite earlier reports to the contrary (Steers, 1965; Jones, 1965; Finger *et al.*, 1966). Assuming that all the serotypic antigens stem from a common ancestral gene, a sizeable deletion or insertion has taken place in the course of the development of the group. What is particularly noteworthy is that this mass difference (amounting to about 400 residues) exists between 51A and 51B. The *DI* and $H\phi_{av}$ of these two molecules show that they are very similar, demonstrating that the amino acid proportions in the 400 residue segment are virtually identical with those in the molecules as a whole. In addition, Preer noted, as we have, that 51A and 51B show distinct serological cross reactions. It appears not unlikely that the deletion or insertion may have been a single event and is the principal difference between these two antigens. While we have no evidence which allows us to favor insertion over deletion, *i.e.*, $B^{51} \rightarrow A^{51}$ rather than $A^{51} \rightarrow B^{51}$, the fact that A is the most stable serotype in stock 51 might be taken as favoring slightly the insertion hypothesis (it should be noted that A^{51} and B^{51} are nonallelic genes).

Analyses of the amino acid contents of these molecules demonstrate two characteristic and outstanding properties. First their cystine content is remarkably high, indeed as high as wool (Chapman and Bradbury, 1968), and second these proteins are very rich in hydroxylamino acids (24–29%) particularly threonine (12–16%). Neither Jones nor Steers found evidence for titratable thiol groups prior to reduction, under conditions which would be expected to disrupt secondary structure, indicating that all the thiol groups present are in the form of disulfide bridges. Thus, for example, 51A contains 164 thiol bridges, *i.e.*, about one disulfide bridge per eight nonpolar residues. Concerning the hydroxylamino acids there is 1/1.8 polar residues and 1 threonine/3.5 polar residues. As Smith points out, the survival of threonine in proteins has always been puzzling, but it is not improbable that it can serve a dual role, the hydroxyl group participating in hydrogen bonding while the methyl group makes a nonpolar contact (Smith, 1968). Thus, in the serotypic antigens it could serve as the principal element in bonding the polar shell to the hydrophobic core (see below).

From the values of the difference indices generated by this group of six antigens it is seen that the amino acid compositions support the intuitive conception that these proteins are closely related molecules (a difference index of 12 falls below the fifth percentile in the study of Metzger *et al.*, 1968). Another feature revealed by the difference index analysis is that the ratio DI_p/DI_{np} is highly variable ranging from 0.51 (51D–178D) to 1.86 (51A–51D and 51B–51D) indicating that the amino acid replacements that have taken place in

TABLE V: Molecular Dimensions of Six Serotypic Antigens Based on Their Amino Acid Compositions.[a]

	Major (a) and Minor (b) Axes (Å)			
	Oblate Ellipsoid		Prolate Ellipsoid	
Antigen	$a \times b$	a/b	$a \times b$	a/b
51A	210 × 15	14.0	1161 × 24	48.4
51B	192 × 16	12.0	963 × 25	38.5
51D	200 × 15	13.3	1096 × 24	45.7
90D	195 × 15	13.0	1029 × 24	42.9
178D	195 × 15	13.0	1089 × 23	47.3
90G	187 × 16	11.7	892 × 25	35.7

[a] The dimensions were obtained by solving Jennings' equation (1968). The molecule is assumed to be an ellipsoid of revolution with a monolayer polar shell of 4-Å diameter surrounding a nonpolar core. Molecular weight (g/mole): 51A, 301,500; 51B, 259,000; 51D, 271,000; 90D, 178D, 90G, 250,000. a/b is the ratio of the major to the minor axis.

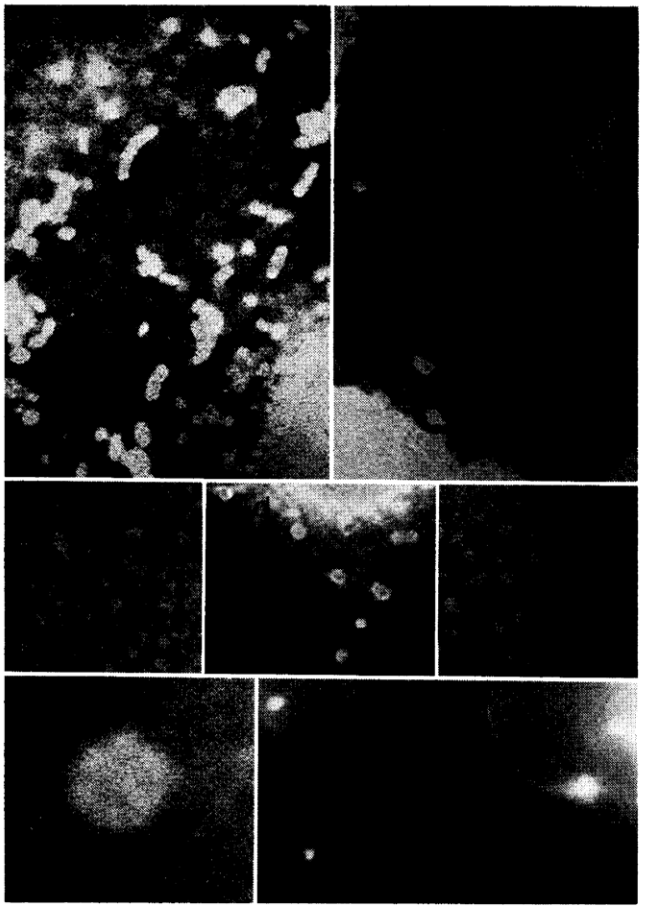

FIGURE 3: Electron microscopy of 51A protein negatively stained with potassium phosphotungstate. Magnification on original plates 50,000×. (a–e) Five different views where some of the molecules appear surrounded by, but not imbedded in the stain and have tended to aggregate. Magnification, 107,500×. (f) 51A molecule, 672,000×. (g) Sample of distilled water treated identically with the 51A solution. Magnification 107,600×.

the system do not tend to be restricted to either the core or the surface of these proteins.

The values obtained for $H\phi_{av}$, *NPS*, and p (Table II) all point to a particular feature of these molecules—they have very small hydrophobic cores, a situation which leads directly to the highly asymmetrical dimensions obtained from the Jennings' equation (1968). As is demonstrated in Figure 3, electron microscopy supports (within the limits of measurement) the value predicted by the Jennings equation for the major axis of an oblate ellipsoid.

The effective hydrodynamic ellipsoids calculated using the Scheraga–Mandelkern equation are prolate and have axial ratios of about 11:1. This finding is in marked contrast to the observation made using the electron microscope and the predictions of the Jennings equation (Table V). However, it should be remembered that Scheraga and Mandelkern (1953) and Scheraga (1961) emphasized that the relationships they developed describe the volume and axial ratio of the *effective hydrodynamic* ellipsoid. Although it has often been implied since, that this ellipsoid is representative of the actual configuration of a protein molecule, such an interpretation was never intended. Recently, Mitchell and Schmidt (1969) showed that although clostridiopeptidase B has a β value of 2.7×10^6 (indicating a prolate ellipsoid with an axial ratio of 24:1) the sedimentation coefficient is nonconcentration dependent and the protein gives a circular image in the electron microscope. They concluded that this enzyme is spherical and that the large β value is obtained because of a large effective hydrodynamic volume. Using their data we calculate $V_e = 8.43 \times 10^4$ Å^3, and $V_e/V_{aM} = 1.55$, a figure similar to those reported in Table II.

The observation that these molecules (51A, 51B, and 51D) show no tendency to aggregate in pure water indicates that there are few exposed hydrophobic regions. In addition the isoelectric points of these molecules demonstrate a considerable net negative charge at physiological pH while electrophoresis of whole cells shows them to have, near neutrality, a net positive charge (Van Wagtendonk *et al.*, 1952). These findings suggest that the serotypic antigens are bound to the pellicular surface electrostatically rather than hydrophobically.

If the serotypic antigens act as surface protectants, their disk-like structure well might be the most efficient way of utilizing the cell's resources in order to synthesize a molecule of large surface area. The 51A molecule possesses over 160 thiol bridges while the other proteins of the group contain about 130 disulfide bonds. This suggests that a relatively high bonding energy is required for the core to maintain its structure—a situation which would be predicted if the core has a large surface to volume ratio, *e.g.*, a disk. The presence of threonine in high concentration in the coat would serve to bond it securely to the core while making the exterior of the molecule, together with serine, highly hydrophilic.

Tooney and Fasman (1968) have pointed out that β-hydroxyls would be expected to destabilize intramolecular hydrogen bonds in the α helix while such interference would be minimized in the β conformation. Our finding that little if any α helix is present in 51A is, therefore, not surprising and we might predict regions of β structure in the coat,

possibly extensive regions. The demonstration by Blake *et al.* (1967) of a segment of lysozyme in β conformation shows that this structure can exist in globular proteins.

Finally, we should like to suggest that a smooth oblate ellipsoid of revolution need not be the best model to apply to these proteins. For example calculation of the total cross-sectional areas of the polar and nonpolar residues of 51A indicates that it would be possible for the polar amino acids to form a monolayer 170 Å in diameter by 25 Å thick which would just surround a circular disk of nonpolar amino acids two residues thick. Thus, while we have no data indicating that these molecules do resemble double bimolecular leaflets, we can say that their amino acid composition does not exclude the possibility.

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