The Macromolecular Properties of Blood-Group Substances

SEDIMENTATION-VELOCITY AND VISCOSITY MEASUREMENTS

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(Received 12 May 1967)

1. Sedimentation-velocity, intrinsic-viscosity and partial-specific-volume measurements on a typical blood-group-specific glycoprotein are reported for a range of environmental conditions. 2. The sedimentation coefficients, S , are strongly concentration-dependent, and follow the reciprocal law; the limiting values at 2°, 25° and 45°, after correction to 25°, show slight dependence on temperature. 3. The intrinsic viscosities, $[\eta]$, at 25° and 45° show more marked temperature-dependence, and correspond to a very asymmetric or very expanded molecular conformation. 4. From the value of the ratio $K/[\eta]$, where $K = S^0 \cdot d(1/S)/dc$, it is concluded that the molecular conformation is roughly spherical; application of the Einstein viscosity equation then suggests an expansion factor of about 60, compatible with a flexible configuration approaching that of a random coil. 5. The sedimentation coefficient is not affected by variation of ionic strength in the range 0.01–0.50, nor by pH in the range 3–10. 6. Sodium dodecyl sulphate at 1.5% produces a small decrease in S ; the effect is greater than would be expected from the observed extent of binding, but is too small to correspond to a significant change in secondary structure; the serological activity is unaffected by sodium dodecyl sulphate. 7. All the properties observed indicate the absence of any secondary structure in blood-group substances.

The water-soluble blood-group-specific substances derived from human ovarian cysts have been the subject of much detailed study (e.g. Morgan, 1960*a*, 1964*a*; Watkins, 1966*a,b*). In particular, their analytical composition has been closely defined (e.g. Pusztai & Morgan, 1961, 1963); typically, a blood-group substance contains about 85% carbohydrate and 15% amino acids, the latter being in the form of peptide. Sialic acid may be present, generally in rather low proportion, but is not essential for activity. Serine and threonine make up about 50% of the amino acids, and this fact, together with the isolation of relatively short chains of carbohydrate units from acid-hydrolysis experiments, enables a model structure for the blood-group substances to be postulated (Morgan, 1959, 1964*b*). This consists of a polypeptide backbone, with chains containing about seven carbohydrate units attached by glycosidic linkages to the serine and threonine hydroxyl groups (Anderson *et al.* 1964; Adams, 1965; Kabat *et al.* 1965). The carbohydrate chains probably consist of five units in a linear arrangement with two fucose residues

as branches; partial structures have been proposed for these chains (Rege, Painter, Watkins & Morgan, 1963). It is recognized that the model is oversimplified, but it is nevertheless useful for interpreting the properties of the substances.

The serological specificity is known to reside largely in the nature of the di- and tri-saccharide units that form the non-reducing end groups of the carbohydrate chains (e.g. Morgan, 1960*a*, 1964*a*; Watkins, 1966*a*); however, it has been established that the native blood-group substances are far more active in haemagglutination-inhibition tests than are partially degraded materials that retain the same carbohydrate units as the original (Morgan, 1960*b*). This fact led Morgan (1960*b*, 1964*a*) to suggest that the role of the peptide component was to maintain the correct steric relationship of the carbohydrate chains. Such a macromolecular structure is most readily understood if the polypeptide core is itself organized in a specific manner, i.e. if the polypeptide component possesses a unique secondary, and perhaps tertiary, structure, as in globular proteins.

Accordingly, the sedimentation and viscosity characteristics of a typical blood-group substance

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have been examined, particular attention being paid to those conditions that should reveal the postulated secondary and tertiary structure. To facilitate interpretation of the sedimentation and viscosity results determinations have been made of the partial specific volume. In its general physical properties, the blood-group substance investigated here resembled the less-fractionated samples examined by Kekwick (1950, 1952*a,b*) and Caspary (1954).

MATERIALS AND METHODS

Blood-group substance. The blood-group-specific substance used throughout this work was a freeze-dried preparation made by Professor W. T. J. Morgan, by standard methods (see e.g. Morgan, 1965); it was an ammonium sulphate-soluble B-active substance, isolated from cyst number 376. It is referred to subsequently as glycoprotein 376.

Preparation of solutions. Glycoprotein 376 dissolved readily and completely in water or buffer to give slightly opalescent solutions; clarification, with loss of some gel-like material, could be accomplished by low-speed centrifugation (approx. 500*g* for 50 min.), and this step was therefore employed in preparing the relatively concentrated stock solutions for density and viscosity measurement. The concentrations of these stock solutions were determined refractometrically with a Svensson & Odengrim (1952) cell on the track of a Rayleigh diffusimeter (Creeth, 1955); the value of the specific-refraction increment was taken as 1.64×10^{-3} dl./g., this value having been found for unclarified solutions of material dried *in vacuo* to constant weight. Since the aggregated material is similar chemically to the dissolved, this procedure probably introduces a negligible error into the specific-refraction increment. Except where otherwise stated, the glycoprotein was dissolved in a buffer consisting of 90 mM-KCl-10 mM-potassium acetate-10 mM-acetic acid, pH 4.6 and *I* 0.10. In the experiments with SDS,* sodium salts were substituted for the KCl and potassium acetate: the SDS was a recrystallized sample (British Drug Houses Ltd., Poole, Dorset: 'sodium lauryl sulphate, specially purified').

Density measurements. Measurements of solution density were made with a pycnometer of 5 ml. capacity, consisting of a bulb fitted with a 1 mm.-diam. capillary neck, 5 cm. long and bearing a fiducial mark. The capillary diameter was calibrated by weighing known lengths of mercury. Excess of dissolved air was removed from the solutions before the apparatus was filled, by a prior period at 26°. The pycnometer was filled with the aid of a syringe fitted with a long steel needle, sufficient liquid being inserted to bring the meniscus to within 0.5 cm. of the mark; the distance from the mark was measured, after equilibration at $25 \pm 0.002^\circ$, with a cathetometer, to 50μ . The pycnometer was weighed against brass weights to 10μ g., and the weights were corrected for air buoyancy. After being weighed, the pycnometer was equilibrated at $45 \pm 0.03^\circ$ in a similar manner and a further determination performed.

Viscosity measurements. Ostwald viscometers of the type described by Schachman (1957) were used, the character-

istics of a typical viscometer being: capacity 1.5 ml., volume of flow 0.5 ml., capillary length 80 cm., diameter 0.5 mm., flow time for water at 25° 150 sec. A micrometer syringe was used for all filling operations; after about six determinations of the flow time at 25° , the viscometer was transferred to the 45° bath and a similar series carried out. Relative viscosity, $\eta_{rel.}$, and specific viscosity, $\eta_{sp.}$, values were determined from the averaged flow times, *t*, and solution densities, ρ , by the equation:

$$\eta_{sp.} = \eta_{rel.} - 1 = (\rho t - \rho_0 t_0) / \rho_0 t_0$$

where subscript zero refers to the buffer solvent; the kinetic energy correction was negligible. All dilutions in a given series were made by weight from a single stock solution. The viscometer was emptied and cleaned with H_2O_2 - H_2SO_4 mixture after each filling; only by this means could reproducible results be obtained.

Sedimentation-coefficient measurements. A Spinco analytical ultracentrifuge was used, the details of the equipment and measurement being identical with those described by Creeth (1964). Plots of $\log r_p$ versus time (where r_p is the radial displacement of the position of the maximum refractive-index gradient) showed very slight upward curvature; slopes were calculated by the method of least squares, however, so that the average obtained refers almost exactly to the mean concentration of the timed part of the experiment. The boundaries were nearly symmetrical, and trial calculations showed that the peak and second moment positions almost coincided; all values of the sedimentation coefficient, *S*, were therefore obtained from the r_p values. Eight or ten frames were used for each value of *S*.

RESULTS

Partial specific volumes. The primary purpose of the measurements of partial specific volume, $\bar{v}$, was to define the buoyancy factor, $(1 - \bar{v}\rho)$. Accordingly, the method of Kraemer (1940) was used; however, insufficient material was available to extend these measurements over the full range of concentration covered by the sedimentation-velocity experiments. In the low-concentration range, the values of $\bar{v}$ showed a small dependence on concentration, ranging from 0.631 ml./g. at concentration (*c*) 0 to 0.634 ml./g. at *c* 1.4 g./dl., at both 25° and 45° ; such a variation is not unexpected in systems where marked solute-solvent interaction occurs (Kraemer, 1941). In computing buoyancy factors for correcting sedimentation coefficients, a constant value for $\bar{v}$ of 0.633 ml./g. was used throughout; this procedure introduces negligible error into the corrected sedimentation coefficients.

Viscosity. It was desired to obtain the intrinsic viscosity and also the Huggins coefficient (see, e.g., Flory, 1953, p. 309), since both these quantities are related to solute configuration. Standard procedures were followed: the intrinsic viscosity, $[\eta]$, was obtained as the common intercept as $c \rightarrow 0$, of graphs of $(\eta_{rel.} - 1)/c$ versus *c* and $(\ln \eta_{rel.})/c$ versus *c*. Denoting the slope of the first of these lines by

* Abbreviation: SDS, sodium dodecyl sulphate.

a_2 , the Huggins coefficient k' is obtained from:

$$k' = a_2/[\eta]^2$$

Two series of experiments were performed, yielding very similar results. Those found in the second series, plotted according to the forms given, are shown in Fig. 1; it is clear that the points fall on straight lines within experimental error. Table 1 lists the intrinsic viscosities at 25° and 45°, the values of the Huggins coefficient and the temperature-dependence of the intrinsic viscosities, expressed as $d\ln[\eta]/dT$. Conditions in the two series were intended to be identical, but the differences between corresponding $[\eta]$ values, which were greater than experimental errors, show that this was not so; it is almost certain that the differences arose from the different extents of separation from the gel layer in the clarification of the solutions. It is therefore apparent that the intrinsic viscosity values themselves are uncertain to perhaps 5%, but that the values at the two temperatures in the same series are strictly comparable. Thus the intrinsic viscosity decreased by 14–15% on the temperature being raised from 25 to 45°. It is also apparent that the values of the Huggins coefficient were within the range 0.35–0.40 for the results at 25°, but are somewhat greater at 45°.

The reversibility of the temperature effect was investigated by determining the flow time of a solution alternately at 25° and 45°; the results (Table 2) show that the ratio of the reduced viscosities at the two temperatures is independent of

the number of cycles, although a small progressive decrease occurs in the absolute values.

Experiments on the same solution in two viscometers of widely differing bore gave identical values for $\eta_{rel.}$, showing the absence of 'structural' or non-Newtonian viscosity; in agreement with this finding, no birefringence of flow could be detected when the effluent from a capillary was examined between crossed polaroids. Thus it may be concluded that molecular orientation does not occur at the moderate shear rates used here, which had a nominal maximum of 270 sec.⁻¹.

Sedimentation. (a) General. Glycoprotein 376 sediments with a single symmetrical boundary in the ultracentrifuge; Fig. 2 shows the series of patterns obtained in an experiment conducted under conditions where boundary-sharpening effects were minimized. At higher speeds and at higher concentrations, 'hypersharp' boundaries persist through a great proportion of the duration

Table 2. *Effect of temperature cycle on the reduced viscosity of glycoprotein 376*

Concentration (c) was 0.437 g./dl.

Temp.	Flow time (sec.)	$\eta_{sp.}/c$ (dl./g.)
25°	223.78	1.05
45	145.14	0.90
25	220.51	1.01
45	144.19	0.88
25	219.20	0.99

Fig. 1. Determination of intrinsic viscosity for glycoprotein 376. ○, $(\eta_{rel.}-1)/c$; ●, $(\ln \eta_{rel.})/c$. Temperatures were as indicated.

Fig. 2. Sedimentation-velocity experiment on glycoprotein 376 at $c = 0.75$ g./dl. Frames were exposed at 16 min. intervals, beginning 5 min. after the centrifuge reached 44770 rev./min. The phase-plate angle was 75° throughout; a 12 mm. double cell was used.

Table 1. *Viscosity characteristics of glycoprotein 376*

Temp., $T...$	Series I		Series II	
	25°	45°	25°	45°
Intrinsic viscosity, $[\eta]$ (dl./g.)	0.94 ± 0.01	0.82 ± 0.01	0.91	0.78
Huggins coefficient, k'	0.40	0.47	0.36	0.44
$d\ln[\eta]/dT$	6.8×10^{-3}		7.7×10^{-3}	

of the experiment (cf. Creeth, 1964); at lower concentrations, spreading is more pronounced, and suggests a moderate degree of polydispersity.

(b) Optical recovery. Since freeze-dried materials generally show incomplete optical recovery, it was necessary to establish this quantity under the conditions employed. The procedure followed was the direct observation of the concentration in the plateau region of the cell; Rayleigh optics were employed in order to follow the time-course of dilution with maximum precision, with intermittent schlieren photographs to allow the simultaneous determination of the sedimentation coefficient.

In highly concentration-dependent systems, the concentration in the plateau region, $c(r_x)$, is not simply related to the time of centrifugation because the dilution affects the value of S . This effect must be allowed for in calculating values of $c(r_x)$ to compare with those observed in the experiment. An appropriate analysis was therefore developed; its mathematical basis is described in Appendix I. In the present connexion it is necessary to realize only that the 'predicted' curves are obtained from an expression that makes full allowance for the complicating effects. For reasons explained in Appendix I, the time, t , must be replaced by the

Fig. 3. Variation of concentration in the plateau region during a sedimentation-velocity experiment on glycoprotein 376 at c^0 1.17 g./dl. Circles represent measurements of the concentration in the plateau region (see the text), expressed logarithmically in terms of S , the number of interference fringes formed in the 12 mm. cell; the abscissa is the time axis in terms of y , where $y = \int_0^t \omega^2 dt$. Curve I represents the predicted dependence for a constant sedimentation coefficient of 5.07s, the value observed over the period $b-d$, and curve II the predicted dependence for a concentration-dependent sedimentation coefficient, as explained in the text. For both curves I and II, S^0 was taken as 38.70. Curve III is the predicted dependence over the period of the sedimentation-velocity measurements, with the observed sedimentation coefficient and allowing for concentration-dependence; S^0 was taken as 32.01. (a) Operating speed attained; (b) and (d) beginning and end of period over which S was determined; (c) mid-point on y scale of period (b)–(d).

variable $\int_0^t \omega^2 dt$, where ω is the angular velocity. This variable is denoted by y .

The results of applying the procedure to a solution of initial nominal concentration 1.17 g./dl. are shown in Fig. 3. Here the ordinate is expressed as S , the number of interference fringes between solution and solvent in a cell of 12 mm. optical path. The initial concentration, based on dry weight and the specific refraction increment (see below), gave S 42.19; after the standard period of low-speed centrifugation, the value of S was 38.70. Accordingly, the predicted curves I and II on the graph were obtained from eqn. (7) (Appendix I) by using S^0 38.70; curve I is that expected for a constant sedimentation coefficient equal to that observed in the experiment (5.07s) and curve II is that found when y is taken as $1.06 \times 10^{11} \text{ sec.}^{-1}$ (the mean value for the whole experiment), which defines a concentration at which S is 4.90s. This involves a successive approximation, in which curve I is used to give the first estimate of S .

Two main points of interest emerge from a comparison of the observed points with the predicted curves. First, about 3% of the material is lost at y values too low to be plotted, and the subsequent loss before the operating speed is attained brings the total to approx. 5% (S 35.39 compared with 37.20 predicted). Secondly, the subsequent decrease in $\log S$ is approximately linear in time, but with a distinctly greater (negative) slope; to enable a precise comparison to be made a third curve (III in Fig. 3) was calculated from eqn. (7) for the value of S^0 existing at the time sedimentation-velocity measurements started and with the value of S observed. It is apparent that the discrepancy in slope persists throughout the experiment. This behaviour, in which the concentration in a region well ahead of the boundary decreases at a greater rate than radial dilution allows, must be due to the presence of a continuous distribution of sedimentation coefficients extending to very high values of S ; i.e. a true plateau region is not attainable with this system because the fastest components traverse the region ' r_x ' during the time of observation. This agrees with the observation that the fringe pattern always had a slight positive slope in the 'plateau' region.

In spite of these anomalies, sedimentation-velocity methods may still provide a useful characterization, provided first that the sedimentation coefficient is related to the observed mean concentration during the part of the experiment in which the velocity is determined, and secondly that the ratio of the mean concentration observed to that predicted by radial dilution is used to define the proportion of the initial distribution that is characterized. In the experiments reported here, it follows that the S measurements refer to the lowest

90% of the fully soluble material and to the lowest 85% of the dried solid. It is probable that the freeze-drying process is responsible for producing the gel-like material.

(c) Concentration-dependence of sedimentation coefficient. Measurements of S were made over the concentration range 0–4 g./dl. at pH 4.6, I 0.10 and temperatures close to 2°, 25° and 45°. In all cases, the sedimentation coefficients were very concentration-dependent, the value at, e.g., 1.0 g./dl. being less than half the extrapolated value at infinite dilution. The results are shown in Fig. 4 as plots of $1/S$ versus c . In addition the results at 2° and 45° have been corrected to 25° in the standard manner; for these calculations, it was assumed that $\bar{v}$ at 2° was the same as at 25° and that the density of the buffer solution varied directly with the density of water. In these experiments, solutions were prepared directly from the dried material, and concentrations were calculated from the initial

stoichiometric value and the mean radial dilution factor.

It is seen that the reciprocal relationship is precisely followed at all three temperatures, and that the values of S measured at 2° and 45°, when corrected to 25°, fall closely, but not exactly, on the line characterizing the results obtained at 25°. This slight difference between the values obtained at different temperatures is also manifested in the extrapolated S^0 values (Table 3). To aid in interpreting the significance of changes in S with temperature, four experiments were performed on a solution of bovine plasma albumin at an initial concentration of 0.841 g./dl. in a tris-potassium chloride buffer, pH 7.5 and I 0.216. The S values were measured at 1°, 25° and 40°, and also at 25° after a 4 hr. period at 40°. The results of these experiments were S (uncorr.) values of 2.303, 4.466, 6.098 and 4.431 s respectively; after allowance had been made for differences in dilution factors, these values become $S_{25,w}$ values of 4.69, 4.66, 4.67 and 4.62 s. A constant value for $\bar{v}$ of 0.736 ml./g. was used in obtaining the corrected values. By comparison with the results for albumin, it must be concluded that the small differences in S^0 for the glycoprotein are significant.

The numerical values characterizing the slopes of the reciprocal plots are also given in Table 3; the concentration-dependence coefficient K is independent of temperature. From the known average optical recovery (see above) a factor of 1.16 should be applied to the 25° value, giving K_{25} 1.46 dl./g. To confirm this value more directly, a second series of seven experiments was performed at 25° with precentrifuged solutions (20000 rev./min. in the Spinco model L ultracentrifuge with rotor no. 40, for 90 min., $\int \omega^2 dt$ $1.7 \times 10^8 \text{ sec.}^{-1}$), the initial concentrations being determined refractometrically. These gave $S^0_{25,w}$ 10.9 s and $K_{25, \text{uncorr.}}$ 1.21 dl./g.; application of the appropriate correction for mean optical recovery then gave the value K_{25} 1.32. Because of the smaller correction this is probably the most reliable mean value characterizing the material, but it is clear that the absolute

Fig. 4. Concentration-dependence of sedimentation coefficients for glycoprotein 376 at different temperatures. Δ , Measurements at 25°; $\circ$, measurements at 2°; $\bullet$, values found at 2° and corrected to 25°; $\square$, measurements at 45°; $\blacksquare$, values found at 45° and corrected to 25°. The concentration scale is based on dry weights, corrected for radial dilution only.

Table 3. Extrapolated sedimentation coefficients and concentration-dependence coefficients for glycoprotein 376

All measurements were made in potassium acetate-KCl buffers, pH 4.6 and I 0.10. The coefficient K is the product $S^0 \cdot d(1/S)/dc$; the concentration terms were evaluated by applying the radial dilution law to the stoichiometric initial concentrations and the K values are therefore subject to correction (see the text).

T	$S^0_{T,w}$ (s)	K (uncorr.) (dl./g.)	$S^0_{25,w}$ (s)
2.0°	5.56	1.20	10.4
25.0	11.5	1.23	11.9
45.0	17.0	1.15	

value of K cannot be specified precisely. Nevertheless, the accurate linearity of the reciprocal plot is one of the most striking physicochemical properties of the glycoprotein, and emphasizes the importance of the parameter K .

(d) Effect of varying pH and ionic strength. This glycoprotein contains 2.5% (w/w) of sialic acid (Pusztai & Morgan, 1964) and appreciable proportions of arginine, lysine, aspartic acid, glutamic acid and histidine (0.67, 0.39, 0.71, 0.90 and 0.44%, w/w, respectively). To determine whether the consequent variation of net charge with pH brought about changes in configuration, the sedimentation coefficient was measured at two fixed concentrations (0.305 and 0.831 g./dl.) at the pH values 3.20, 3.76, 4.61, 6.08, 7.60, 8.43 and 9.88, at I 0.10. No trend was detectable in the results, the sedimentation coefficients falling within the extremes 8.58–8.83s and 5.85–5.97s at the lower and the higher concentration respectively. The possibility of configurational change with ionic strength was also investigated by similar means; sedimentation coefficients were measured at pH 4.6 and at two fixed concentrations (0.273 and 0.780 g./dl.) at I 0.01, 0.02, 0.05, 0.10, 0.20 and 0.50. Again no trends were evident, the values falling within the extremes 8.82–8.97s and 6.03–6.09s at the concentrations quoted.

(e) Effect of SDS. As indicated in the introduction, the structure postulated for the glycoprotein clearly allows scope for hydrophobic bonding and hydrogen-bonding in the peptide core, and, although a tertiary structure is eliminated by the viscosity and sedimentation characteristics described, there remained the possibility of the occurrence of regions containing a specific secondary structure. SDS is of proved effectiveness in disrupting both secondary and tertiary structures in proteins, and accordingly its effect was investigated.

The sedimentation coefficient of glycoprotein 376, at a fixed mean concentration of 0.40 g./dl., was found to vary linearly with concentration (C) of SDS up to 1.5 g./dl.; the equation:

$$S_{25,w} = 7.75(1 - 0.049C) \text{ s}$$

was followed. Thus the change in S is only 7–8% at the maximum, and therefore a major change in secondary structure cannot have occurred. However, because binding of the detergent by the glycoprotein could tend to mask a configurational change, it was thought desirable to analyse the effect as fully as possible, by determining the extent of binding, α , and finding quantitative relations for the corresponding changes in sedimentation coefficient. The equations relating S and α are of general application, and their derivation is given in Appendix II.

Determination of α , the proportion of SDS bound.

This quantity was measured by experiments using the boundary-forming cell of the ultracentrifuge. Pure buffer was layered over a solution whose initial concentration in terms of glycoprotein and SDS was known. During the experiment (see Fig. 5), a fast peak, corresponding to glycoprotein + bound SDS, was resolved from a slow peak, corresponding to free SDS. Determination of the areas of these peaks by planimetric measurements on $8\times$ enlargements, followed by correction for radial dilution and the Johnston-Ogston effect, allowed the determination of α . The method of Trautman, Schumaker, Harrington & Schachman (1954) was followed in correcting for the latter effect. When allowance is made for the incomplete optical recovery of the glycoprotein, values of α may also be determined from the characteristics of the fast peak, so permitting a check on those values determined more directly from the slow peak alone; the results of this series of experiments are shown in Table 4.

It is seen that, in the limited range of conditions in which experiments were possible, the degree of binding was nearly constant at about 0.3 g. of detergent/g. of glycoprotein; slightly more is bound at high ionic strengths. Application of the appropriate equations then shows that the maximum change in sedimentation coefficient on the 'hard-sphere' model is about -0.2 s and on the 'random-coil' model about -0.3 s for this degree of binding. It therefore follows that only part of the observed decrease in S (totalling about 0.6s) is due to binding: the remaining part must correspond to a minor configurational change. It must be concluded that SDS, even at 1.5 g./dl., has only a trivial effect on the macromolecular structure.

Fig. 5. Sedimentation-velocity experiment on glycoprotein 376-detergent mixture, to determine extent of binding. Buffer was layered over a solution of glycoprotein (c^0 0.75 g./dl.) and SDS (c^0 1.50 g./dl.). The time (min.) at full speed (59780 rev./min.) is shown under each frame; the slow peak represents free detergent, and the fast peak the glycoprotein-detergent complex. The base line for the area determinations is provided by the first frame; the phase-plate angle was 75° throughout.

Table 4. *Binding of SDS by glycoprotein 376*

The symbols x_s and x_f refer respectively to degrees of binding calculated from the slow and fast peaks. Calculations of x_f for the two lowest ionic-strength values were not possible because of base-line uncertainties and skew peaks.

Concn. of glycoprotein (g./dl.)	Concn. of SDS (g./dl.)	Ionic strength (M)	Amount bound (g. of SDS/g. of glycoprotein)	
			x_s	x_f
0.669	0.90	0.10	0.28	0.27
0.669	1.50	0.10	0.29	0.32
0.750	1.50	0.40	0.37	0.40
0.750	1.50	0.25	0.32	0.30
0.775	1.50	0.10	0.27	0.28
0.775	1.50	0.05	0.31	—
0.775	1.50	0.01	0.13	—

Table 5. *Haemagglutination-inhibition activity of SDS-treated glycoprotein 376 against human anti-B serum*

The glycoprotein control had not been exposed to SDS. The treatment with SDS consisted of mixing the glycoprotein with the detergent to give a concentration of SDS of 1.5 g./dl., followed by removal of the SDS by dialysis. The SDS control contained no glycoprotein, but was otherwise treated identically.

Solution	Ionic strength (M)	Inhibition end point	
		Sample 1	Sample 2
Glycoprotein control	0.40	1:800000	—
	0.10	1:800000	—
SDS-treated glycoprotein	0.40	1:800000	1:200000
	0.10	1:800000	1:400000
SDS control	0.40	1:32000	1:8000
	0.10	1:64000	1:32000

Effect of SDS on biological activity. The most sensitive means of detecting changes in organization of the macromolecule is presumably by measurement of its specific biological activity before and after treatment with the detergent. Solutions containing 1.0 g. of glycoprotein/dl. and 1.50 g. of SDS/dl. were prepared and, after standing for about 1 hr. at 20°, were exhaustively dialysed against buffer over several days to remove the SDS as completely as possible. Control experiments in which either glycoprotein or SDS was omitted were also performed. The haemagglutination-inhibition activity of each of these solutions was then determined by the method of Morgan & King (1943). The results shown in Table 5 were obtained. It is seen that the biological activity of the blood-group substance is unaffected by the treatment with SDS; the small activities shown by the SDS controls are presumably due to residual traces of SDS not removed by dialysis and undetectable by the physical method employed, based on refractive index.

DISCUSSION

The values of the intrinsic viscosity quoted in Table 1 indicate that the glycoprotein molecules are either very asymmetric or greatly expanded. For example, following Simha's (1940) formulation in terms of rigid ellipsoids of revolution of axial ratio p :

$$[\eta] = \nu(p) \cdot v_e$$

If v_e , the effective hydrodynamic volume, is taken as equal to $\bar{v}$, as it would be for a rigid impenetrable particle, $\nu(p)$, the viscosity increment, is about 150, equivalent to p about 40–50; alternatively, for the other extreme of spherical symmetry, when ν is 2.5, v_e becomes approx. 37 ml./g. The ratio $v_e/\bar{v}$ may be regarded as an expansion factor; here it has a value of about 60. No choice between these extremes may be made from the viscosity data alone. However, the influence of temperature in decreasing $[\eta]$ is most directly interpreted in terms

of a partially flexible structure; the greater kinetic energy at the higher temperature allows some of the potential energy barriers corresponding to hindered rotation to be overcome. Thus the molecule is allowed to take up a more truly random (and thus more nearly spherical) configuration. This behaviour is analogous to that of some cellulose derivatives (Flory, Spurr & Carpenter, 1958; Mandelkern & Flory, 1952), where a similar value of $d \ln [\eta] / dt$ was interpreted as a decrease in mean square end-to-end distance. The effect of a cycle of changes in temperature shows that the configurational change is reversible. Further, the values of the Huggins coefficient k' (0.36–0.44) are characteristic of a random-coil polymer in a good solvent (0.35–0.40; Flory, 1953, p. 310).

The effect of temperature on the sedimentation coefficients also suggests a partially flexible structure; although the effects are small, they indicate a frictional coefficient that decreases with rise in temperature. It must be remembered that the sedimentation coefficient is only one-third as sensitive as the intrinsic viscosity to change in configuration; thus the magnitude of the change in S is approximately as expected from the viscosity determinations.

Independent evidence relating to configuration may also be obtained from the coefficient of concentration-dependence of sedimentation coefficient, K , through its relation to $[\eta]$. In a survey of about 50 macromolecular substances, Creeth & Knight (1965) pointed out that the ratio $K/[\eta]$ has a value of about 1.6 for substances of approximately spherical conformation whether compact or expanded in form, whereas smaller values of the ratio indicate either increasing asymmetry or increasing rigidity with consequently smaller solvent entrainment. Thus, although the theoretical basis for this relationship is not rigorous, it appears that the value of the ratio is a function of molecular shape alone. For the glycoprotein 376 the values quoted in the results section give $K/[\eta]$ ratios 1.4–1.5 at 25° and 1.6–1.7 at 45°. From this, it is concluded that the molecular configuration is approximately spherical; this conclusion allows the viscosity values to be interpreted directly in terms of the degree of expansion of a particle possessing spherical symmetry, and thus indicates that the second interpretation given above is probably correct. That is, the substance has a very expanded form in aqueous solution, arising from a high degree of flexibility in the molecular structure.

For spherical particles, it was shown by Creeth & Knight (1965) that the value of K defines an independent value of the degree of expansion. The semi-empirical expression is:

$$v_e = K/4.1$$

from which one finds v_e 30 ml./g. approximately, at 25°.

Thus both the viscosity and the concentration-dependence coefficient agree in indicating a value of 50–60 for the expansion factor. Although this can only have qualitative significance, there can be no doubt that a large volume of water is immobilized in the macromolecular domain. This conformation is clearly incompatible with the presence of a tertiary structure, and indicates that any secondary structure can only be of limited extent.

This representation of the macromolecular structure of the blood-group substance receives further support from the results of the study of the action of the detergent. With proteins, detergents disrupt the native structure through solubilization of non-polar residues and through the mutual repulsion of adsorbed detergent ions. The significant binding of SDS by the glycoprotein shows that the detergent is adsorbed, as is to be expected from the content of proline, leucine, isoleucine and valine (Pusztai & Morgan, 1963). This binding, however, is not accompanied by an appreciable change in configuration; it follows that there can be no significant degree of secondary structure in the peptide moiety, and therefore that the carbohydrate side chains are probably not organized in any regular manner. This conclusion is in agreement with the results of studies of the optical rotatory dispersion, which demonstrated the absence of peptide α -helix (Beychok & Kabat, 1965). The greater activity of the blood-group substance relative to simple sugars of the same specificity is therefore not likely to arise from a specific steric arrangement of the terminal carbohydrate residues; it may be due simply to the large size of the blood-group substance (mol.wt. in the range 5×10^5 – 10×10^5) and the multiplicity of specific carbohydrate chains.

The flexible structure for blood-group substances suggested by the present work is in general agreement with the supposed structures of other glycoproteins. For example, bovine cervical glycoproteins, which have a similar chemical composition (Gibbons, 1959) and analogous physical properties (Gibbons & Glover, 1959), have random-coil-like structures. Ovine submaxillary-gland glycoprotein has a similar polypeptide backbone, but shorter carbohydrate chains (e.g. Pigman & Gottschalk, 1966); on the basis of its high intrinsic viscosity and the value of K , Gottschalk & McKenzie (1961) proposed a random-coil structure with some rigidity. Recent measurements of some physical properties of the blood-group NN and Me-Vg antigens (Bezkerovainy, Springer & Hotta, 1966) gave high values for the frictional ratio, and were tentatively interpreted as indicating pronounced

asymmetry; they are equally consistent, however, with a random-coil model.

The simple structure postulated in the introduction for the blood-group substances clearly has fairly wide possibilities for polydispersity of molecular weight, if the carbohydrate side chains are of irregular length, are irregularly distributed or involve appreciable cross-linking. Only qualitative evidence relating to polydispersity has been obtained in this investigation, but there can be no doubt that the absence of a true plateau region in the sedimentation-velocity experiments indicates a considerable spread of molecular weight, at least on the high side of the distribution.

The authors are very grateful to Professor W. T. J. Morgan, F.R.S., for supplying the material and carrying out the haemagglutination-inhibition tests, and for many useful discussions, and to Professor R. A. Kekwick, F.R.S., for criticism of the manuscript. Mr H. S. Murray and Mr E. J. Gaughran gave skilled technical assistance. Grateful acknowledgement is made to the Medical Research Council for a supporting grant to C.G.K.

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APPENDIX I

Variation of the Plateau-Region Concentration with Time in Sedimentation-Velocity Experiments on Concentration-Dependent Systems

The purpose of this analysis is to allow ready comparison to be made between the predicted and the observed concentrations in the plateau region,

$c(r_x)$, in experiments where S varies significantly with c .

The continuity equation (e.g. Williams, Van

Holde, Baldwin & Fujita, 1958, p. 747) has the form:

$$\left(\frac{\partial c}{\partial t}\right)_{r=r_x} = -2\omega^2 c_x S(c_x) \quad (1)$$

Here r_x is the radial displacement of the arbitrary surface considered, and for simplicity $c(r_x)$ is written c_x . The other symbols have their usual significance. Dilution occurs during the period in which the speed varies as well as when it is constant, so that the function y is defined:

$$y = \int_0^t \omega^2 dt \quad (2)$$

It is then clear that S is a function of c^0 and y only, and for a given solution therefore $S=S(y)$. Eqn. (1) therefore becomes:

$$\frac{d \ln c}{dy} = -2S(y) \quad (3)$$

For small differences between $S(y)$ and $S(\bar{y})$, where $\bar{y}$ is the mean value of y during the experiment, one may write:

$$S(y) = S(\bar{y}) + (y - \bar{y}) \left(\frac{dS}{dy} \right)_{y=\bar{y}} + \dots \quad (4)$$

The derivative required follows from the concentration-dependence expression for S , which in this case follows the reciprocal law:

$$1/S = (1/S^0)(1 + Kc) \quad (5)$$

One therefore has $dS/dc = -S^2 K/S^0$, and from eqn. (3) $dc/dy = -2Sc(y)$. Substitution in eqn. (4) yields:

$$S(y) = S(\bar{y}) + 2Kc(\bar{y})(y - \bar{y})[S(\bar{y})]^3/S^0 + \dots \quad (6)$$

Eqn. (3) may now be integrated to give the result:

$$\ln c = \ln c^0 - 2yS(\bar{y}) + 4Kc(\bar{y})[S(\bar{y})]^3 y(y - \bar{y})/S^0 + \dots \quad (7)$$

which is explicit for $c(r_x)$ in terms of experimentally observable quantities.

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APPENDIX II

Effect of Binding a Third Component on the Sedimentation Coefficient of a Macromolecular Substance

Schachman (1959) has summarized expressions relating the sedimentation coefficient of a complex to the molecular weights and partial specific volumes of the components and the degree of binding. However, the expressions involve the frictional coefficient of the complex, which is generally unknown. In connexion with the effects of denaturing agents, one frequently wishes to know if an observed change in sedimentation coefficient indicates a configurational change or is merely the result of binding the added agent; for this purpose series expressions in which the first term represents the properties of the original macromolecule are most useful, and the fact that they give approximate solutions only is unimportant. Expressions for the two limiting cases of a compact macromolecule and a flexible-coil macromolecule have therefore been derived.

(a) *Compact macromolecules.* Where the initial configuration is compact (but not necessarily spherical) the conventional representation, based on an impermeable sphere obeying Stokes' Law (e.g. Svedberg & Pedersen, 1940), may be expressed as:

$$S^0 = k_1 M_p^{\frac{1}{2}} (1 - \bar{v}_{p0})/\bar{v}_p^{\frac{1}{2}} \quad (8)$$

where p represents the macromolecule and

$$k_1 = \frac{1}{6\eta_0(f/f_0)} \left(\frac{4}{3N^2\pi^2} \right)^{\frac{1}{2}} \quad (9)$$

An expression for the variation of S^0 with extent of binding, x (g. of reagent/g. of macromolecule), then follows:

$$\frac{dS^0}{dx} = \left(\frac{\partial S^0}{\partial M_p} \right)_{\bar{v}_p, (f/f_0)} \cdot \frac{dM}{dx} + \left(\frac{\partial S^0}{\partial \bar{v}_p} \right)_{M_p, (f/f_0)} \cdot \frac{d\bar{v}}{dx} \quad (10)$$

The partial derivatives are obtained from eqn. (8); the molecular weight and partial specific volume of the complex (denoted by subscript x) are given by the relations:

$$M_x = M_p(1 + x) \quad (11)$$

$$\bar{v}_x = (\bar{v} + x\bar{v}_A)/(1 + x) \quad (12)$$

where subscript A represents the SDS or similar component. These equations enable the two total

derivatives to be found. On substituting the results of the differentiations into eqn. (10) one obtains:

$$\left(\frac{dS^0}{dx}\right)_{x \rightarrow 0} = \frac{S^0}{3} \left[2 - \left(\frac{\bar{v}_A}{\bar{v}_p} - 1 \right) \cdot \frac{1 + 2\bar{v}_p \rho_0}{1 - \bar{v}_p \rho_0} \right] \quad (13)$$

This is the required expression for the change in S^0 [or, appropriately, $S(c)$ for fixed c] brought about by binding alone, that is, unaccompanied by a change in configuration.

(b) *Flexible-coil macromolecules.* For flexible linear polymer chains, the corresponding equation for S^0 (see, e.g., Tanford, 1961) is:

$$S^0 = M_p(1 - \bar{v}_p \rho_0) / 6\pi\eta_0 N \xi_t R_G \quad (14)$$

where R_G is the radius of gyration and ξ_t is a numerical factor relating R_G to the radius of the equivalent sphere. R_G is given by the expression:

$$R_G^2 = \alpha^2 \beta^2 M_p / 6M_0 \quad (15)$$

where α and β are constants related to the configuration of the macromolecule and M is the monomer molecular weight. For our purposes, α and β must be taken as constant, so that eqn. (15) becomes:

$$S^0 = k_2 M_p^{\frac{1}{2}} (1 - \bar{v}_p \rho_0) \quad (16)$$

where $k_2 = (M_0/6)^{\frac{1}{2}} / \pi\eta_0 N \xi_t \alpha \beta$. Following a route similar to that used before, one finds for this case:

$$\left(\frac{dS^0}{dx}\right)_{x \rightarrow 0} = \frac{S^0}{2} \cdot \left[1 + \frac{\rho_0(\bar{v}_p - 2\bar{v}_A)}{1 - \bar{v}_p \rho_0} \right] \quad (17)$$

Once again $S(c)$ may replace S^0 for experiments at fixed c ; the eqns. (13) and (17) are used to determine the expected change in $S(c)$ for a given (small) x from the relation:

$$S(c, x) = S(c, 0) + x(dS/dx) + \dots \quad (18)$$

Eqns. (13) and (17) give formal recognition to the fact that binding an agent of higher partial volume (e.g. $\bar{v}_{SDS}$ 0.863 ml./g.; Anacker, Rush & Johnson, 1964) may either increase or decrease the sedimentation coefficient of the macromolecule, depending on its $\bar{v}$; for example, with proteins in aqueous solutions of density close to 1, (dS/dx) is positive, whereas for the blood-group substance, it is negative, on either model.

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