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SOME PHYSICAL STUDIES ON THE TWO α-GLYCOPROTEINS OF FOETAL CALF SERUM

K. J. TURNER*

Commonwealth Serum Laboratories, Melbourne (Australia)

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SUMMARY

The 2 α-glycoproteins of foetal calf serum have been shown to differ widely in physico-chemical properties. The major component had a sedimentation coefficient of 3.4 S and a molecular weight of 47000. The minor component, a macroglobulin, had a sedimentation coefficient of 20 S and a molecular weight of 990000. It is suggested that the macroglobulin may be an aggregate of the 3.4-S fraction.

INTRODUCTION

The glycoproteins of foetal calf serum were first described by PEDERSEN[1-3] and termed fetuin[1]. They represented 45% of the total serum proteins and formed the major globulin component. The sedimentation constant for fetuin was found to be markedly concentration dependent and at infinite dilution varied with the origin of the fetuin. A detailed physico-chemical characterization was not attempted as the preparations were contaminated with a macroglobulin of sedimentation constant approx. 20 S. However, PEDERSEN drew attention to the isoelectric point, pH 3.5, which was exceptionally low for a plasma protein.

DEUTSCH[4] isolated fetuin by precipitation in trichloroacetate buffer and obtained, as with the ammonium sulphate fractionation[1-3], a major component of 3.2 S and a minor component of approx. 20 S. This minor component accounted for from 5 to 10% of the total glycoprotein. The low-temperature ethanol fractionation in the presence of Ba^{2+} and Zn^{2+} used by SPIRO[5] yielded a preparation of fetuin of a high degree of purity, being homogeneous both by electrophoresis and in the ultracentrifuge over a wide pH range.

FISHER *et al.*[8] showed that the fetuin prepared by ammonium sulphate fractionation had the ability to promote the stretching and attachment of cultured mammalian cells. No such ability could be demonstrated in the preparation obtained by SPIRO[5]. Since the ammonium sulphate procedure yielded both 3.2-S and a 20-S components, the inference could be that the macroglobulin was the biologically active protein. While the 3.2-S glycoprotein has been isolated by SPIRO with a high degree of purity, so far no attempt has been made to isolate the macroglubolin.

MARR *et al.*[8] have modified the method of PEDERSEN by the use of high-speed

* Present address: Department of Microbiology, University of Adelaide, Adelaide, (Australia).

centrifugation and have obtained homogeneous preparations of both the 3.2-S and the 20-S α-glycoproteins. This publication reports some physico-chemical studies done on these fractions and contrasts the results obtained with the data of other authors.

METHODS

The two α-glycoproteins from foetal calf serum were prepared by Mr. A. G. M. MARR[8] and shall be referred to in this paper by their $s_{20,w}$ values. Thus 3.4 S refers to the major fraction of low molecular weight generally referred to in the literature as "fetuin" and 20 S refers to the minor component a macroglobulin of high molecular weight.

The following buffers of ionic strength 0.1 were used for all physical measurements:

pH	*Buffer composition*
2.5	0.05 M NaCl + 0.029 M glycine — 0.021 M HCl
3.0	0.05 M NaCl + 0.039 M glycine — 0.011 M HCl
3.5	0.05 M NaCl + 0.046 M glycine — 0.004 M HCl
4.0	0.05 M NaCl + 0.05 M sodium acetate + 0.015 M acetic acid
5.0	0.05 M sodium acetate + 0.010 M acetic acid
7.0	0.05 M NaCl + 0.018 M sodium acetate
8.5	0.05 M NaCl + 0.013 M HCl — 0.053 M sodium veronal
9.0	0.05 M NaCl + 0.005 M HCl — 0.053 M sodium veronal
10.0	0.05 M NaCl + 0.036 M glycine — 0.014 M NaOH

The concentration of dissolved solids was determined by drying aliquots to constant weight at 60° *in vacuo*.

Partial specific volumes were measured at 20 ± 0.01° using pyknometers of 50 ml volume calibrated with distilled water at that temperature. Viscosity determinations were made at 20° in an "Ostwald-Fenske" capillary-type viscometer with a flow time for water of 202 sec. The solutions and solvents were filtered prior to measurement through a sintered-glass filter.

Sedimentation-velocity experiments were performed at about 260000 × *g* in a Spinco Model-E ultracentrifuge over a range of concentrations that permitted extrapolation to zero concentration. Paper electrophoresis was carried out with a Spinco hanging-strip apparatus under the normal operative conditions of veronal buffer pH 8.6 and ionic strength 0.075. Papers were stained for proteins with bromophenol blue and for carbohydrate with Schiffs acid fuchsin.

RESULTS

The results of the physical measurements on both 3.4-S and 20-S fractions are summarized in Table I.

The partial specific volumes ($\bar{v}$) were measured at pH 7.0 on a single preparation at three independent concentrations. Since there was no evidence of concentration dependence the reported values are the means of the separate measurements. The value of $\bar{v}$ for the 3.4-S fraction is low (0.70 ml/g) and the buoyancy factor ($1 - \bar{v}\rho$) in the sedimentation equation corresponding large (0.30). The corresponding values for the 20-S component (0.73 ml/g and 0.27) are closer to those generally found for proteins.

The 3.4-S glycoprotein sedimented as a single symmetrical peak with constant sedimentation coefficients over a range of ionic strength from $I = 0.05$ to 0.25 (Fig. 1). The 20-S glycoprotein, however, contained traces of contaminants representing less than 10 % of the total protein and sedimenting either side of the main peak. The sedimentation coefficient of the major component was likewise independent

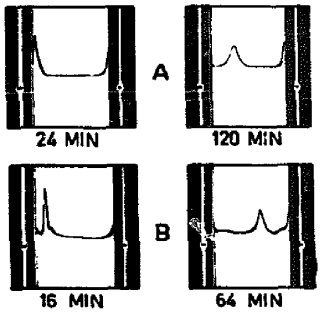

Fig. 1. Sedimentation patterns of the 3.4-S (A) and 20-S (B) glycoproteins of foetal calf serum. The solute concentration in both cases was 0.8 % in acetatebuffer (I 0.1, pH 7.0). The times refer to the duration in min after the rotor had reached the maximum speed of 59780 rev./min (A) and 42040 rev./min (B).

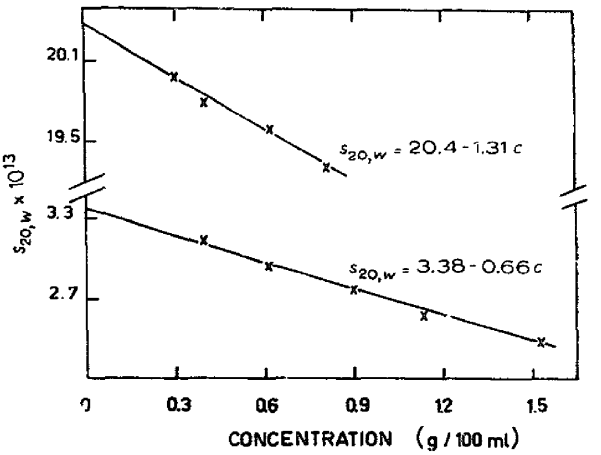

Fig. 2. Sedimentation measurements on the α-glycoproteins of foetal calf serum in acetate buffer (I 0.1, pH 7.0).

of ionic strength over the range studied. A plot of sedimentation coefficient *versus* concentration at pH 7.0 is shown in Fig. 2, the plot being made by the method of least squares. Both fractions exhibited pronounced concentration dependence. From the slopes of the lines and their intercepts the following relationships were obtained: $s_{20,w} = 3.38 - 0.66c$ for the 3.4-S fraction and $s_{20,w} = 20.4 - 1.31c$ for the 20-S fraction. $s_{20,w}$ is in Svedberg units and c represents the protein concentration in grams per 100 ml. The sedimentation coefficient of the 3.4-S fraction was constant over the pH range 4–10 but increased by a factor 1.7 at pH 3.0. This increase in sedimentation coefficient below the isoelectric pH was accompanied by the appearance of a smaller faster sedimenting component of $s_{20,w}$ three-fold that of the major component. At all other pH values the protein was found to be homogeneous. The 20-S component on the other hand was homogeneous over the entire pH range studied and showed no significant variation of sedimentation constant with pH.

The values for the intrinsic viscosities of the 3.4-S and 20-S fractions at pH 7.0 were 6.9 and $10.2 \cdot 10^{-2}$ ml/g respectively. The values of the viscosity increment (ν) were found to be 9.78 and 13.9 respectively. Assuming the molecules to be unhydrated ellipsoids of revolution the axial ratios from SIMHA's[9] equation become 7.8 and 10.2 for a prolate ellipsoid and 12.8 and 18.7 for an oblate ellipsoid.

Paper electrophoresis analysis showed that both preparations were homogeneous and migrated as α-globulins at pH 8.6. Both gave positive staining with both Schiff's acid fuchsin and bromophenol blue confirming the presence of a glycoprotein. A plot

off-band displacement against pH over the range of from pH 2.5 to 8.5 established the isoelectric pH at 4.1 and 4.7 for 3.4-S and 20-S fractions respectively. In the original fetuin mixture both fractions migrated together at pH 8.6 but were resolved at lower pH values.

TABLE I

SUMMARY OF PHYSICAL MEASUREMENTS ON THE TWO α-GLYCOPROTEINS OF FOETAL CALF SERUM

Property	*3.4-S fraction*	*20-S fraction*
$\bar{v}$ (ml/g)	0.702	0.733
$[\eta] \times 10^2$ ml/g	6.86	10.2
ν	9.78	13.9
f^*	7.8	10.2
f^{**}	12.8	18.7
$s^\circ_{20,w}$(Svedbergs)	3.38	20.4
Isoelectric pH	4.1	4.7
Mol. wt. from $s^\circ_{20,w}$ and $[\eta]$	47 000	990 000

* Prolate ellipsoid of revolution.
** Oblate ellipsoid of revolution.

The molecular weight of each fraction when calculated by using the formula of SCHERAGA AND MANDELKERN[10] gave values of 47 000 and 990 000. In these calculations a β coefficient of $2.16 \cdot 10^6$ was used for each fraction as proposed by SCHACHMAN[11]. The modified equation becomes:

$$M = \frac{4690\ (s^\circ_{20,w})^{1.5}\ [\eta]^{0.5}}{(1 - \bar{v}\rho)^{1.5}} \qquad (1)$$

where M is the molecular weight, $s^\circ_{20,w}$ the sedimentation coefficient in Svedbergs at zero concentration, $[\eta]$ the intrinsic viscosity in $(g/100\ ml)^{-1}$, $\bar{v}$ the partical specific volume in ml/g.

DISCUSSION

The physical measurements of the glycoproteins from foetal calf serum (Table I) are generally in good agreement with the results of PEDERSEN[1-3] and more recently SPIRO[5]. While the method of preparation used by PEDERSEN[1-3] was essentially reproduced in this present work, the final preparations used here were considerably more homogeneous. This arose from the use of high-speed centrifugation as a further step in the fractionation process. PEDERSEN[1-3] reported values of 3.09–3.28 for sedimentation coefficient, 0.693–0.712 for partial specific volume and 3.5 for the isoelectric pH. SPIRO[5] found values of 3.47, 0.696 and 3.3 respectively for these measurements. While the results of DEUTSCH[4] (0.712 for partial specific volume and 3.4 for isoelectric pH) are in close agreement with the above, the sedimentation coefficient at infinite dilution of 2.8, obtained by this author, is considerably lower. The mol. wt. of 45000 obtained by the combination of sedimentation and diffusion data is likewise lower than that obtained by either PEDERSEN[1-3] (49000) or SPIRO[5] (44000) using the same techniques. It is difficult to assess what contribution the trichloroacetic acid used in the Deutsch preparation made towards these lowered physical constants.

As alternative methods for the measurement of molecular weight were unavailable, an estimate was obtained by the combination of sedimentation and viscosity data. A value of $2.16 \cdot 10^6$ was assumed for the coefficient in the Scheraga–Mandelkern relationship as direct measurement of this constant is uncertain due to it being extremely sensitive to slight experimental errors. The correct assessment of β would be greater than $2.16 \cdot 10^6$, since the concentration dependence of the sedimentation coefficient and the axial ratios calculated from the viscosity data indicated that both molecules were asymmetric. In practice this means that the mol. wt. of 47000 and 990000 reported in Table I are slightly higher than the true values. Nevertheless, the result for the 3.4-S glycoprotein compares favourably with a mol. wt. of 50300 for fetuin calculated from the corresponding data presented by SPIRO[5].

It has been shown that the acid nature of some glycoproteins is attributable to the sialic acid content. SPIRO[5] has demonstrated that this is likewise the case with fetuin. The shift in isoelectric pH towards the alkaline pH's shown by our preparations, when compared with those of previous authors, reflects the lowered sialic acid content of our material. Taking Spiro's fetuin and its sialic acid-free counterpart as extremes a linear plot of isoelectric pH. *vs.* sialic acid content can be shown to include both glycoprotein preparations employed in this study.

Fetuin has a tendency to aggregate particularly at acid pH (see ref. 4). In the ultracentrifuge this is revealed at pH 3.0 by an increase in the sedimentation coefficient of the major component (3.4-S fraction) of fetuin and the appearance of a second faster sedimenting component. DEUTSCH[4] has claimed the difficulties encountered in obtaining consistent results in diffusion experiments may be attributed to the formation of higher-molecular-weight aggregates. Lower values for the diffusion constant were found near the isoelectric-point region than at neutrality. SPIRO[5], on the other hand, found that the increased sedimentation around the isoelectric point is accompanied by a decrease in viscosity suggesting the likelihood of a change in molecular shape rather than an increase in molecular weight. He does not, however, report the presence of additional components as has generally been found. Removal of sialic acid by dilute-acid treatment[5] gave proteins which sedimented at about the same rate as the untreated protein. In addition a small amount of a faster sedimenting component was evident. While the latter may be attributed to heat coagulation, it seems more likely in view of the work of DEUTSCH[4] that it is a true aggregate arising out of the decreased sialic acid content. In fact, the treatments which have caused

TABLE II

CHEMICAL COMPOSITION OF THE TWO α-GLYCOPROTEINS OF FOETAL CALF SERUM

	*Content (%)**		*Residues per mole***		*Molar ratios*	
	3.2-S fraction	*20-S fraction*	*3.2-S fraction*	*20-S fraction*	*3.2-S fraction*	*20-S fraction*
Hexose	5.3	2.7	14.0	150	2	2.8
Hexosamines	3.3	1.2	7.0	54	1	1.0
Sialic acid	5.6	0.7	8.5	22	1.2	0.4

* See MARR *et al.*[8].

** Calculated at the basis of a mol. wt. of 47000 for the the 3.2-S fraction and 990000 for the 20-S fraction.

fetuin to aggregate, namely heating at elevated temperatures or subjection to low pH are those which are known to result in loss of sialic acid[4,5].

If loss in sialic acid results in partial aggregation of fetuin, might not the 20-S component reported here be itself an aggregate of the more widely characterized 3.4-S fraction? The mol. wt. of the 20-S component obtained by sedimentation–viscosity data is 990000 and the sialic acid, hexosamine, and hexose composition in terms of moles/unit based on this value is reported in Table II, together with the corresponding data for the 3.4-S fraction. The molar ratios for sialic acid: hexosamine: hexose are 1.2:1:2 for the 3.4-S fraction and 0.4:1:3 for the 20-S fraction. It is apparent that these molar ratios are very similar with the exception of the sialic acid figures. GRAHAM[14] has established the order of the prosthetic group of fetuin as sialic acid–hexosamine–hexose–peptide. It seems possible therefore that sialic acid, due to its exposed position, would be the most labile component of the prosthetic group. Partial removal of the sialic acid may, possibly due to its altered surface change, result in limited aggregation of the 3.4-S glycoprotein to produce the 20-S glycoprotein. While the evidence for this relationship is rather speculative it does provide a mechanism for establishing possible relationships between globulins and their macroglobulin counterparts existing in both normal adult and foetal sera.

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