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Behavior of Glycopolypeptides with Empirical Molecular Weight Estimation Methods. 2. In Random Coil Producing Solvents†

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ABSTRACT: The effect of oligosaccharide branch chains on the hydrodynamic behavior of reduced glycopolypeptides was examined by gel chromatography in random coil producing solvents. This entailed a comparison of the gel chromatographic behavior in the presence of concentrated guanidinium chloride of 16 glycopolypeptides of known physical and chemical properties to that of regular polypeptides. For most of the glycopolypeptides employed, the presence of oligosaccharide branches sufficiently perturbed the dimensions of the unfolded glycopolypeptide such that its effective hydrodynamic radius was the same as that of a linear polypeptide of the same total mass. For this reason, gel chromatography in random coil producing solvents appears to be the most reliable empirical method to obtain a first approximation of the molecular weight of a glycopolypeptide. Glycopolypeptides rich in *N*-acetylneuraminic acid, and thus possessing low isoionic points, exhibited more pronounced deviations in their electrophoretic behavior in the presence of 8 M urea than those glycopolypeptides whose ionic properties were similar to those of the polypeptide standards employed.

Gel chromatography, as an empirical method for molecular size discrimination, is frequently employed in denaturing solvents for the estimation of molecular weights of reduced polypeptide chains. Two denaturing solvent systems which are frequently employed for this purpose are 8 M urea and 6 M guanidinium chloride[1] (Davison, 1968; Fish et al., 1969). The latter of these solvents is more generally utilized by reason of its greater denaturing power (Tanford, 1968). However, more important is the fact that the gross conformation of the polypeptide products which result from these denaturants' actions on proteins has been rigorously defined. That is, reduced polypeptide chains in either of these solvent systems behave hydrodynamically as randomly coiled, linear homopolymers (Tanford, 1968). As a random coil, the hydrodynamic size assumed by a polypeptide free of constraint by cross-links is a function of polypeptide chain length, hence molecular weight.

As has been repeatedly emphasized, one of the fundamental assumptions implicit to gel chromatography in denaturing solvents as an empirical molecular weight estimation method is that standards and unknowns possess congruent shapes (Fish, 1975). Thus, it was of interest to attempt to gain a quantitative measure of the differences in the hydrodynamic behaviors between polypeptides as randomly coiled linear pseudo-homopolymers and glycopolypeptides as randomly coiled branched-chain heteropolymers by investigating their gel chromatographic properties in 6 M GdmCl and their disc gel electrophoretic properties in 8 M urea. The purpose of this paper is to present the results of those studies.

Materials and Methods

Glycopolypeptides. The glycopolypeptides utilized in this investigation are listed in Table I. Detailed information about source, physical properties, and chemical properties of each glycoprotein follows or is presented in the preceding paper (Leach et al., 1980).

Fetuin was obtained from Sigma (lot 33C-1390). This preparation was further purified by gel chromatography of the disulfide cross-linked material in 6 M GdmCl. Three chromatographic species were obtained; the major component

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[1] Abbreviations used: GdmCl, guanidinium chloride; $NaDodSO_4$, sodium dodecyl sulfate; R_e, equivalent hydrodynamic (or Stokes) radius; DNP, dinitrophenyl.

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(~60%) eluted at an apparent R_e[1] of 55 ± 2 Å. It was the only component which possessed carbohydrate as evaluated by monitoring fractions with the phenol–sulfuric acid assay (Dubois et al., 1956), and its amino acid composition confirmed the presence of a proportion of proline higher than that nominally present in other plasma proteins but exhibited by fetuin (Spiro & Spiro, 1962). For these reasons, this chromatographic fraction was taken as fetuin. The molecular weight estimated for this preparation by sedimentation equilibrium was 44 000 ± 1000 when a $\bar{v}$ of a 0.70 mL/g was used (Spiro, 1960; Green & Kay, 1963) in conjunction with our sedimentation equilibrium data. This is in good agreement with literature values. A value of 23% was used for the carbohydrate content of fetuin (Spiro & Bhoyroo, 1974; Baenziger & Fiete, 1979; Nilsson et al., 1979), and an $E_{278}^{mg/mL}$ of 0.41 was employed for concentration estimations of fetuin (Spiro, 1960). Hydrodynamic measurements for the Sigma fetuin were later duplicated with a preparation from Grand Island Biological Co. which was >90% fetuin as evaluated by electrophoresis.

Ovomucoid was purchased from Worthington (lot 0I 91A). This preparation was further purified by gel chromatography of the disulfide cross-linked material in 6 M GdmCl. Of the four chromatographic species which were obtained, only one (of R_e = 34.4 Å) exhibited trypsin inhibition after the fractions were refolded in dilute buffer. This species, which amounted to ~50% of the commercial preparation, also possessed carbohydrate. The molecular weight estimated by sedimentation equilibrium for this preparation of ovomucoid in both 6 M GdmCl and in dilute buffer was 27 000 ± 500. This is in excellent agreement with previously reported values (Davis et al., 1971; Kato et al., 1976). A partial specific volume of 0.70 mL/g, an $E_{278}^{mg/mL}$ of 0.41 (Davis et al., 1971), and a nominal carbohydrate content of 23% (Lin & Feeney, 1972; Chatterjee & Montgomery, 1962) were used for this ovomucoid preparation.

Glucose oxidase from *Aspergillus niger* (EC 1.1.34) was obtained from Sigma (type V, lot 16C-0312). For this preparation, the major electrophoretic component (>95%) which stained for protein also stained for carbohydrate and for oxidase activity (Cohen, 1974). No contaminating proteolytic activity could be detected in the preparation (Schumacher & Schill, 1972). Sedimentation velocity measurements on the native protein yielded a single boundary of $s^0_{20,w}$ = 7.9 S. This corresponds well with previously reported values (Bodmann & Walter, 1965; Swoboda & Massey, 1965). Sedimentation equilibrium measurements on the native enzyme yielded a weight average molecular weight of 145 000. Sedimentation equilibrium measurements were also performed on both unreduced and reduced glucose oxidase in 6 M GdmCl. In both cases, depending upon the value chosen for the ϕ' of the protein in the solvent system, the weight-average molecular weight estimated for the glycopolypeptide after correction for solution nonideality was 67 000–75 000. Similarly, a reduced, carbaminomethylated sample from the 6% agarose gel chromatographic column equilibrated in 6 M GdmCl yielded a weight-average molecular weight by sedimentation equilibrium of 65 000–72 000. These results are consistent with a model for the glycopolypeptide chain composition of native glucose oxidase of two chains of about 72 000 molecular weight each. This supports an earlier report of two constituent glycopolypeptide chains in the native protein (O'Malley & Weaver, 1972), although our estimates for the molecular weights of the native enzyme and its glycopolypeptides are about 10% lower than those previously reported (Bodmann & Walter, 1965; Swoboda & Massey, 1965; O'Malley & Weaver, 1972). A $\bar{v}$ of 0.711 mL/g (Bodmann & Walter, 1965) and a carbohydrate content of 16.5% (Swoboda & Massey, 1965) were employed.

Human chorionic gonadotropin (Sigma, lot 102C-0360) was used without further purification. Gel chromatography of the unreduced protein in 6 M GdmCl yielded two solute chromatographic zones, one of apparent R_e ~ 40 Å (~65%) and the other of apparent R_e ~ 30 Å (~35%). Each chromatographic species had significant amounts of carbohydrate associated with it. Chromatography of the reduced, carbaminomethylated protein in 6 M GdmCl again yielded two zones, one of apparent R_e = 46 Å and the other of apparent R_e = 35.6 Å. Again, carbohydrate was associated with each species. Two species were also observed by gel chromatography or by gel electrophoresis in $NaDodSO_4$.[1] The smaller chromatographic species was assumed to be the α subunit of human chorionic gonadotropin. Values of 14 900 for the molecular weight and 32% for the carbohydrate content were used for this species. These are based on the amino acid sequence and chemical composition reported for the α subunit (Bellisario et al., 1973; Morgan et al., 1975; Bahl, 1969). The amino acid sequence and chemical composition published for the β subunit, which we presumed to be the chromatographic species of larger hydrodynamic size, yield a molecular weight of 23 000 and 30% carbohydrate (Carlsen et al., 1973; Morgan et al., 1975; Bahl, 1969).

Bovine thyrotropin was obtained from Sigma (lot 122C-2240) and used without further purification. Only a single, rather broad, elution zone was observed for the reduced, carbaminomethylated protein when it was subjected to gel chromatography in 6 M GdmCl. From the published amino acid sequence and the chemical analyses, the molecular weights and carbohydrate percentages used for each of the chains were thyrotropin-α, 13 600, 21%, and thyrotropin-β, 14 700, 12% (Liao & Pierce, 1970, 1971). Since our gel chromatography column was unable to separate solutes of such similar size and since we could not predict the hydrodynamic contributions made by the polypeptide and oligosaccharide moieties beforehand, we used the weighted-average molecular weight and carbohydrate content for the mixture of the α and β chains, i.e., 14 100 and 16.3%, respectively.

1,4-β-D-Glucan cellobiohydrolase C (EC 3.2.1.91) was a gift of Drs. R. D. Brown, Jr., and E. K. Gum, Jr., of Virginia Polytechnic Institute and State University. This enzyme contains 13% carbohydrate bound through 16–18 linear chains, 1–5 residues in length (Gum & Brown, 1976). An $E_{280}^{mg/mL}$ of 1.42 was employed for concentration estimations, and a $\bar{v}$ of 0.699 mL/g was estimated from its chemical composition. Sedimentation equilibrium measurements on the native protein preparation yielded results suggestive of contamination by <10% of a lower molecular weight component. A molecular weight of 58 000 ± 2500 was estimated for the heavier species from a total of five runs on the native preparation. Gel chromatography of the reduced, carbaminomethylated enzyme preparation either in 6 M GdmCl or in $NaDodSO_4$ yielded two zones. In each case, carbohydrate was associated with the major component (~90%). The minor component (~10%), apparent molecular weight ~38 000, had no carbohydrate associated with it. Sedimentation equilibrium measurements were made on the major chromatographic species in each denaturing solvent system, and the appropriate corrections were made for nonideality. The molecular weight estimated for cellobiohydrolase C from these sedimentation equilibrium measurements together with the measurements

in dilute buffer was 57 000 ± 3000.

Fetuin and α_1-acid glycoprotein were treated with neuraminidase (Sigma type V) by the procedure of Spiro (1962). Quantitation of *N*-acetylneuraminic acid associated with the glycoproteins before and after neuraminidase treatment was by gas–liquid chromatography of the trimethylsilyl derivatives of the acid hydrolysates. These determinations were kindly performed by Dr. Robert Lovins.

Ultracentrifugation. Sedimentation velocity and equilibrium measurements were performed in a Beckman Model E analytical ultracentrifuge equipped with Schlieren and interference optics. The optics were aligned by the procedure of Rees et al. (1974), and alignment was regularly confirmed by the method of Dyson (1970). Equilibrium measurements employed either the high-speed meniscus depletion method (Yphantis, 1964), the long-column meniscus depletion method (Chervenka, 1970), or the low-speed sedimentation equilibrium method as outlined by Richards et al. (1968). Data treatments of low-speed experiments were performed as outlined by Richards et al. (1968), by the method of Nazarian (1968), and by the method of Rowe & Rowe (1970).

Gel Chromatography in 6 M GdmCl. Six percent agarose (Bio-Rad Laboratories or Pharmacia) which was equilibrated in 6 M GdmCl (Sigma, Grade I) was employed in a 1.6 × 100 cm Pharmacia column. Gel preparation, column packing, and sample denaturation, reduction, and carbaminomethylation were performed according to previously published procedures (Mann & Fish, 1972). A flow rate of 1.4 g h^{-1} cm^{-2} was used, and ~1-g fractions were collected. The elution positions of polypeptides and glycopolypeptides were determined by absorbance at 280 nm and expressed in cumulative eluate weight. The elution positions of the glycopolypeptides were also verified by monitoring individual fractions for carbohydrate (Dubois et al., 1956). A low, but constant, level of oligosaccharides continually leached from the agarose column. This produced a weak background color in the carbohydrate assay for which a correction was made. The elution position of each glycopolypeptide was measured at least twice, once with the glycopolypeptide alone and once with standard polypeptides which were selected to elute on either side of it. Additionally, a mixture of three to five standard polypeptides was utilized every third run as a precaution against undetected shifts in the column calibration curve. Blue Dextran was employed as the column void volume marker and DNP-glycine was employed as the column internal volume marker. Gel chromatographic data were treated in the common fashion for molecular weight estimation (Fish et al., 1969), while the data treatment of Ackers (1967) was employed for estimation of R_e values. In this latter treatment, values of R_e used for the randomly coiled polypeptide standards were based on intrinsic viscosity. The intrinsic viscosity (η), the R_e, and the radius of gyration (R_G) of a flexible polymer may be expressed in terms of the properties of the polymer by the relations (Flory, 1953; Tanford, 1961):

$$[\eta] = \frac{10\pi N\alpha^3\beta^3\xi^3 M^{1/2}}{3(6M_0)^{3/2}} = \frac{10\pi N}{3M}\xi^3 R_G{}^3 = \frac{10\pi N}{3M}R_e{}^3$$

In these equations, α is a polymer expansion factor which takes into account any attractive forces between segments of the polymer or between solvent and polymer, β is the effective bond length per monomer unit and is a constant characteristic of the nature of the polymer, ξ is the ratio between R_e and R_G of the polymer, M is the molecular weight of the polymer, M_0 is the mean residue (or polymer unit) molecular weight, and N is Avagadro's number. The number of units, n, in the polymer can be used in place of M by substituting the relation $M = nM_0$.

For linear polypeptide chains in 6 M GdmCl[2] at 25 °C, the effective bond length per monomer unit is about 8.4 Å, and the polymer expansion factor can be related to the polypeptide chain length by the relation

$$\alpha = 0.85(n)^{0.055}$$

where α is the expansion factor and n is the number of amino acid residues in the polypeptide chain.

Gel Electrophoresis in 8 M Urea. The low pH, urea-containing polyacrylamide disc gel electrophoresis system designed by Panyim & Chalkley (1969) was employed. The methodology which was followed was that outlined by Poole et al. (1974) for the analysis of reduced, carbaminomethylated polypeptides in 8 M urea.

Standard polypeptides and glycopolypeptides were detected in the gels by the same procedure as employed in the preceding paper (Leach et al., 1980).

Results

Gel Chromatography. For examination of the effects exerted by oligosaccharide branches on the hydrodynamic behavior of randomly coiled polypeptide chains, the elution positions of the reduced glycopolypeptides were determined on a gel chromatography column which was first calibrated with reduced polypeptides of known chain length. The elution position of each glycopolypeptide was converted to an apparent molecular weight based on a calibration curve generated with the standard polypeptides. The percent difference between the apparent molecular weight of each glycopolypeptide and its molecular weight estimated by sedimentation equilibrium or amino acid sequence and carbohydrate composition is presented in Figure 1 as a function of the percentage carbohydrate in the glycopolypeptide. The dimensions of the rectangles in the figure represent the approximate confidence intervals within which the molecular weight and carbohydrate content of each of glycopolypeptide are known. The precision of ±1% to which the gel chromatographic partition coefficients were measured falls well within the aforementioned confidence limits. As illustrated by Figure 1, the disparity between the hydrodynamic volume of a glycopolypeptide and that of a linear polypeptide of the same mass is vanishingly small for 14 of the 16 glycopolypeptides examined. These rather surprising results covered a group of glycopolypeptides ranging in carbohydrate content from 3% to 30%. Only α_1-acid glycoprotein and its neuraminidase-treated form (rectangles 6 and 7 of Figure 1) exhibited significantly smaller hydrodynamic volumes than would be exhibited by linear polypeptides of equal mass. Furthermore, the extent of the deviation represented by α_1-acid glycoprotein is not simply a function of the fractional amount of carbohydrate in the molecule, but it must depend in some fashion on the numbers, locations of attach-

[2] Calculated from the data of Tanford et al. (1967) and Lapanje & Tanford (1967) according to the relations given in Tanford (1961, 1968). When the effective bond length, β, of the randomly coiled polypeptide chain in 6 M GdmCl is compared with a bond length, l_{av}, of 3.8 Å per peptide unit (Ramachandran & Sasisekharan, 1968), the ratio β/l_{av} = 2.2 suggests a nominal stiffness of the polypeptide chain. A final constant, ξ = 0.789, the ratio between the effective hydrodynamic radius and the average radius of gyration, allows the interconversion of the intrinsic viscosity, polypeptide chain length, hydrodynamic radius, and average radius of gyration for linear polypeptides in 6 M GdmCl. For randomly coiled polypeptides in 8 M urea (Lapanje, 1969), the values of β and ξ are the same as in 6 M GdmCl, while α may be found by the relation $\alpha = 0.919(n)^{0.038}$.

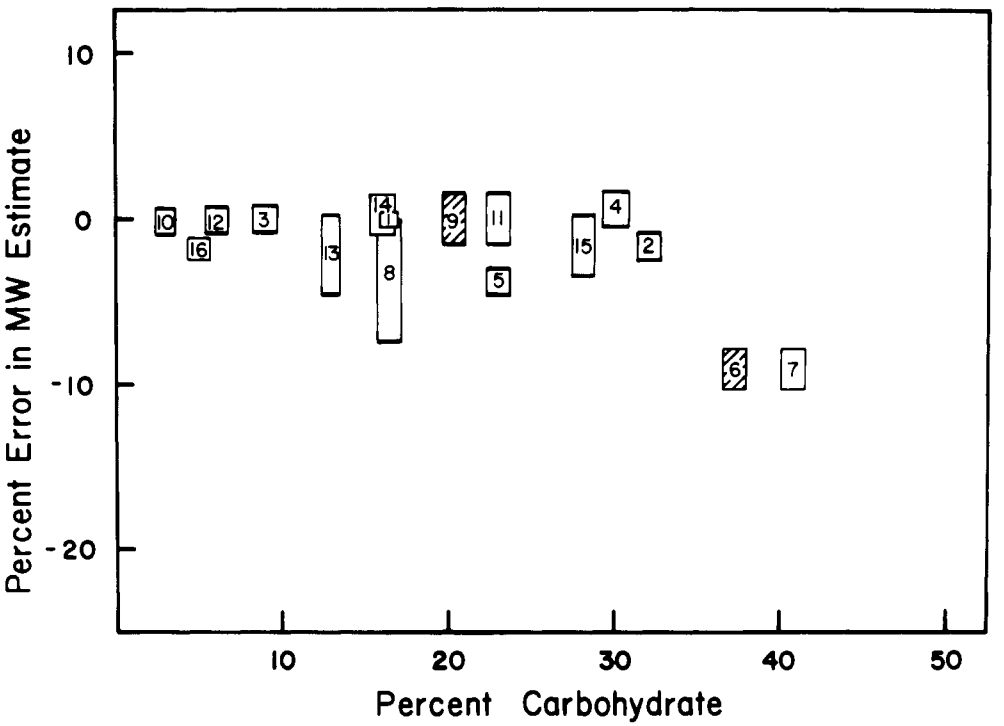

FIGURE 1: Gel chromatographic behavior of reduced, carbaminomethylated glycopolypeptides in 6 M GdmCl. The size of each rectangle approximates the confidence limits of our sedimentation equilibrium estimates and literature values for the molecular weight and carbohydrate content of the glycopolypeptide. The glycopolypeptides are numbered according to increasing molecular weight; their specific identities are given in Table I. The shaded rectangle indicates a glycopolypeptide from a neuraminidase-treated glycoprotein.

ments, chain lengths, and extents of branching of the oligosaccharide moieties. For example, cellobiohydrolase C, which possesses more of a mucin-like oligosaccharide complement (Gum & Brown, 1976), exhibits a hydrodynamic behavior similar to those glycopolypeptides which possess a limited number of rather large oligosaccharide branches [e.g., human chorionic gonadotropin, β chain (Carlsen et al., 1973; Bahl, 1969), and ovomucoid (Kato et al., 1976; Chatterjee & Montgomery, 1962)]. On the other hand, α_1-acid glycoprotein (Schmid et al., 1973; Fournet et al., 1978) and the α chain of chorionic gonadotropin (Bellisario et al., 1973; Bahl, 1969; Liao & Pierce, 1971) exhibit somewhat different hydrodynamic behavior, yet both possess a sizable proportion of carbohydrate which is incorporated as a small number of rather large oligosaccharide chains located in a terminal half of the polypeptide chain.

Further credence is given to the reliability of our gel chromatographic data when the values for the equivalent hydrodynamic radii of the glycopolypeptides estimated by this method are compared to corresponding values of R_e calculated from intrinsic viscosities reported in the literature (Table I). The excellent agreement for ovalbumin and for transferrin is expected because of the low carbohydrate contents of these two glycoproteins. The gel chromatographic estimate of the hydrodynamic volume of Tamm–Horsfall glycopolypeptide, which possesses about 28% carbohydrate branched from its polypeptide chain, agrees well with that estimated by viscosity (Stevenson & Kent, 1970). Furthermore, the R_e of 85–86 Å (corresponding to an $[\eta]$ = 51–53 mL/g) measured for this glycopolypeptide is substantially larger than that predicted (an R_e of 71.4 Å and an $[\eta]$ = 42 mL/g) if the oligosaccharide branches exerted absolutely no influence on the hydrodynamic behavior of the polypeptide moiety. The disparities between the values of R_e estimated for ovomucoid and glucose oxidase in this investigation and those calculated from literature values of the intrinsic viscosities possibly reflect errors in the viscosity measurements because the reported viscosity of each glycopolypeptide is less than that which would be predicted for its polypeptide chain alone. For glucose oxidase, $[\eta]_{\text{literature}}$ equals 30.0 mL/g (O'Malley & Weaver, 1972) and $[\eta]_{\text{polypeptide}}$ should equal 44.7 mL/g. For ovomucoid, $[\eta]_{\text{literature}}$ equals 16.0 mL/g (Ahmad & Salahuddin, 1975) and $[\eta]_{\text{polypeptide}}$ should equal 22.4 mL/g. Certainly, more recent viscosity measurements on disulfide cross-linked ovomucoid in 6 M GdmCl (Baig & Salahuddin, 1978) suggest that the preparation upon which the earlier intrinsic viscosity was determined contained appreciable quantities of lysozyme; this indeed would have yielded too low an estimate for the intrinsic viscosity. The intrinsic viscosity estimated by Baig & Salahuddin (1978) for disulfide cross-linked ovomucoid, $[\eta]$ = 9.4 mL/g, yields an R_e of 34.3 Å; this value is identical with our estimate by gel chromatography (see Materials and Methods).

Gel Electrophoresis. Because electrophoresis in the presence of concentrated urea is frequently employed for the analysis of protein mixtures containing glycoproteins (Panyim & Chalkley, 1969; Senior & Mac Lennan, 1970; Hillyard et al., 1972) and because the electrophoretic behavior of a polyelectrolyte depends upon its charge as well as its size, it was of interest to examine the electrophoretic behaviors of some of the glycopolypeptides employed in this investigation.

This examination was accomplished by making comparisons with the electrophoretic behaviors of a limited number of common polypeptides of isoelectric or isoionic points reported to be between 4.7 and 6.5. The results are illustrated in Figure 2. As demonstrated previously, the electrophoretic mobilities of polypeptides of similar ionic properties approximate a linear function of the logarithms of their molecular weights (Poole et al., 1974). Quite obviously, the glycopolypeptides neither obey this relationship nor exhibit adherence to any consistent pattern of behavior. This may be explained by the widely disparate ionic properties among the glycopolypeptides. Ovalbumin (pI = 4.7; Conway-Jacobs & Lewin, 1971) and

Table I: Glycopolypeptides Subjected to Gel Chromatography in 6 M Guanidinium Chloride

no.[a]	glycopolypeptide	mol wt[b]	carbohydrate[c] (%)	exptl R_e (Å)[d]	lit. R_e (Å)[e]
1	thyrotropic hormone, $\alpha + \beta$ chains	14 100	16.3	35.0	
2	human chorionic gonadotropin, α chain	14 900	32	35.6	
3	glyco alpha-lactalbumin	15 700	9	35.4	
4	human chorionic gonadotropin, β-chain	23 000	30	46.0	
5	ovomucoid	27 000	23	49.0	41[f]
6	neuraminidase-treated α_1-acid glycoprotein	33 700	37	51.6	
7	α_1-acid glycoprotein	36 000	41	54.2	
8	horseradish peroxidase	42 000	16.5	60.0	
9	neuraminidase-treated fetuin	43 000	21	62.0	
10	ovalbumin	43 500	3	62.0	62[g]
11	fetuin	44 000	23	63.5	
12	immunoglobulin G, heavy chain	50 000	6	69.0	
13	cellobiohydrolase C	57 000	13	71.5	
14	glucose oxidase	72 000	16.5	87.0	70[h]
15	Tamm-Horsfall urinary glycoprotein	76 000	28	85.0	86[i]
16	transferrin	81 400	5	85.0	87[j]

[a] The glycopolypeptides are listed in order of increasing molecular weight. The number designated for a glycopolypeptide is employed in Figure 1 and may be used for means of identification. [b] We have determined or verified these values by sedimentation equilibrium except for human chorionic gonadotropin and thyrotropic hormone. [c] The most commonly accepted value from the literature. [d] Experimentally estimated from a standard curve which was generated with the usual polypeptide standards (Fish, 1975). [e] Calculated according to eq 4 of Reynolds & Tanford (1970) by using intrinsic viscosities reported in the literature for the reduced glycopolypeptide and the appropriate molecular weight from this table. [f] $[\eta]$ = 16.0 mL/g (Ahmad & Salahuddin, 1975). [g] $[\eta]$ = 34.6 mL/g (Castellino & Barker, 1968). [h] $[\eta]$ = 30 mL/g (O'Malley & Weaver, 1972). [i] $[\eta]$ = 52.9 mL/g (Stevenson & Kent, 1970). [j] $[\eta]$ = 50.8 mL/g (Mann et al., 1970).

IgG heavy chain (pI = 5.8–7.3; Phelps & Putnam, 1960) possess ionic properties similar to those of the reference polypeptides. On the other hand, because of the appreciable *N*-acetylneuraminic acid content of each, α_1-acid glycoprotein (pI = 2.7; Schmid, 1953), fetuin (pI = 3.3; Spiro, 1960), and ovomucoid (pI = 4.3; Bier et al., 1953) would be expected to possess distinctly different electrostatic charges at pH 3.3, the pH at which the electrophoresis was performed.

Discussion

This investigation yields rather unexpected results, i.e., that the hydrodynamic volumes of many reduced glycopolypeptides in 6 M GdmCl are equivalent to the hydrodynamic volumes of linear polypeptides of corresponding molecular weight. The reason that these results are somewhat surprising is that statistical calculations for various forms of branching by a homopolymer predict that the dimensions of a given molecular weight polymer will decrease with increased branching (Zimm & Stockmayer, 1949). Thus, it appears that in the case of glycopolypeptide heteropolymers in 6 M GdmCl this anticipated diminution as a result of branching is fortuitously compensated for by an expansive effect produced by the greater bond lengths and/or polymer expansion factors of the oligosaccharide chains. The values of these polymer parameters have not been determined for any types of carbohydrate polymers in concentrated urea or GdmCl solutions and would be necessary before attempting a quantitative description of the hydrodynamic effects of oligosaccharide branches extending from a randomly coiled linear polypeptide. Accordingly then, it appears that attempts at explaining our empirical observations made with reference to the polymer dimensions of glycopolypeptides in 6 M GdmCl must await examination of the hydrodynamic behavior of model polysaccharides in concentrated GdmCl.

Lest it be interpreted that gel chromatography in concentrated GdmCl is offered as a panacea for the estimation of glycopolypeptide molecular weights, Table II presents repre-

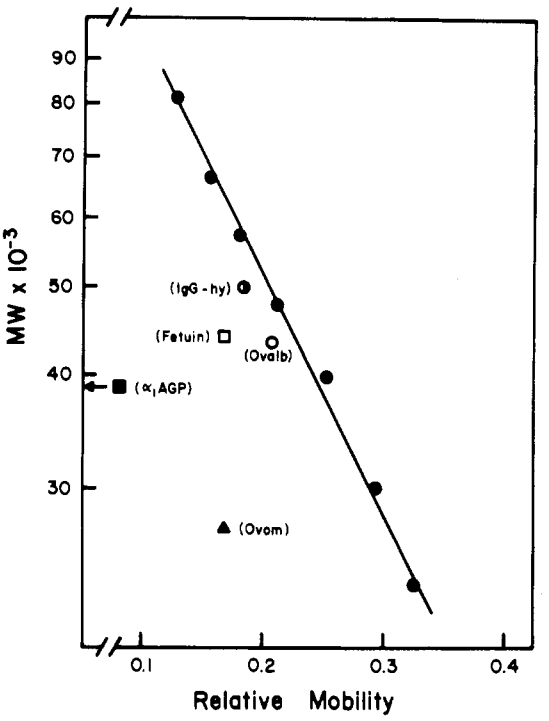

FIGURE 2: Electrophoretic behavior of glycopolypeptides in 8 M urea, pH 3.3. The relative electrophoretic mobilites on 10% polyacrylamide gels for a number of common polypeptide standards (●), immunoglobulin G heavy chain (IgG-hy) (◑), fetuin (□), ovalbumin (ovalb) (○), α_1-acid glycoprotein (α_1-AGP) (■), and ovomucoid (Ovom) (▲).

Table II: Errors in Molecular Weight Estimates of Glycopolypeptides by Gel Chromatography in 6 M Guanidinium Chloride

glycopolypeptide	carbohydrate (%)	"true" mol wt[a]	error in gel chromatography estimate	ref
glycophorin	55	29 000	> +100%; it aggregates	Grefrath & Reynolds, 1974
ceruloplasmin	7.5	134 000	−13%	Rydén, 1972
taipoxin γ chain	15	21 700	−14%	Fohlman et al., 1976
myrosinase, mustard	18	75 500	−12.5%	Björkman & Janson, 1972
AMV gp85[b]	45[c]	80 000[c]	−12.5%	Fleissner, 1971

[a] Determined by sequence analysis or by sedimentation equilibrium measurements. [b] Avain myeloblastosis virus glycoprotein-85. [c] R. Green, personal communication.

sentative examples from the literature in which rather pronounced disparaties occur. Two types of deviations are observed. The behavior of glycophorin illustrates the failure to meet a conformational requirement that is not concerned with oligosaccharide branches but which must be met when empirical methods of this type are utilized; that is, the (glyco)protein must be susceptible to the denaturing system employed. This particular problem appears to be more common with integral membrane proteins and is a manifestation of the unique properties of the protein portion of the molecule. As illustrated by the hydrophilic glycopolypeptide examples when they occur, deviations from the "true" molecular weight are always negative. The sense of these deviations is consistent with polymer theory (Zimm & Stockmayer, 1949), but the sporadity of their occurrence presently cannot be explained.

In conclusion, it appears that gel chromatography in 6 M GdmCl is the more reliable empirical method to employ as a first approximation of the molecular weight of glycopolypeptides. It must be emphasized, however, that a molecular weight estimated by this procedure should serve only as an approximation which is subordinate to a rigorous method such as sedimentation equilibrium.

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A Unique, Pepsin-Sensitive Collagen Synthesized by Aortic Endothelial Cells in Culture†

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ABSTRACT: A unique collagen, designated EC, has been isolated from the culture medium of adult bovine aortic endothelial cells. After diethylaminoethylcellulose chromatography of [^{3}H]proline-labeled culture medium, three non-disulfide-bonded bacterial collagenase-sensitive components with apparent M_r of 177 000 (EC 1), 125 000 (EC 2), and 100 000 (EC 3) were demonstrated. Molecular sieve chromatography, cyanogen bromide cleavage, and two-dimensional peptide mapping of radioiodinated EC fragments produced by protease digestion suggest that the lower molecular weight components originate from EC 1. Both EC 1 and EC 2 were digested by pepsin within 10 min to products of less than 60 000 molecular weight, under conditions which supported only limited proteolysis of other native collagens. A pepsin-resistant fragment of M_r 50 000, derived from a digest of EC 2, contained equal amounts of hydroxyproline and proline, suggesting that at least a portion of the endothelial collagen contains a stable, collagen-like triple helix. Comparative mapping using mast cell protease and cyanogen bromide cleavage, followed by polyacrylamide gel electrophoresis, indicates that the primary structure of this collagen differs from that of other known collagen types.

Endothelial cells line vascular channels and provide an effective permeability barrier between the blood and connective tissue stroma. These cells are disposed as a contact-inhibited monolayer which adheres to the subendothelial matrix. Vascular endothelial cells from several species have been established in culture, and these cells maintain the typical morphology of endothelial cells in vivo [Lewis et al., 1963; Jaffe et al., 1973b; Booyse et al., 1975; for a review, see Gimbrone (1976)]. Synthesis and/or specific binding of several biologically important compounds by endothelial cells, including plasminogen activator (Loskutoff & Edgington, 1977), factor VIII antigen (Jaffe et al., 1973a), prostacyclin (Weksler et al., 1977), angiotensin-converting enzyme (Hial et al., 1979), low-density lipoprotein (Stein & Stein, 1976), and glycosaminoglycans (Buonassissi & Root, 1975) have been studied in detail. Recent studies have shown that, in addition to the major biosynthetic product, fibronectin (Macarak et al., 1978; Birdwell et al., 1978; Jaffe & Mosher, 1978), the principal collagenous component secreted into the culture medium by bovine aortic endothelial cells is type III procollagen (Sage et al., 1979a). Other collagen types including type V appear to be restricted to the cell layer (Sage et al., 1979a; Sage et al., 1981a). However, different results have been obtained by other workers studying both bovine aortic endothelial cells (Howard et al., 1976) and cells from other species (Jaffe et al., 1976; Barnes et al., 1978; Kay et al., 1979).

Conflicting reports of collagen biosynthesis by endothelial cells should be resolved since questions regarding this process have important implications for several cellular functions, such as (a) platelet adhesion and production of a thrombogenic surface following endothelial injury, (b) synthesis of basal lamina, (c) attachment to and elaboration of an extracellular matrix, (d) polarity of secretion, and (e) apparent modulation of protein synthesis in vitro, including collagen type switching (Cotta-Pereira et al., 1980). In this study we present evidence for the synthesis and secretion of a unique collagen type by cultures of bovine aortic endothelial cells.

Materials and Methods

Cell Culture and Metabolic Labeling. Adult bovine aortic endothelial cells were isolated, [^{3}H]thymidine selected, and subcultured according to procedures described by Schwartz (1978). Further subcultivation was performed as previously described (Sage et al., 1979a), except that in the later stages of this work the cells were maintained in DMEM[1] containing 16% VSP bovine serum (Biocell Laboratories). Cultures ranging from primary to fourteenth passage were labeled, but those used for preparative-scale procedures were between the seventh and twelfth passage. The effects of different sera including PDS, growth factors such as FGF, and [^{3}H]thymidine selection on collagen synthesis by these cells were tested as previously described (Sage et al., 1979a).

Cultures which had just reached confluence were labeled for 21–24 h by using 50 μCi/mL L-[2,3-^{3}H]proline (35 Ci/mmol; New England Nuclear) in serum-free DMEM containing 50 μg/mL sodium ascorbate and 80 μg/mL β-APN.

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[1] Abbreviations used: DMEM, Dulbecco–Vogt modified Eagle's medium; EC, endothelial collagen; VSP, viable serum protein; PDS, bovine serum which is deficient in the platelet-derived growth factor; FGF, fibroblast growth factor; β-APN, β-aminopropionitrile fumarate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; CM, carboxymethyl; DEAE, diethylaminoethyl.

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