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# Behavior of Glycopolypeptides with Empirical Molecular Weight Estimation Methods. 1. In Sodium Dodecyl Sulfate†

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ABSTRACT: The influence of the presence of oligosaccharide branches was examined with respect to the behavior of glycopolypeptides in empirical molecular weight estimation methods in the presence of sodium dodecyl sulfate ($NaDodSO_4$). This examination was conducted by comparing the gel chromatographic and gel electrophoretic behaviors in the presence of $NaDodSO_4$ of 13 glycopolypeptides of known chemical and physical properties to those of regular polypeptides. Errors in the gel chromatographic molecular weight for glycopolypeptides in $NaDodSO_4$ varied from −22% to +10% and indicated that the hydrodynamic behavior of the glycopolypeptide–$NaDodSO_4$ complex could not be correlated with the amount of carbohydrate in the glycopolypeptide. $NaDodSO_4$ binding measurements on a number of the glycopolypeptides suggest that the polypeptide moiety binds the nominal weight ratio of $NaDodSO_4$, while the carbohydrate portion exhibits little or no $NaDodSO_4$ binding. As has been reported by others, the polyacrylamide gel electrophoretic behavior of glycopolypeptide–$NaDodSO_4$ complexes yielded abnormally high molecular weight estimates. In general, the error of these estimates diminished with decreasing porosity of the gel; however, each glycopolypeptide behaved in a unique fashion. Treatment of the electrophoretic data by any of several empirical means provided no reliable way to correct for the glycopolypeptides' aberrant behavior.

Only recently have we recognized the ubiquitous distribution of glycoproteins throughout biological systems. As a first step in gaining an accurate understanding of the functional importance of the covalently attached carbohydrate chains, the glycoprotein must be accurately characterized chemically and physically. Physical characterization includes, of course, the most fundamental property, molecular weight. Empirical methods for polypeptide chain molecular weight estimation currently enjoy widespread use; however, for the results to be reliable, these empirical methods demand strict adherence to certain requirements (Fish, 1975). The primary of these requirements is that the effective hydrodynamic size of a polypeptide be a unique function of its chain length and, hence, its mass. Gel electrophoresis in the presence of sodium dodecyl sulfate[1] additionally requires that the electrostatic charge on the polypeptide–detergent complex must be proportional to the mass of the polypeptide. These conditions are generally met by *linear polypeptides* complexed in the normal fashion with $NaDodSO_4$ (Reynolds & Tanford, 1970a,b). However, by their very chemical nature, glycopolypeptides as branched-chain polymers cannot meet this primary conformational requirement. In spite of this fact, attempts to estimate the molecular weights of glycopolypeptides by making empirical corrections to $NaDodSO_4$–gel electrophoresis data are frequently made.

Quantitatively, it is not known just how much the structural and chemical incongruity of glycopolypeptides affects their behavior in $NaDodSO_4$ denaturing solvent systems for empirical molecular weight estimations. In an attempt to obtain information relative to this problem, we have compared the gel chromatographic and gel electrophoretic behaviors of a number of glycopolypeptides of known physical and chemical properties to those of regular polypeptides. The results of these comparisons are presented herein.

## Materials and Methods

*Glycopolypeptides.* $\alpha_1$-Acid glycoprotein was obtained from Miles Laboratories as human glycoprotein, fraction VI (lot II). The preparation exhibited a single electrophoretic zone

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; DNP, dinitrophenyl.

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in dilute buffer, pH 9, or in NaDodSO$_4$. Furthermore, the zone stained for both protein and carbohydrate. That this preparation was truly $\alpha_1$-acid glycoprotein was verified by immunodiffusion and immunoelectrophoresis against rabbit anti-human $\alpha_1$-acid glycoprotein and rabbit anti-human serum. Before the glycoprotein was employed in any experiments, it was necessary to remove large quantities of divalent cations by initial treatment first with a mixed-bed resin (Bio-Rad AG 501-X8) and then with a cation-exchange resin (Bio-Rad AG 50W-X2). The molecular weight used for $\alpha_1$-acid glycoprotein was 36 000. This value was based on the amino acid sequence of the protein portion (Schmid et al., 1973) together with the oligosaccharide compositions and sequences (Fournet et al., 1978). These data yield a carbohydrate content for the protein of 41%. Sedimentation equilibrium measurements on the preparation we utilized yielded a value of $M(1 - \bar{v}\rho)$ for the protein, identical with that reported by Kawahara et al. (1973). An $E_{278}^{mg/mL}$ of 0.893 was employed for concentration estimations of $\alpha_1$-acid glycoprotein (Schmid, 1953).

Human Tamm–Horsfall urinary glycoprotein was isolated from the urine of a single donor according to the procedure of Fletcher (1972). The preparation exhibited the same amino acid composition and associating properties in dilute buffer as those previously reported for the protein (Fletcher, 1972). A molecular weight of 76 000 ± 4000 was used for the glycopolypeptide (Hamlin & Fish, 1977). A carbohydrate content of 28% was used for the glycoprotein (Fletcher et al., 1970), and an $E_{278}^{mg/mL}$ of 1.08 was employed for concentration estimations (Finnigan et al., 1971).

Horseradish peroxidase (Sigma type VI, lot 44C-9570) was used without further purification. It exhibited only a single zone by gel chromatography or gel electrophoresis under dissociating conditions. A partial specific volume of 0.716 mL/g was estimated for the protein from the chemical composition of its isozymes; the relative amounts of the various constituents were weighted according to the percent of activity contributed by each isozyme (Shannon et al., 1966). The native enzyme exhibited a time-dependent aggregation by sedimentation equilibrium analysis. Our estimate for its monomeric molecular weight from the data of four sedimentation equilibrium runs was 42 000 ± 2000. This is in reasonable agreement with a minimal molecular weight of 40 000 estimated from quantitation of the heme content of the enzyme (Keilin & Hartree, 1951) and estimates nearer 44 000 from sedimentation velocity and diffusion measurements (Maehly, 1955; Cecil & Ogston, 1951; Theorell, 1942; Shannon et al., 1966). A carbohydrate content of 16.5% (Shannon et al., 1966) and an $E_{277}^{mg/mL}$ of 0.3 were employed for the glycoprotein. These values are weighted averages based on the proportions of the various isozymes (Shannon et al., 1966).

Glyco-$\alpha$-lactalbumin was isolated from a commercial preparation of bovine $\alpha$-lactalbumin (Miles laboratories). The chemical and biological properties of this preparation[2] were similar to those previously reported (Barman, 1970). The molecular weight we estimated by sedimentation equilibrium for the native or desialized protein was 15 700 ± 500. The $\bar{v}$ of 0.70 mL/g, which was used for the molecular weight estimation, was obtained by making a weighted combination of the measured $\bar{v}$ for $\alpha$-lactalbumin, 0.704 mL/g (Lee & Timasheff, 1974), and the volume contribution calculated for the carbohydrate in the usual manner. The amount of carbohydrate in the glycoprotein, 9%, was estimated from the chemical composition reported for glyco-$\alpha$-lactalbumin and $\alpha$-lactalbumin (Barman, 1970; Brew et al., 1970).

The physical and chemical properties used for ovalbumin were those reported by Castellino & Barker (1968) and by Spiro (1973). The properties assumed for transferrin were those reported by MacGillivray et al. (1977). Physical and chemical properties employed for immunoglobulin G heavy chain were taken from Björk & Tanford (1971) and from Spiro (1973).

Sources, physical properties, and chemical properties for the other glycopolypeptides employed in this investigation are described in detail in the following paper (Leach et al., 1980).

*Gel Chromatography in NaDodSO$_4$.* Bio-Gel P-200 was equilibrated with 0.1% NaDodSO$_4$ and 0.02% sodium azide in sodium phosphate buffer of $I$ = 0.1, pH 7.2. This gel chromatography medium was employed in a 1.6 × 100 cm Pharmacia column. Chromatographic support media which are carbohydrate polymers work equally well in NaDodSO$_4$ for polypeptides (Tanford et al., 1974; Nozaki et al., 1976) but were not employed for these experiments in order to minimize the potential for glycopolypeptide–gel matrix interactions. A height differential of 25 cm was used between the reservoir (Mariott Flask) and the tip of the column delivery tubing; this produced a flow rate of 1.25–1.5 h$^{-1}$ cm$^{-2}$. Fractions of 0.8–1 g were collected. The elution positions of polypeptides and glycopolypeptides were determined by weight and were monitored by absorbance at 280 nm. Blue Dextran was employed as the column void volume marker and DNP-glycine was employed as the column internal volume marker. The elution positions of the glycopolypeptides were also verified by monitoring individual fractions for carbohydrate with the phenol–sulfuric acid assay (Dubois et al., 1956). The gel chromatography column was standardized in the usual manner with 14 polypeptide standards which ranged in molecular weight from 15 000 to 80 000 (Fish, 1975).

Before application of the (glyco)polypeptide–NaDodSO$_4$ complex to the gel chromatography column, samples were prepared as follows. Proteins or glycoproteins were dissolved in column buffer to a concentration of about 5–7 mg/mL for each (glyco)protein. An amount of NaDodSO$_4$ which equaled about 3–5 times the total weight of protein and sufficient 2-mercaptoethanol to be 0.1–0.2 M was added to the protein solution. The pH of the solution was adjusted to 8 with 1 N NaOH. The solution was then heated in a boiling water bath to 90+ °C for 10 min. Small amounts of Blue Dextran and DNP-glycine and sucrose to about 5% were added immediately before application to the column. A sample volume of between 0.2 and 0.5 mL was applied to the column.

*NaDodSO$_4$–Gel Electrophoresis.* The method of sample preparation and the NaDodSO$_4$ electrophoresis procedure essentially followed those procedures outlined by Weber & Osborn (1969) and Weber et al. (1972). Identical treatment of all samples was found to be imperative; for example, the NaDodSO$_4$ electrophoretic mobility of a protein–NaDodSO$_4$ complex prepared in a great excess of NaDodSO$_4$ (10–20% NaDodSO$_4$) was different from that of the same protein–NaDodSO$_4$ complex after gel chromatography, hence equilibration, in 0.1% NaDodSO$_4$. These mobility differences apparently depend upon the amount of NaDodSO$_4$ present in excess of the (glyco)polypeptide–NaDodSO$_4$ complex and the ionic composition of the buffer. The NaDodSO$_4$ gels possessed a 37:1 ratio of acrylamide to methylenebis(acrylamide) and were polymerized in the presence of sodium phosphate buffer, pH 7.2, $I$ = 0.2, and 0.2% in NaDodSO$_4$. The tray buffer was sodium phosphate, pH 7.2, $I$ = 0.4, and 0.1% in NaDodSO$_4$. A limited number of corroborative runs were performed on

[2] K. Olson and W. Fish, unpublished results.

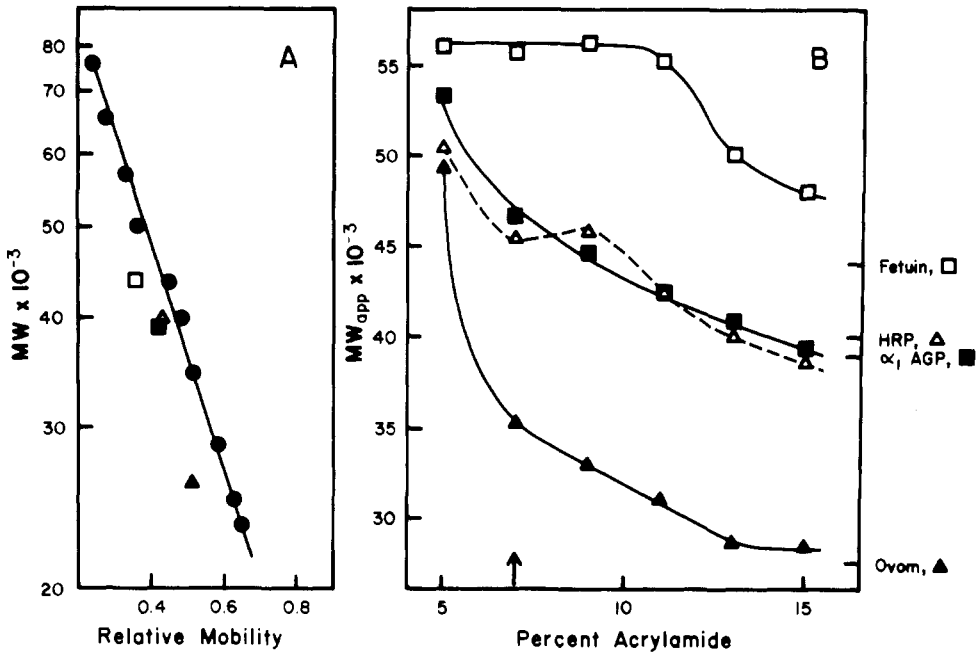


FIGURE 1: Electrophoretic behavior of glycopolypeptides in $NaDodSO_4$. (A) Electrophoretic mobilities on 7% acrylamide gels for fetuin (□), horseradish peroxidase (△), $\alpha_1$-acid glycoprotein (■), and ovomucoid (▲) relative to a series of polypeptide standards (●). Data are plotted as log $M_r$ vs. relative mobility. (B) Electrophoretic mobilities as a function of acrylamide percentage for fetuin (□), horseradish peroxidase (HRP) (△), $\alpha_1$-acid glycoprotein ($\alpha_1$-AGP) (■), and ovomucoid (Ovom) (▲). Data are plotted according to Segrest et al. (1971). The arrow indicates the data taken from part A.

a discontinuous thin-slab gel system (Laemmli, 1970).

Standard polypeptides were detected in the gels by fixation and staining with Coomassie Blue (Weber & Osborn, 1969). Glycopolypeptides were routinely detected in the same manner, but the presence of each was also verified at least once by scanning the unstained gel at 280 nm and by staining for carbohydrate.

The commonly employed methods for fixation of glycopolypeptides in $NaDodSO_4$ gels (Fairbanks et al., 1971; Glossmann & Neville, 1971; Zacharius et al., 1969) failed to fix some of the glycopolypeptides in adequate quantities to be detected by the basic fuschin stain for carbohydrate which is normally employed for acrylamide gels (McGuckin & McKenzie, 1958). We subsequently developed the following procedure for glycopolypeptide fixation and staining. After removal from the tubes, gels were allowed to stand in test tubes in 5% phosphotungstic acid in 2 N HCl at room temperature for 1.5 h. Excess $NaDodSO_4$ was removed by placing the gels in perforated polyethylene tubes and dialyzing for 2 h vs. two changes of 500 mL of 7% methanol–14% acetic acid. Oxidation was then carried out on ice and in the dark with 1% periodic acid in 7% trichloroacetic acid for 1 h. Excess periodic acid was reduced by washing the gels at room temperature in 0.5% sodium metabisulfite in 0.1 N HCl for 1 h. Immediately upon their exposure to the metabisulfite, $I_2$ is formed and the gels develop an amber color. Reduction of iodine to iodide as evidenced by the disappearance of the amber color is generally complete by the end of 1 h. Incubation in the commonly employed Schiff stain (McGuckin & McKenzie, 1958) was carried out on ice in the dark. Color development was gradual but stable for several days. The minimum amount of detectable carbohydrate for a nominal glycopolypeptide by this procedure was 3–4 $\mu$g.

Apparent glycopolypeptide molecular weights were estimated from the comparison of their mobilities to those of polypeptide standards in the usual fashion (Weber & Osborn, 1969). Apparent glycopolypeptide molecular weight estimations were made on 5, 7, 9, 11, 13, and 15% acrylamide gels. For each acrylamide percentage, all samples were run simultaneously. Data from these runs were also plotted according to the empirical plot of Ferguson (Ferguson, 1964) or the empirical plot of Segrest et al. (1971).

*$NaDodSO_4$ Binding.* The quantity of $NaDodSO_4$ bound to selected polypeptides and glycopolypeptides was estimated by the procedure of Reynolds & Tanford (1970a) or of Hayashi (1975) on fractions eluted from the P-200–$NaDodSO_4$–gel chromatography column. In each case before binding measurements were performed, it was experimentally determined that the elution position of the (glyco)polypeptide did not overlap with that of $NaDodSO_4$ (as micelles) which was applied to the column with the (glyco)polypeptide and which represented an excess over the $NaDodSO_4$ level in the column eluant.

Protein concentrations were estimated by the UV absorbance of each (glyco)polypeptide by using the wavelength of its maximum absorbance near 280 nm and the extinction coefficient of its native form at that wavelength. Difference spectral measurements suggest that this approach introduces an error in the estimate of the protein concentration of less than 10%.

## Results

*Gel Electrophoresis.* When subjected to $NaDodSO_4$–gel electrophoresis, glycopolypeptides routinely migrate slower than polypeptides of the same molecular weight (Figure 1A; Glossman & Neville, 1971; Schubert, 1970; Segrest et al., 1971). As illustrated in Figure 1B and as has been observed by others (Segrest et al., 1971), the disparity between the actual molecular weight of a glycopolypeptide and its apparent molecular weight estimated by $NaDodSO_4$–gel electrophoresis diminishes as the percentage acrylamide in the gels is increased; however, these data also suggest that each glycopolypeptide behaves differently and that no uniform correction can be applied.

Figure 2 illustrates the effect that removal of sialic acid residues has on the electrophoretic behavior of glycopoly-

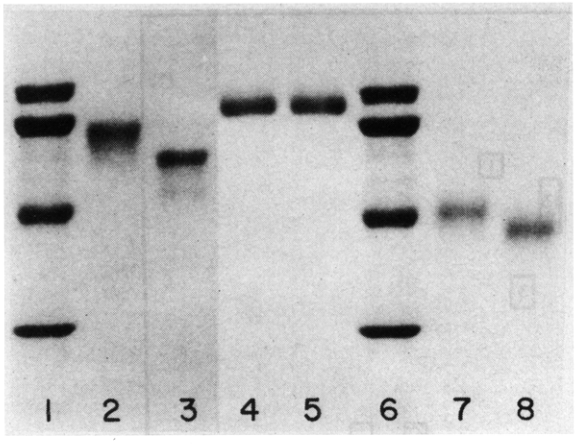


FIGURE 2: The effect of *N*-acetylneuraminic acid removal on the NaDodSO$_4$–gel electrophoretic mobility of glycopolypeptides. Samples were run on a 7.5%–10%–12.5% step-gradient polyacrylamide gel which was equilibrated in 0.1% NaDodSO$_4$ (Laemmli, 1970). Sample 1, reference (glyco)polypeptides (from the top, i.e., cathode end) transferrin, bovine serum albumin, ovalbumin, and chymotrypsinogen A; sample 2, fetuin; sample 3, neuraminidase-treated fetuin; sample 4, glucose oxidase; sample 5, neuraminidase-treated glucose oxidase; sample 6, reference (glyco)polypeptides (see sample 1); sample 7, $\alpha_1$-acid glycoprotein; sample 8, neuraminidase-treated $\alpha_1$-acid glycoprotein.

peptides in NaDodSO$_4$. Removal of half of the sialic acid residues from $\alpha_1$-acid glycoprotein or fetuin by neuraminidase treatment elicited a slight increase in their electrophoretic mobilities. Glucose oxidase, which possesses no sialic acid (Swoboda & Massey, 1965), exhibited no change in its electrophoretic mobility after neuraminidase treatment.

As shown in Figure 3, these electrophoretic data were also treated according to a Ferguson plot (Ferguson, 1964). This type of data treatment presumably separates the relative electrophoretic mobility of a solute into a function of its free electrophoretic mobility and a function of the sieving effect exerted on the solute by the gel matrix (Neville, 1971). As demonstrated by the representative data in Figure 3 for ten common polypeptides and as has been previously reported (Neville, 1971; Banker & Cotman, 1972), the limiting relative mobility (as defined by Shirahama et al., 1974) is constant for the usual polypeptide–NaDodSO$_4$ complexes. On the other hand, the six glycopolypeptides we examined by this procedure exhibited anomalous behavior in both the slope and intercept parameters of a Ferguson plot (four are illustrated in Figure 3).

*Gel Chromatography.* Gel chromatography was employed to examine the effect of the oligosaccharide side chains on the hydrodynamic behavior of glycopolypeptide–NaDodSO$_4$ complexes. To accomplish this, the gel chromatography column was first calibrated with reduced polypeptides of known chain length which had been complexed with NaDodSO$_4$ in the usual stoichiometry (Reynolds & Tanford, 1970b). In our lab, the deviation of the apparent molecular weights of the calibrating polypeptides about the standard curve is less than ±3%. The gel chromatographic behaviors of the glycopolypeptides are summarized in Figure 4 in terms of the percentage deviation between the apparent and the actual molecular weights of each glycopolypeptide vs. its carbohydrate content. The dimensions of the rectangles in Figure 4 represent the uncertainties in the glycopolypeptides' actual molecular weights and carbohydrate contents. The precision of the measurements of the gel chromatographic partition coefficients, which is about ±1%, is well within these uncertainties. It is obvious from this figure that no correlation can be drawn between the hydrodynamic behavior of the glycopolypeptide–NaDodSO$_4$ complexes and the amount of glycopolypeptide-associated carbohydrate.

*NaDodSO$_4$ Binding.* The binding of NaDodSO$_4$ by a number of the glycopolypeptides was performed after their elution from the gel chromatography column. The results of these measurements are summarized in Table I. BSA,[1] whose binding properties with NaDodSO$_4$ are well documented under similar solution conditions (Reynolds & Tanford, 1970a; Allen, 1974), was included as a polypeptide control, and a commercial preparation of glycogen was included as a polysaccharide control. The same binding ratios were obtained whether the reduced (glyco)polypeptides were applied to the column in an

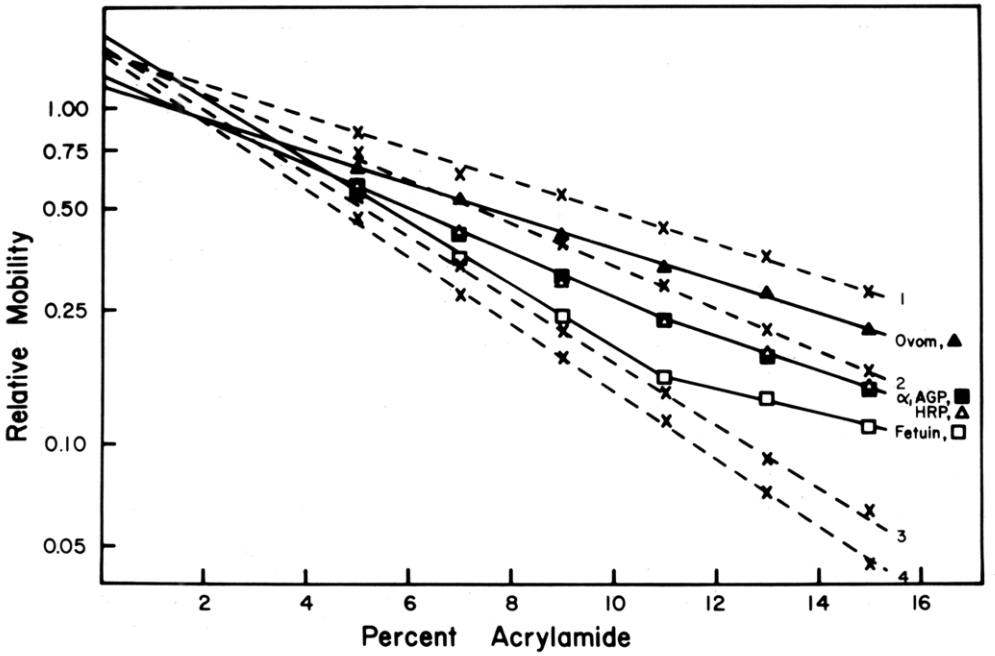


FIGURE 3: Ferguson (1964) plot of NaDodSO$_4$–gel electrophoretic data for glycopolypeptides and polypeptide standards. (×) Representative data from a total of ten polypeptides. The data for all ten were linear and all extrapolated to the same intercept. Curve 1, IgG light chain; curve 2, aldolase; curve 3, catalase; curve 4, bovine serum albumin. (—) Data for four glycopolypeptides. (▲) Ovomucoid (Ovom), (■) $\alpha_1$-acid glycoprotein ($\alpha_1$-AGP), (△) horseradish peroxidase (HRP), and (□) fetuin.

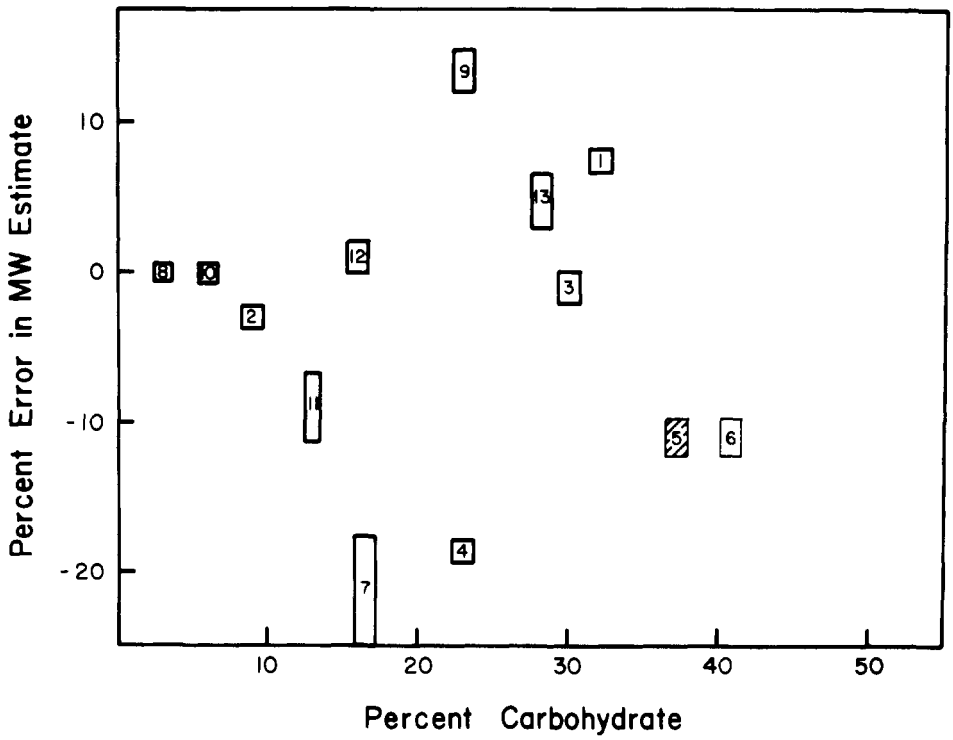


FIGURE 4: Gel chromatographic behavior of reduced glycopolypeptides in $NaDodSO_4$. The size of each rectangle approximates the confidence limits of our experimental estimates and literature values for the molecular weight and carbohydrate content of the glycopolypeptide. No differentiation was made among the glycopolypeptides with respect to numbers, lengths, locations, and extent of branching of the oligosaccharide side chains. The glycopolypeptides are numbered according to increasing molecular weight: (1) $\alpha$ chain of human chorionic gonadotropin, (2) glyco-$\alpha$-lactalbumin, (3) $\beta$ chain of human chorionic gonadotropin, (4) ovomucoid, (5) neuraminidase-treated $\alpha_1$-acid glycoprotein, (6) $\alpha_1$-acid glycoprotein, (7) horseradish peroxidase, (8) ovalbumin, (9) fetuin, (10) immunoglobulin G heavy chain, (11) cellobiohydrolase C, (12) glucose oxidase, and (13) Tamm–Horsfall glycoprotein.

Table I: $NaDodSO_4$ Binding by Glycopolypeptides[a]

| molecule | CHO (%) | g of $NaDodSO_4$ per g of molecule[b] | g of $NaDodSO_4$ per g of polypeptide |
|---|---|---|---|
| BSA | 0 | 1.45 ± 0.15 (15)[c] | 1.45 ± 0.15 (15) |
| glycogen | 100 | <0.03[d] | |
| $\alpha_1$-acid glycoprotein | 41 | 0.70 | 1.28 |
| Tamm–Horsfall urinary glycoprotein | 28 | 0.99 | 1.37 |
| ovomucoid | 23 | 1.10 | 1.43 |
| fetuin | 23 | 1.23 | 1.60 |
| cellobiohydrolase C | 13 | 1.28 | 1.46 |

[a] Reduced and equilibrated in 0.1% $NaDodSO_4$ in phosphate buffer of $I$ = 0.1, pH 7.2. [b] The concentration of each (glyco)-polypeptide in $NaDodSO_4$ was estimated from its UV absorbance and the extinction coefficient of the native protein. [c] These are the mean and standard deviation of 15 separate experiments carried out on BSA by three different experimenters during the course of this investigation. [d] The concentration of glycogen in $NaDodSO_4$ was estimated by the method of Dubois et al. (1956) with glycogen which was gravimetrically prepared in the $NaDodSO_4$ solvent used as the standard.

excess of $NaDodSO_4$ or in a deficiency of $NaDodSO_4$. The $NaDodSO_4$ binding data for the 5 glycopolypeptides suggest that the polypeptide moiety binds the nominal ratio of $NaDodSO_4$ while the carbohydrate portion exhibits little or no $NaDodSO_4$ binding. Certainly glycogen, which was treated in a fashion identical to the polypeptide and glycopolypeptides, bound an almost undetectable amount of detergent. The $NaDodSO_4$ binding by peptides and glycopolypeptides whose hydrodynamic size was smaller than that of ovomucoid could not be measured by the methods we employed because the elution position of the micelle of excess (or depleted) $NaDodSO_4$ overlapped with the elution positions of these smaller (glyco)polypeptides.

## Discussion

The results of this investigation demonstrate that both deviant hydrodynamic characteristics and atypical charge properties contribute to the aberrant behavior which is commonly observed for glycopolypeptides subjected to $NaDodSO_4$–gel electrophoresis.

Expression of the gel chromatographic behaviors of the glycopolypeptide–$NaDodSO_4$ complexes in terms of equivalent hydrodynamic radii (Reynolds & Tanford, 1970b) and comparison of these values with the hydrodynamic radii predicted for the respective polypeptide moieties (eq 4.4 of Fish, 1975) indicate that in all cases the hydrodynamic volume swept out by the glycopolypeptide–$NaDodSO_4$ complex is greater than that anticipated for only the polypeptide portion complexed with $NaDodSO_4$. Beyond this expected observation, however, no general pattern of hydrodynamic behavior is observed for glycopolypeptides in $NaDodSO_4$ when compared to that of linear polypeptides. In fact, what is observed is an array of hydrodynamic behaviors which cannot be correlated to the size of the glycopolypeptide or its chemical composition (Figure 4). The hydrodynamic behavior of the $NaDodSO_4$ complexes of three (glyco)polypeptides of nearly the same mass illustrates this point: aldolase, no carbohydrate and $M_r$ = 40 000 (Kawahara & Tanford, 1966); $\alpha_1$-acid glycoprotein, 41% carbohydrate and $M_r$ = 36 000; and fetuin, 23% carbohydrate and $M_r$ = 44 000. Complexed with $NaDodSO_4$, reduced $\alpha_1$-acid glycoprotein exhibits an apparent $R_e$ of 48.5 Å while reduced fetuin exhibits an $R_e$ of 67 Å. Thus, the behavior of these two glycopolypeptides deviates in opposite directions when compared to the aldolase-$NaDodSO_4$ complex whose $R_e$ is esti-

mated at 57 Å. This also means that the hydrodynamic volume contribution per residue by the oligosaccharide side chains of fetuin must be over twice that for $\alpha_1$-acid glycoprotein (that, of course, assumes that the polypeptide backbone of each interacts with NaDodSO$_4$ in the usual fashion to produce the normal complex).

Since the gross conformation nominally adopted by polypeptides complexed with NaDodSO$_4$ has not been established and a rather diverse number of models have been proposed (Reynolds & Tanford, 1970b; Shirahama et al., 1974; Wright et al., 1975; Rowe & Steinhardt, 1976; Mattice et al., 1976), it is impossible to make any specific conformational interpretations from these gel chromatographic results. It appears, however, that the sizes, numbers, locations, and perhaps extent of branching of the oligosaccharide side chains are strikingly manifested by their influence on the hydrodynamic behavior of glycopolypeptide–NaDodSO$_4$ complexes.

The NaDodSO$_4$-binding results of Table I indicate that reduced detergent binding by glycopolypeptides is a second factor which contributes to their commonly observed aberrant electrophoretic behavior. As a first step in an attempt to quantitate the effect that abnormal NaDodSO$_4$ binding might exert on the NaDodSO$_4$–gel electrophoretic behavior of a (glyco)polypeptide, the charge properties of a complex are expressed in terms of a (glyco)polypeptide's intrinsic charge properties and its detergent binding properties. The average net charge, $\bar{Q}$, on a (glyco)polypeptide–NaDodSO$_4$ complex may be expressed as

$$\bar{Q} = \delta_D M Z_D / M_D + \bar{Z}_{pp}$$

where $\delta_D$ is the binding ratio in grams of NaDodSO$_4$ per gram of (glyco)polypeptide, $Z_D$ is the charge per NaDodSO$_4$ molecule, $M$ is the molecular weight of the (glyco)polypeptide, $M_D$ is the molecular weight of the NaDodSO$_4$, and $\bar{Z}_{pp}$ is the average net charge intrinsic to the (glyco)polypeptide. The assumption implicit in NaDodSO$_4$–gel electrophoresis is that the quantities of anions present at the nominally high detergent binding ratio will render insignificant any net charge intrinsic to the protein, and thus $\bar{Q}$ will be proportional to the size of the polypeptide, i.e.

$$\bar{Q} \simeq \delta_D M Z_D / M_D$$

Thus, this equation stresses that the ratio $\bar{Q}/M$ must remain constant among standards and unknowns as a premise for NaDodSO$_4$–gel electrophoretic molecular weight estimations. Clearly, a significant contribution to the overall charge of the complex by the intrinsic charge of the (glyco)polypeptide or an atypical binding ratio between detergent and (glyco)polypeptide will perturb $\bar{Q}/M$ to a value outside the ratio nominally exhibited by the common polypeptide standards. This is illustrated in Table II. The calculated values presented in this table assume there is no binding of counterions to the anionic portion of the detergent as it exists in the (glyco)polypeptide–NaDodSO$_4$ complex. There is no experimental evidence to confirm or refute this assumption, although there is evidence for counterion binding to surface charges on NaDodSO$_4$ micelles (Emerson & Holtzer, 1967; Tanford, 1973).

The potential effect on $\bar{Q}/M$ which results solely from the ionic properties of the polypeptide is exemplified by the data for histone H1 and ferridoxin. An abnormally rapid rate of migration has been observed for the ferridoxins in the conventional NaDodSO$_4$ system (Williams & Gratzer, 1971); this aberrant behavior could be eliminated by esterification of the ferridoxin's carboxyl groups. Conversely, the histones migrate on NaDodSO$_4$ gels slower than predicted from their molecular weights and the migration rates of the usual standard proteins (Panyim & Chalkley, 1971), and this is consistent with the abnormally low value calculated for $\bar{Q}/M$ of histone H1.

Table II: Charge to Mass Ratio of Various Proteins Complexed with NaDodSO$_4$

| proteins | $\delta_D$[a] | $Z_{PP}$[b] | $\bar{Q}$ | $\bar{Q}/M \times 10^3$ |
|---|---|---|---|---|
| average of 12 common hydrophilic proteins | 1.4 ± 0.1[c] | | | −4.7 ± 0.4 |
| histone H1 (lysine-rich)[d] | 1.4 | +56 | −102 | −2.2 |
| ferridoxin[e] | 1.4 | −11 | −30 | −6.6 |
| $\alpha_1$-acid glycoprotein[f] | 0.7 | −26 | −95 | −3.1 |
| fetuin[f] | 1.23 | −21 | −183 | −4.6 |
| Tamm-Horsfall urinary glycoprotein[f] | 0.99 | −12 | −261 | −3.6 |
| ovomucoid[f] | 1.10 | −4 | −105 | −4.0 |
| $p$-κ-casein[g] | 2.0 | +4 | −81 | −6.6 |
| glycophorin[h] | 0.52 | −26 | −78 | −2.7 |

[a] Grams of NaDodSO$_4$ bound per gram of (glyco)polypeptide. [b] Intrinsic charge on the (glyco)polypeptide at pH 7.2. These values are shown for the individual (glyco)polypeptides but were also employed in the calculation of $\bar{Q}$ for each of the 12 common hydrophilic proteins. [c] Binding ratios are principally from Reynolds & Tanford (1970a) and Pitt-Rivers & Impiombato (1968). [d] Amino acid composition data of Kinkade & Cole (1966); binding data of Reynolds & Tanford (1970a). [e] Amino acid sequence data of Tanaka et al. (1964). A NaDodSO$_4$ binding ratio of 1.4 is assumed. [f] NaDodSO$_4$ binding data from this investigation. [g] Chemical composition from the data of Mercier et al. (1973); NaDodSO$_4$ binding data that of Makino & Niki (1977). [h] Chemical composition from the data of Grefrath & Reynolds (1974); NaDodSO$_4$ binding data that of Mimms & Glasgow (1980) and J. A. Reynolds, personal communication.

The effect exerted on $\bar{Q}$ by the reduced NaDodSO$_4$ binding ratio of hydrophilic glycopolypeptides (Table I; Pitt-Rivers & Impiombato, 1968) is illustrated in Table II by the data for five glycopolypeptides examined in this study. It is observed that the amount of NaDodSO$_4$ bound to hydrophilic glycopolypeptides on a gram per gram basis is reduced by about the same percentage as the weight percentage of their constituent carbohydrate. This suggests that the polypeptide moiety binds the nominal ratio of NaDodSO$_4$ while the carbohydrate portion exhibits little or no NaDodSO$_4$ binding. This overall reduced NaDodSO$_4$ binding is probably the predominant factor, particularly in lower percentage acrylamide gels, which causes glycopolypeptides to migrate at a slower rate during NaDodSO$_4$–gel electrophoresis than standard polypeptides of the same total mass (Glossman & Neville, 1971; Schubert, 1970; Segrest et al., 1971; Figure 1A). The slight increase in electrophoretic mobility which is observed upon removal of sialic acid residues suggests that the retarding effects of the frictional drag offered by these residues is manifested to a greater extent than is the Coulombic driving force of their negative charge. Abnormal NaDodSO$_4$ binding is also the reason why glycopolypeptides exhibit atypical behavior upon treatment of NaDodSO$_4$ electrophoretic results in terms of a Ferguson plot (Banker & Cotman, 1972; Figure 3). As suggested by Figure 3, a deviative limiting relative mobility may be of aid in diagnosing charge/size anomalies of polypeptide–NaDodSO$_4$ complexes, but this approach must await further testing. Additionally, the utilization of "appropriate" glycopolypeptide standards for NaDodSO$_4$–gel electrophoresis or NaDodSO$_4$–gel chromatography appears to be unfeasible since the numbers and locations of oligosaccharide attachments to the polypeptide chain, as well as the lengths and extent of branching of the oligosaccharide chains themselves, vary from one glycoprotein to another, and these variables appear to affect the behavior of the glyco-

polypeptide–$NaDodSO_4$ complex (Figures 1B and 4).

An approach which has been proposed as a means to correct for the aberrant electrophoretic behavior of glycopolypeptides and to ultimately yield a more reliable molecular weight estimation has been offered by Segrest et al. (1971; Segrest & Jackson, 1972). They observed that the magnitude of the errors in the glycopolypeptide molecular weights estimated electrophoretically diminished as the percentage acrylamide in the gels was increased. They further observed that the estimated molecular weights made an asymptotic approach to some fixed value near the true molecular weight. At variance with this observation, we observe that the apparent molecular weight will not always approach the true value in an asymptotic fashion (Figure 1B). Though our results with a limited number of glycopolypeptides concur with those of Segrest et al. (1971) in that the errors in the molecular weights estimated by $NaDodSO_4$–gel electrophoresis are smaller with higher percentage acrylamide gels, molecular weights estimated in this fashion have little reason to be credible, reports to the contrary notwithstanding (Lambin, 1978).

The effect on $\bar{Q}/M$ produced by the increased amounts of $NaDodSO_4$ bound by hydrophobic (glyco)polypeptides is exemplified in Table II by the data for $p$-$\kappa$-casein (Makino & Niki, 1977). It would appear that the enhanced binding of $NaDodSO_4$ to hydrophobic domains of amphipathic proteins creates $NaDodSO_4$–gel electrophoretic anomalies whether the protein possesses carbohydrate or not (Robinson & Tanford, 1975; Makino & Niki, 1977).

Finally, the membrane protein glycophorin illustrates yet another potential problem inherent to empirical molecular weight estimation methods. The $R_e$ reported for the glycopolypeptide–$NaDodSO_4$ complex (Grefrath & Reynolds, 1974) is close to that predicted for a $NaDodSO_4$ complex of a polypeptide of equal mass, and thus, gel chromatography would fortuitously yield a molecular weight estimate of reasonable accuracy. On the other hand, the smaller amount of $NaDodSO_4$ bound by glycophorin (Mimms & Glasgow, 1980; J. A. Reynolds, personal communication) yields an abnormally low $\bar{Q}/M$ and suggests that gel electrophoretic estimates of its molecular weight would be high. In practice, electrophoresis in $NaDodSO_4$ yields molecular weight estimates for glycophorin which are about twice the true value (Segrest et al., 1971). However, recent studies indicate that at the glycophorin concentrations routinely employed for electrophoresis, the glycopolypeptide aggregates in $NaDodSO_4$, and this turns out to be the major contributing factor to glycophorin's anomalous electrophoretic behavior in $NaDodSO_4$ (Mimms & Glasgow, 1980).

In conclusion, the data obtained with a limited number of hydrophilic glycoproteins demonstrate that by the very nature of their chemical structure glycopolypeptides are not amenable to reliable empirical molecular weight estimations or corrections in $NaDodSO_4$.

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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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