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Methods for the Isolation, Purification and Analysis of Glycoproteins—a Brief Review*

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The glycoproteins, a very large group of conjugated proteins, which are widely distributed in nature, have recently become a focus of study in biochemical research ([1-30] refer to review articles on this subject). Glycoproteins are defined in this review as proteins that carry covalently-bound sugars. It is the convention to exclude the nucleoproteins from this group of macromolecules.[31] The sugars often found in glycoproteins are galactose, mannose, fucose, glucose, glucosamine, galactosamine and sialic acids. Bacteria and plants contain in addition meuramic acid and many others sugars.[32, 32a-f] The amino group of glucosamine and galactosamine is generally acetylated, whereas sialic acid possesses one or more acetyl and/or glycolyl residues. Sialic acid is usually terminally located and often linked to galactose**. The carboxylic group of sialic acid, whose average pK is 2.6,[33] is free and contributes greatly to the acidic nature of glycoproteins. The pK value of this acidic residue may vary greatly.[34] It should be realized that certain sugar-containing proteins are devoid of fucose (fetuin,[35] fibrinogen,[36]), while others are free of sialic acid (Gc-components[37]) and still others are lacking galactosamine (α_1-acid glycoprotein[38]). Hexuronic acids are generally not found in this class of proteins, unless the mucopolysaccharide-protein complexes are considered as a special subgroup of the glycoproteins. The polypeptide moiety is composed of the known amino acids of proteins. Further, the collagens contain, in addition, hydroxyproline and hydroxylysine.

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** It is not yet known whether the linkage to this penultimate sugar is of the α- or β-type.

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[15] T. C. LAURENT and B. BLOMBÄCK, *Acta Chem. Scand. 12* (1958) 1875.
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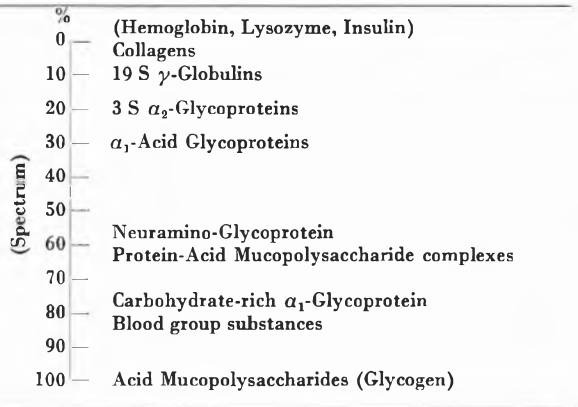
With regard to the percentage of the carbohydrate moiety of glycoproteins, there exists a very wide range indeed (Table 1). In fact the percentage of carbohydrate covers essentially the entire spectrum from 0 to 100%. For instance, insulin is devoid of sugars and glycogen is free of amino acids. The blood group substances and acid mucopolysaccharides possess polypeptide chains that account for only a small percentage of the total weight and should not be classified as glycoproteins. Thus, the question arises which of these macromolecules should be termed glycoproteins and which polysaccharides or mucocompounds.[38a] In order to qualify some of these macromolecules as glycoproteins, two further criteria ought to be applied.

(1) The typical properties of a protein must be retained and are not depressed or practically eliminated by the carbohydrate moiety.

(2) The carbohydrate moiety of glycoproteins does not contain uronic acids, except the protein "complexes" of acid mucopolysaccharides, as mentioned above, or sulfate. However, the polypeptide moiety may contain sulfate or phosphate esters in its tyrosyl residues, such as the fibrinogens.[39–41]

Table 1. Carbohydrate Content of Glycoproteins



Isolation

Progress in any field of research depends upon several factors, one of which is the development of new methods. The aim of this brief review is not to discuss all techniques employed for the isolation of and the methods utilized for the measurement of the constituents of glycoproteins, but to indicate some general guide lines as to how the problem of isolating and characterizing glycoproteins should be solved. Important handbooks and chapters on isolation and characterization of these macromolecules are listed as references.[1, 8, 42–70]

If the human plasma proteins are studied, almost all of which appear to be glycoproteins except, for instance, albumin, 3 S γ-globulins[71] and insulin, the starting material contains all proteins in the soluble state. However, if other tissues are investigated whose proteins are often in part in the insoluble state, the investigator must apply or invent conditions under which some or all proteins are rendered soluble.[42] Moreover, for the study of proteins it is a prerequisite to avoid conditions which might lead to denaturation of these macromolecules. Such conditions are heat, relatively extreme acidity or alkalinity, organic

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[39] B. Blombäck and A. Henschen, *Klin. Wschr. 41* (1963) 78.
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[71] S. Takahashi and K. Schmid, *Biochim. Biophys. Acta 63* (1962) 343.

solvents, light, or detergents, to mention the most important ones. The glycoproteins should be preserved in their natural state. They should not be altered in their primary and secondary structure or, in the extreme case, grossly denatured.

It should be pointed out that, in contrast to certain beliefs, the carbohydrate moiety (units) of the glycoproteins does not render these proteins stable or prevent their denaturation. Glycoproteins are not sturdier than any other proteins just because some of them are not rendered insoluble when their aqueous solutions are brought to a boil! Also the solubility of these macromolecules is not increased by the carbohydrate units as compared with non-conjugated proteins of comparable molecular weights and composition. It is true, however, that many glycoproteins are very soluble at neutral *p*H's. The reason for this is that they carry a relatively large number of sialic acid residues which provide a high electrostatic net charge at neutrality. It is also true that some of these proteins are soluble, even if they are grossly denatured. α_1-Acid glycoprotein denatured in a water-ethanol-ether mixture (1:1 v/v) remains soluble in this solvent.[72]

The problem of solubilizing certain extracellular proteins[42] may be illustrated by the following examples. To render collagens and keratins soluble, general rules derived from the study of the physical chemical properties of proteins are considered. The collagen of the skin of a growing normal animal is present in four different forms. Physiological NaCl solution will remove the most recently produced collagen. Slightly matured collagen can be brought in solution by increasing the ionic strenght ($\Gamma/2$) of the extracting solvent to about 0.5. (Globulins are rendered soluble by addition of salt, "salting-in"!) A further part of the still insoluble collagen can be solubilized by changing the *p*H from 7 to 3.5 effecting a great increase in the electrostatic net charge. It is noted that the change in *p*H is more effective than the increase of $\Gamma/2$ of the solvent. The remaining collagen is solubilized subsequently by its transformation to gelatin[73–76]. Keratin can be dissolved on either side of its isoelectric point but, because of its special properties, solution takes place only below *p*H 2.5 and above 12. As secondary reactions may occur at *p*H 12 the extraction is best carried out near *p*H 2.5.[77, 78]

A different problem is posed by bone and teeth.[79] Apatite must be removed first in order to make the matrix acessible for further study. Since the introduction of EDTA by SCHWARZENBACH, apatite can be dissolved to the largest extent at neutrality. Under such conditions the proteins are thought to be preserved in their natural state. Collagen is isolated subsequently as indicated above.

Cells and tissues require a different approach for the study of their constituents. Bacteria and yeast are known to have cell walls that are difficult to rupture. Besides various grinding techniques, treatment with ultrasonic vibrators and homogenizers, extrusion under high pressure appears to be most effective for this purpose.[42] Animal and plant tissues can be homogenized much easier than bacteria and yeast. For large-scale preparations it is best to use the pressure technique. In general, insoluble cell wall particles and the cell contents are obtained after separation by centrifugation.[42, 43] The cell wall fractions can be further degraded, chemically or enzymatically, to solubilize its constituents and make them accessible to further chemical studies. The cell contents consist of organelles (nuclei, mitochondria, microsomes, ribosomes, lysozomes and others) and the cytoplasmic soluble proteins. The use of density gradient in the separation of these particulates from each other proved to be particularly useful.

Fractionation

The fractionation serves to separate glycoproteins or groups of similar glycoproteins from each other. The fractionation or separation of proteins is based on the difference in one of their properties of which the most important ones are solubility, isoelectric point, electrostatic net charge, size, sedimentation coefficient, density and adsorption. The most frequently utilized fractionation techniques, classical or modern, are matched with these properties in Table 2. Two points of general interest are worth mentioning: (1) The most effective separation of

Table 2. Fractionation of Proteins

Difference in property on which procedure is based	Fractionation Procedure
Solubility	Salting-out (precipitation) Salting-in (extraction) COHN's Methods
Isoelectric point	Isoelectric precipitation Zone electrophoresis
Electrostatic net charge	Zone electrophoresis
Size	Gel filtration (Sephadex)
Sedimentation coefficient	Ultracentrifugation
Density	Flotation
Adsorption	Adsorption-Elution

[72] K. SCHMID, unpublished data.
[73] J. GROSS, in *Connective Tissue* (Intercellular Macromolecules), p. 63, Little Brown & Company, Boston 1964.
[74] J. GROSS, in *Comparative Biochemistry* (A Comprehensive Treatise) (M. FLORKIN and H. S. MASON, eds.), p. 307, Academic Press, New York 1963.
[75] P. M. GALLOP, in *Connective Tissue* (Intercellular Macromolecules), p. 79, Little Brown & Company, Boston 1964.
[76] W. GRASSMANN, K. HANNIG and J. ENGEL, *Z. physiol. Chem. 324* (1961) 284.
[77] A. G. MATOLTSY, *Nature 201* (1964) 1130.
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[79] M. J. GLIMCHER, in *Biophysical Science* (A Study Program) (J. L. ONCLEY, ed.), p. 359, John Wiley & Sons, New York 1959.

proteins from each other and, therefore, the most effective purification is obtained if a combination of procedures is employed that is based on different principles. For instance: the initial separation by fractional precipitation with $AmSO_4$ may be followed by ion exchange chromatography on DEAE-cellulose and then by gel filtration on Sephadex or by zone electrophoresis. (2) No method exists which permits the separation of glycoproteins from each other on the basis of differences in their carbohydrate content. Therefore, the preparative methods used in fractionating glycoproteins are those employed in protein chemistry in general.

Some of the classical procedures which were introduced fifty to sixty years ago are still in use. It is of great importance that only reagents may be added to a protein solution that can be removed subsequently without harming the protein. For instance, it has been known for many years that heavy metal ions form insoluble complexes with proteins, but because these metal ions could not be separated from the protein precipitate, one did not know whether or not the proteins had been denatured. Only through the relatively recent introduction of chelating agents, such as EDTA and ion exchange resins, has it become possible to remove such ions and to study further the precipitated proteins.

Fractional precipitation by high concentration of neutral salts such as $(NH_4)_2SO_4$, Na_2SO_4 and phosphates, referred to as "salting-out," is often used with significant success, particularly as an initial fractionation. Nevertheless, it should be mentioned that the final purification of myoglobin by recrystallization, for instance, was achieved in high ionic strength *p*H 6 phosphate.[80] The pitfalls of the "high salt" methods have recently been discussed by DIXON and WEBB.[44] Mention should be made that dialysis against water often utilized for the removal of salt occasionally leads to denaturation. Dialysis should be carried out against very dilute salt solutions, for instance against 0.001 M NaCl. Glycine appears to be another stabilizer for certain proteins.[81] Lyophilization may also lead to denaturation which may be prevented by addition of stabilizing agents such as dextran-10[81a] or must be replaced, if possible, by concentration by negative pressure dialysis.

A much better separation generally results if the fractionation, that is to say precipitation or extraction, is carried out at low $\Gamma/2$ in contrast to the relatively gross separation obtained by precipitation at high $\Gamma/2$. This was realized by COHN and coworkers and by KECKWICK whose procedures have been discussed in detail by EDSALL,[45] HUGHES,[46] ONCLEY[47] and PENNELL.[70] The solubility of a protein, like its electrostatic net charge, was found to be a function of *p*H, ionic strength, temperature, dielectric constant of the solvent, the presence of organic solvent and of specific ions. Since the possibility exists that in presence of ethanol, even at low temperature, certain proteins might be altered, COHN eliminated ethanol completely in Method 12[82] and replaced it by heavy metal ions such as those of $Zn(Ala)_2$ and $Hg(Ala)_2$. Ions of heavy metals form relatively insoluble complexes with proteins at concentrations between 10—20 mM, and often a surprisingly high specificity is observed. For instance, the low molecular weight, 3 S human plasma α_2-glycoproteins could be separated from each other with the aid of Ba^{++}-ions (in presence of 25% ethanol at *p*H 6 and $-5\,^{\circ}C$ to reduce the solubility).[83] At concentrations below 1 mM and above 30 mM these heavy metal ions effect salting-in of the protein.[81]

Instead of heavy metal ions, increasing concentrations of O-polyphosphate[84] or rivanol[85] have also been used successfully for the fractionation and isolation of proteins. Another group of procedures, very frequently employed in enzymology, is based on adsorption, especially on Al_2O_3, Ca-phosphates and $BaSO_4$.

Perchloric acid precipitation has been used widely for special purposes in the study of blood. The seromucoid fraction comprises the serum proteins that are soluble in perchloric acid and are precipitated with phosphotungstic acid.[50] The major component of this fraction is α_1-acid glycoprotein,[38] the minor components include haptoglobins, 3 S α_2-glycoproteins and many other proteins.[86] Because of the ease in preparing this protein fraction, the blood level of the seromucoid fraction has been determined in a very large number of disease states. It could be demonstrated that the changes in the blood concentration of this protein mixture are not of diagnostic value. For the sake of completeness, mention should be made that the term "glycoprotein" is occasionally used to refer to unknown serum proteins.

The modern methods are much more sophisticated. They are based to a large extent on the relatively detailed knowledge of proteins. Of the modern techniques, ion exchange chromatography on modified celluloses, which largely takes advantage of differences in the electrostatic net charge and was introduced by SOBER,[87] and gel filtration through Sephadex which takes advantage of differences in size of proteins and was introduced by PORATH and FLODIN,[88, 89] are probably the most important ones. The advantages of these well known procedures, such as high capacity, mild conditions, suitability

[80] J. KEILIN and K. SCHMID, *Nature 162* (1948) 496.
[81] J. PORATH and N. UI, private communication (1964).
[81a] *Brochure on Dextrans, Dextransulfates and DEAE-Dextrans for Laboratory Use*, Pharmacia Fine Chemicals, Inc., New York 1964.
[82] D. M. SURGENOR, R. B. PENNELL, E. L. ALAMERI, W. H. BATCHELOR, R. K. BROWN, M. J. HUNTER and V. L. MANNICK, *Vox Sang. 5* (1960) 272.
[83] K. SCHMID, *J. Amer. Chem. Soc. 77* (1955) 742.
[84] H. NITSCHMANN, E. RICKLI and P. KISTLER, *Vox Sang. 5* (1960) 231.
[85] E. W. BOETTCHER, P. KISTLER and H. NITSCHMANN, *Nature 181* (1958) 490.
[86] H. E. SCHULTZE, K. HEIDE and H. HAUPT, *Clin. Chim. Acta 7* (1962) 854.
[87] E. A. PETERSON and H. A. SOBER, in *Methods in Enzymology* (S. P. COLOWICK and N. O. KAPLAN, eds.), Vol. 5, p. 3, Academic Press, New York 1962.
[88] J. PORATH and P. FLODIN, *Nature 183* (1959) 1657.
[89] J. PORATH, *Advances Protein Chem. 17* (1962) 209.

for any scale, have been discussed extensively. Chromatography on Amberlite IRC-50 is often used with excellent results,[90] although the capacity of this resin to retain protein is far below that of DEAE-cellulose. Amberlite IRC-50 is particularly useful for the chromatography of relatively low molecular weight proteins (independent of their isoelectric points).[91, 92] By selecting the conditions of chromatography so that the protein to be purified is not retained and the proteinaceous impurities are adsorbed, the recovery may be as high as 95%.[90] Zone electrophoresis, a separation procedure which is also based on differences in electrostatic net charge of the proteins, is widely employed for preparative purposes in small and medium scale operations.[92] As supporting media paper powder, starch granules, agar and polyvinyl chloride, etc. are used. Precaution must be taken that the protein derived from starch or the amino acids from paper are not analyzed! Paper electropherograms used for analytical purposes may be stained for the polypeptide moiety, preferably with amidoblack 10B (because the color of this dye is independent of *p*H), for the carbohydrate moiety, namely its sialic acid residues, by the periodic acid-Schiff stain[93] and for lipids with oil red or Sudan black. Electrophoresis in gels, such as starch gel and cyanogum, are also utilized primarily for analytical purposes, although attemps to employ these procedures for preparative purposes have been described.[93a] The resolution of these methods is so high that genetically determined variants[93b, 93c] such as those of isoenzymes,[94] α_1-acid glycoproteins[94a] and haptoglobins[95], can be separated from each other. Of particular interest for analysis[96–98] is the micromodification of starch gel electrophoresis requiring approximately 0.02 mg of a protein mixture. Ultracentrifugation, although extensively employed, often yields a poor resolution. Normal human plasma which contains over 150 different proteins[99] resolves into only three peaks! However, removal of large particles such as ribosomes, viruses, macroglobulins, etc. is easily achieved by this technique. The use of cetyl-pyridinium bromide for precipitating high molecular weight mucoproteins and mucopolysaccharides offers particular advantages.

[90] K. SCHMID, M. B. MACNAIR and A. F. BÜRGI, *J. Biol. Chem. 230* (1958) 853

[91] C. H. W. HIRS, in *Methods in Enzymology* (S. P. COLOWICK and N. O. KAPLAN, eds.), Vol. 1, p. 113, Academic Press, New York 1955.

[92] P. FLODIN and J. PORATH, *Biochim. Biophys. Acta 13* (1954) 175.

[93] W. MACGUCKIN and B. F. MCKENZIE, *Clin. Chem. 4* (1958) 476.

[93a] K. MARRAY, *Anal. Biochem. 3* (1962) 415.

[93b] H. CLEVE and G. A. BEARN, *Progress in Medical Genetics 3* (1962) 64.

[93c] H. BAITSCH, H. RITTER, H. W. GOEDDE and K. ALTLAND, *Vox Sang. 8* (1963) 594.

[94] *Ann. N. Y. Acad. Sci. 94* (1961) 655.

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[95] O. SMITHIES, *Advances Protein Chem. 14* (1959) 65.

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[97] H. MOURAY, J. MORETTI and J. M. FINE, *Bull. Soc. Chim. Biol. 43* (1961) 993.

[98] L. KORNGOLD, *Anal. Biochem. 6* (1963) 47.

[99] H. E. SCHULTZE, in *Sitzungsbericht der Gesellschaft zur Förderung der gesamten Naturwissenschaften zu Marburg, 1963*, p. 1.

If the solution contains lipoproteins which are known to posses mannose, galactose, hexosamine, sialic acid and fucose,[35] its density is increased to such a value that all or a desired fraction of the lipoproteins are floated by ultracentrifugation. For the subsequent separation of the lipoproteins from each other, distribution in a density gradient is employed.[100–102]

With regard to fractionation of proteins, the following question should be answered. Considering the problem of separating proteins from each other, one certainly has to reflect upon the method which should be selected. Obviously, one cannot expect to try out all available procedures. After a clear formulation of the problem and some experience, one usually will have an idea as to which methods should be tried first. Based on the obtained results it is often easily possible to make appropriate corrections. The progress of purification is judged by the change of a property of the protein fractions isolated at different stages of separation, such as the increase of an enzymatic or hormonal activity, the decrease of the electrophoretic heterogeneity or the change in content of Fe or C- and N-terminal amino acids.

Criteria of Purity of a Protein Preparation

A protein preparation which is believed to be pure must be analyzed for homogeneity. Further, it must be established if the protein exists in its native form. Because of the complexity of the protein molecules, as many criteria of purity as possible must be applied.[45, 47] Based on chemical properties the protein preparation must appear homogeneous as judged by analyses for terminal amino acid and sugar residues. Based on physical chemical properties, the preparation must appear monodisperse as judged by ultracentrifugation and electrophosoresis and by chromatography within the *p*H range of the stability of the protein. It should be noted that these three techniques effect a relatively limited resolution power. If starch gel electrophoresis is used, impurities can easily be distinguished from variants of certain protein preparations. Immuno- and gel electrophoresis are probably much more sensitive than analysis by solubility for the detection of proteinaceous impurities. Purification must be carried on until, for instance, maximum possible biological activity is obtained. Crystallization, often difficult to achieve and of extreme importance for x-ray analysis, is not a criteria of purity.

The chief procedures by which it is possible to establish whether a protein is native or denatured are briefly discussed in the following. In general, denaturation is accompanied by aggregation of the protein molecules. Con-

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Table 3. Hydrolysis of Glycoproteins

Ranges of Reasonable Hydrolysis Conditions	
Sialic acids	0.025 – 0.2 N H_2SO_4, 80°, 1 hr
Fucose	0.1 – 1.0 N H_2SO_4, 100°, 30–60 min (4 hrs)
Galactose, Mannose	1 N H_2SO_4, 100°, 1–8 hrs
Hexosamines	2–4 N HCl, 100°, 4–16 hrs
Amino Acids	6 N HCl, 100°, 24–72 hrs

sequently, components with high sedimentation coefficients are observed on ultracentrifugation. The solubility is decreased, the viscosity and the negative optical rotation are increased. The trough at 233 mμ due to the Cotton effect, an expression of the α-helix, disappears.[102a] On starch gel electrophoresis a poor separation into several bands may be observed. Finally, the biological activity is decreased or lost. However, in general, no change in the chemical composition takes place during denaturation. With regard to the special proteins, the collagens, denaturation represents transition to gelatin or, more precisely, rupture into the α-, β- and γ-components[73–75] and, thus, reduction in molecular size. Therefore, the viscosity and negative optical rotation decrease greatly, whereas the sedimentation coefficient increases slightly.

Analysis of Glycoproteins

For the qualitative analyses of the constituents of glycoproteins, hydrolysis with increasing concentrations of acid for increasing periods of time is carried out.

The ranges of conditions for the liberation of the different constituents are given in Table 3. Sialic acid is released first. Depending on the linkage of the sialic acid residue, the stability toward hydrolysis is known to vary greatly.[103] Increasingly stronger conditions are required for the release of fucose, galactose, mannose and hexosamine. As can easily be seen, overlapping occurs in liberating the different sugars. Occasionally, in order to quickly determine the number of different monosaccharides present in a glycoprotein, hydrolysis in 1 N H_2SO_4, at 100 °C for 5 hours is employed. Enzymes which cleave off these monosaccharides have been described by many investigators. Neuraminidases are the most widely used at present. Fucosidase, galactosidase, mannosidase and N-acetyl glucosaminidase preparations have been employed. Even acetyl residues can be removed enzymatically from certain compounds.[104] Some of the peptides, particularly those of valine and isoleucine, require the most drastic conditions for complete release of their amino acids.[105] Proteolytic enzymes with extremely broad specificity, such as pronase, are known to be capable of completely splitting the polypeptide chain of certain proteins to free amino acids and small peptides.

The obtained series of hydrolysates are analyzed qualitatively for the different monosaccharides.[51] This can be achieved by chromatography on paper[106–108] or on columns[109] or by gas liquid partition chromatography.[110] The different sialic acids may be separated from each other by column chromatography.[29] The procedure of Stoffyn and Jeanloz for the identification of certain amino sugars should be mentioned.[111] Further, some hexosamines can be separated by the procedures of Gardell[112] and Yosizawa,[113] by paper chromatography[114] or by chromatography of the acetyl derivatives on paper[115, 116] impregnated with Na-tetraborate.[117] Thin layer chromatography and thin layer electrophoresis become more and more important for the separation of sugars and amino acids.[118–121] After identification of the constituents of glycoproteins, the hydrolysis conditions are determined that lead to the maximum recovery of each monosaccharide and amino acid.

For the quantitative analysis of the glycoprotein, methods based on color reactions are utilized. These procedures are described in detail in the handbooks indicated above.[8, 42, 43, 45–68] Critical evaluation of such techniques, and particularly discussions as to their specificity, are presented in the corresponding chapters. Four points should be emphasized: (1) In general, these color reactions are relatively unspecific. An exception is perhaps the thiobarbituric acid method for the determination of sialic acid (see below). (2) Nevertheless, satisfactory specificity of some of these procedures is achieved when applied to glycoproteins, because the carbohydrate moiety of these proteins consists of a very limited number of types of monosaccharides. (3) In addition, separation of the constituents to be analyzed (amino acids, peptides and other substances) is often carried out prior to actual quantitation. (4) The quantitative determination is further improved if appropriate standards are employed, as it is known that the intensity of color

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Table 4. Quantitative Analysis of the Constituents of Glycoproteins. Recommended Methods

Constituents	Methods
Sialic acids	Thiobarbituric acid Resorcinol Direct Ehrlich
Fucose	Cysteine-H_2SO_4
Neutral hexoses	Anthrone Orcinol SOMOGYI-NELSON
Hexosamines	ELSON-MORGAN HNO_2-indole
Acetyl	Methylester-Hydroxylamine-$FeCl_3$
Polypeptide moiety	Biuret LOWRY-FOLIN Extinction coefficient Automatic amino acid analyzers

produced by a given procedure differs greatly for the different sugars.[122] If carbohydrates are to be determined in a dialysate, it should be realized that Visking dialysis tubings had released glycerol which leads to erroneous results, if the orcinol or resorcinol-disulfonic acid procedures are utilized. These findings point again to the necessity of employing the most specific techniques available.[124a]

The procedures which are often used for the quantitative determination of sugars and amino acids are indicated in Table 4. As mentioned before, sialic acid may be measured by the highly, but not absolutely specific, thiobarbituric acid method.[123, 124] However, since N-glycolyl-neuraminic acid gives a much lower color yield, 62%,[103] than the N-acetyl compound, the total content of neuraminic acid is estimated by the direct Ehrlich's reaction[125] or by the direct resorcinol reaction.[126] These methods have been discussed by GOTTSCHALK.[29]. It should be noted further that certain breakdown products of sialic acid[103, 127–129] and other substances, including ascorbic acid and deoxyribose will interfere with the thiobarbituric acid method. The glycolyl and acetyl residues are determined by special procedures.[29, 103, 130, 131] Fucose is assessed by DISCHE's cystein-H_2SO_4 technique.[50, 51] The total amount of neutral hexoses is measured either by the anthrone, orcinol, or the SOMOGYI-NELSON method.[35, 132, 133] Paper and column chromatography are used for the determination of the ratio of certain neutral hexoses.[38] For the measurement of the hexosamines the ELSON-MORGAN[124a] and DISCHE-BORENFREUND[134]-YOSIZAWA[135] techniques are usually employed. Special procedures for the determination of certain carbohydrate constituents are found in the mentioned handbooks (especially[42, 51]). The content of each sugar should be determined, whenever possible, by two independent techniques. The analysis of the carbohydrate moiety is usually reported as percentage of free monosaccharides. However, an effort should be made to express such results as monosaccharide residues, as is the custom in reporting the amino acid analysis. The determination of the sugars of glycoproteins is further complicated because on acid hydrolysis the monosaccharides are liberated at greatly different rates and destroyed to varying percentages. Moreover, the destruction of the free sugars also depends on the amino acid composition of the polypeptide moiety. To resolve this problem of the over-all recovery, the use of isotopically labelled free sugars and kinetic treatment of the sugar analyses have been proposed.[138a] The polypeptide moiety is determined by the biuret[136] or LOWRY-FOLIN method.[35, 137, 138] The biuret value of certain proteins may vary greatly.[106] For example, collagen yields only 50% and α_1-acid glycoprotein 66%[38] of the color produced by the same amount of bovine serum albumin. The amino acids are quantitated by an automatic amino acid analyzer, based on the principle of MOORE and STEIN, or by gas chromatography.[137–139] The ratio of tyrosine to tryptophan can be determined from the absorption curve of a protein dissolved in 0.1 N NaOH.[140,141] Free sulfhydryl groups are measured by titration with *p*-hydroxy-mercuri-benzoate or Ag-ions.[141, 142] Cystine (and cysteine) is best quantitated after performic acid oxidation by the automatic amino acid analyzer.

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