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The carbohydrates of glycoproteins

Living matter consists largely of water and complex polymers of amino acids, lipids, nucleotides and carbohydrates. Carbohydrate polymers are special in that they are usually associated with the three other polymers. They are stably linked with amino acid polymers (proteins) or with lipids as glycolipids that participate in non-covalently-linked associations, the lipid bilayer of biological membranes. DNA and RNA are, in essence, polymers of D-ribose-phosphate and 2-deoxy-D-ribose-phosphate to which are attached purine and pyrimidine bases at the C-1 reducing position. While the synthesis of proteins and nucleic acids is guided by a template to maximize fidelity of product formation, synthesis of carbohydrate and lipid membrane polymers, which perform a more structural function, is not. As will be discussed, a variability and relative lack of quality control in the making of lipid and carbohydrate polymers may have its special role in the operation of organisms in an environment that is variable and unpredictable.

Sugars almost certainly existed before life itself appeared on Earth. It has been known for over a century that many sugars can be formed from formaldehyde in alkaline solution. Condensation of formaldehyde, most probably a prebiotic constituent, has been carried out in the laboratory to form glycoaldehyde, trioses, tetroses, pentoses, and hexoses. Polymerization of formaldehyde can be catalyzed by surfaces provided by insoluble silicates and carbonates. UV light, electric discharge and ionizing radiation at the right pH and temperature may also promote synthesis of sugars. The phosphorylation of sugars is another indispensable step in the assembly of the building blocks of living matter. Cyanogen, a likely prebiotic constituent, is capable of catalyzing the synthesis

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of glucose mono- and diphosphate from glucose and orthophosphate.

Several alternative, synthetic pathways and reaction conditions have been proposed but specific details will probably never be established. However, it is highly probable that there was a relative abundance of various sugars and their phosphates in the prebiotic world. In this soup, the basic building blocks must have polymerized and assembled, ultimately to form a self-sustaining, self-reproducing, adaptive entity. Our credulity is strained in accepting that such an awesome sequence took place, but, over inconceivably great periods of time, it did occur. The possibility that carbohydrates will be found in fossils is almost zero because they are relatively unstable, capable of being dehydrated and of combining with other molecules. At higher temperatures they caramelize and char.

The structural diversity possible by linking the different, common sugars is immense: theoretically far greater than that of proteins which largely consist of 22 amino acids linked by a single type of union the peptide bond. Linkages between sugars can occur through a glycosidic linkage between the anomeric, first carbon of a sugar in either an α or β configuration with any of a variety of hydroxyl groups on the adjacent sugar. The number of possible forms that can be produced from two or three of the same sugars or amino acids is shown in Table 1. In fact, many possible combinations of sugars do not seem to exist.

Recurring structural patterns are found that are probably dictated by the limited specificities of sugar transferases and the stringent prohibitions of structural requirements. While polypeptides are linear and unbranched, sugar polymers may branch in many ways, so that the total number of surface conformations of saccharides and the information they bear can be enormous. In general, the components of sugar polymers are fixed with little or no rotation about glycosidic linkages. Because of these properties, saccharides bound to protein and lipid are often immunological epitopes and are the target for combination with lectins.

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Table 1. *Diversity of dimeric and trimeric forms of saccharides and peptides*

	Number of isomers	
Composition	Saccharides	Peptides
a-a (dimer)	11	1
a-a-a (trimer)	176	1
a-b-c (trimer)	1056	6

Note: From Clamp, 1974.

They are involved in receptor activities for hormones, viruses, bacteria, and toxins.

History[1]

Human beings have always been aware of sticky, slimy and viscous substances in animal and vegetable matter. They were described in Latin works and were seriously investigated and discussed by 18th century scholars. As early as 1747, Beccaria, an Italian priest, found a gluten in wheat which was shown by Taddei in 1819 to consist of several components, one of which was a 'schliemige Materie'. Workers in the field distinguished this material from others that were essentially protein. This important distinction was reinforced by German workers who found that mucus, but not protein, could be precipitated at low temperature by acetic acid. In 1805, John Bostock published the results of systematic experiments on the nature of mucins, as well as albumen and gelatin.

In 1865, Eichwald discovered that sugar was a component of mucin. Mucins from ovarian cysts and a wide variety of tissue, when hydrolyzed with mineral acid, released a reducing substance that was considered to be glucose. In the next 25 years, a clearer

[1] Much of this section is based on material in Gottschalk, 1960, 1972.

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view of the nature of mucins emerged from the work of Hoppe-Seyler, Giacosa and others, as it became apparent that there was more than one sugar (glucose) present. Some contained nitrogen and had acidic properties that were probably due to sialic acid. It was also shown that the sugar and protein components were stably linked by a covalent bond. With the development of new, analytic methods for carbohydrates and the evolution of organic chemistry, the structures of hexoses, hexosamines, uronic acids and, eventually, sialic acids were established.

However, progress was slow in this difficult and obscure field because too much groundwork had to be done. The general lack of interest in the area was certainly recognized by one of the outstanding workers in the field, the late Karl Meyer, when he stated 'I work on the gunk and muck that others throw away'. Meyer, and his colleagues before him, worked on complex, multicomponent, unknown, heterogeneous materials, using methods that were poorly understood; this frequently led to false interpretation and error, even by the greatest of researchers. Despite the importance and beauty of carbohydrate biochemistry, the image of groups of complicated sugar molecules with hydroxyl and amino groups sticking out in every direction, has discouraged interest and has even frightened people away from the field.

The nature of both the carbohydrate and protein polymers and their linkages had to be clarified. It was only in 1930 that Sorensen clearly established that the carbohydrate component of the glycoprotein ovalbumin was heterogeneous. By fractional crystallization of highly purified ovalbumin, he obtained more soluble preparations that contained 50 times more carbohydrate per mole than early-crystallizing, less soluble fractions. Heterogeneity of pure carbohydrate polymers was always a source of confusion.

Brilliant studies on the dynamic nature of cell constituents and the elucidation of metabolic pathways (glycolysis, Kreb's cycle, the synthesis of amino acids, purine and pyrimidines, lipids, etc.) were of direct relevance to the glycoprotein field. The identification and synthesis of activated sugars by the Leloir group triggered

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an explosion of investigations of general interest on the biosynthesis and interconversions of sugars and on the step-by-step synthesis of sugar polymers.

Although structural work was not of broad concern to the scientific community, there were two lines of investigation that drew attention to protein-bound carbohydrates. Work on blood group substances, an amalgam of glycoprotein chemistry with immunology and genetics, was widely followed by the scientific community. While blood group substances were discovered by Landsteiner at the turn of the century, 50 years passed before their carbohydrate specificities were elucidated by Morgan and Watkins. The complete sequences of the enzymes responsible for specificity have been revealed only in the last decade of the 20th century.

There was general interest as well in the study of influenza virus interacting with red blood cells which led to the discovery of neuraminidase (sialidase) and the characterization of the sialic acids. More recently, the surface membrane of cells, rich in carbohydrate-containing glycoprotein that undergoes characteristic changes in malignancy and other diseases, has been studied intensively. In recent years, major advances have been made in our understanding of intercellular adhesiveness and the molecules participating in the process.

Another major chapter is that concerning proteoglycan (mucopolysaccharide) biochemistry, with work on the structure of chitin from which glucosamine is derived. This hexosamine was also found in ovarian cysts, ovomucoid and seromucoid from horse serum. Galactosamine and D-glucuronic acid were shown to be major components of chondroitin sulfuric acid from cartilage. Over many years, heparin, heparan sulfate, hyaluronic acid and chondroitin A, B and C, keratan sulfate, dermatan sulfate, and other heteropolysaccharides have been identified and chemically characterized. A good understanding of their synthesis and function has been achieved.

Our present knowledge of mucins and glycoprotein biochemistry had to await the development of fractionation and purification

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methods, sensitive analytic procedures for amino acids and sugars, and methods for the determination of molecular weights of macromolecules. Major advances have been made with the advent of amino acid sequencing techniques, isotopic methods, gas and high-performance liquid chromatography, electrophoretic methods, fast atom bombardment and high-resolution NMR spectroscopy, and the use of both exo- and endoglycosidases. The molecular biological revolution has had a growing, enormous impact on glycobiology both in its structural and functional aspects, providing information and insights that were undreamt of even two decades ago.

One of the liveliest areas of research, at the present time, is on glycoproteins in and on the surface membrane of the cell which function as receptors for hormones and drugs and as components of the adhesive process. The structure of the molecules and their function have been delineated by immunocytological and powerful molecular biological methods such as site-directed mutagenesis.

Microheterogeneity of bound carbohydrates

Most membrane and extracellular proteins bear sugar groups. However, some notable exceptions are serum albumin, trypsin and chymotrypsin. Analysis of purified glycoproteins reveals that the variety of individual molecular species with a common polypeptide backbone can be enormous. The groups may be bound to the hydroxyl group of serine, threonine or hydroxyproline by a glycosidic bond or to the amide group of asparagine by a glycosylamine linkage. There are a few, rare instances of *S*-glycosidic bonds such as in digalactosyl cysteine found in human urine or triglucosyl cysteine in human red blood cells. Other relatively uncommon linkages are L-fucose linked by an α-glycosidic bond to the hydroxyl group of serine or threonine and mannose linked to these amino acids in yeast and fungi. Direct linkage of D-glucose to serine and threonine, characteristic of animal cell nuclei, has also been found (Hart *et al.*, 1989). The same amino acids may not be

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glycosylated in members of the same glycoprotein species. The number of groups may vary from one, as in ribonuclease and ovalbumin, to hundreds, as in mucins, and the groups themselves may consist of two to 30 sugars or may, in fact, be large glycosaminoglycans (Baker *et al.*, 1980). In many instances, groups of sugars may be bound to both serine and asparagine in the same polypeptide, as in glycophorin. Here there are 15 groups of the former and one of the latter (Tomita and Marchesi, 1975).

Groups attached to a specific amino acid may differ in structure. Exhaustive digestion of a purified glycoprotein by proteolytic enzymes may produce far more glycopeptide species than there are binding sites on the polypeptide. Quantitative analysis of the sugars comprising a glycopeptide almost invariably results in ratios of sugar that are not integral, which can only mean that the glycopeptides are heterogeneous in their carbohydrate component.

Although porcine ribonuclease has only one glycosylation site, eight forms of the molecule can be found in pancreatic secretion differing in their oligosaccharide. One form of ribonuclease is devoid of carbohydrate while one other contains sialic acid (Plummer and Hirs, 1964; Beintema *et al.*, 1976). Although ovalbumin also has only one glycosylation site, an array of glycopeptides can be derived from purified ovalbumin from one egg (Lush, 1961). The same groups are found in various proportions in ovalbumin from ostriches, gulls, turkeys, and ducks (Lush and Conchie, 1966). A third example of heterogeneity of carbohydrate group can be found in the surface membrane glycoproteins of the baby hamster kidney cell BHK21/C-13 (Baker *et al.*, 1980). At least 12 carbohydrate groups can be identified from a single glycoprotein. If all were present on each polypeptide, their combined molecular weights would be almost that of the entire glycoprotein instead of a known 10% of the molecular weight. Obviously, not all carbohydrate groups are present on each polypeptide. These are but a few of many examples of microheterogeneity.

Microheterogeneity does not result from random degradation of glycoproteins during their isolation. In many studies, every pre-

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caution was deliberately taken to avoid degradation. During synthesis carbohydrate polymers are assembled in the absence of a guiding template. Fidelity is dependent upon enzyme specificity and this may not be absolute. Since conditions such as cation and anion concentrations, intracellular pH, availability of substrate, and other variable factors can affect enzyme activity, the final products at the end of a multienzyme process may not be identical. The relative amounts of competing enzymes can determine the pathway to be followed. Greater variation may occur toward the non-reducing ends of the chains, which often involves L-fucose and sialic acids which are 'cappers' or chain terminators.

However troubling heterogeneity is to the analyst, it appears to be a fundamental characteristic of carbohydrate polymers. The cell usually shows a remarkable tolerance to variations in the carbohydrate component of glycoproteins. The proposal will be made that the synthesis of carbohydrate groups, responding to environmental conditions in their broadest sense, may be the basis of a plastic, adaptive, survival process operating through a shifting polymorphism.

Function of bound carbohydrates

In recent years, our knowledge of the structure and function of the bound carbohydrates has been increasing at an alarming rate. Large numbers of protein-bound carbohydrates have been described. Their roles in nature are complex and cannot be simply categorized and, frequently, experimentalists are unable to assign any function to this component. In some instances, their function is associated with the general properties inherent in their molecular structure: for instance, they constitute a hydrophilic mass that may be charged with sulfate, phosphate or sialic acid residues that readily form hydrogen bonds and increase the solubility of proteins. In globular proteins, they protrude into the aqueous phase at the surface of the polypeptide that is folded in on itself and is stabilized in the folded form by hydrophobic interactions between inte-

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rior amino acids. This latter interaction is the major factor responsible for folding. Depending on their size and nature, the external carbohydrate groups could modify, promote or deter folding. Programed glycosylation of nascent polypeptides within the Golgi could have some effect on folding, resulting in a desired conformation. Specific conformations may prevent certain amino acids from being further glycosylated, or render others available for glycosylation. Variation in polypeptide folding induced by carbohydrate groups could possibly be the basis for small shifts of functional activities (see p. 20). The carbohydrate groups may be in essence multiple, covalently bound, allosteric effectors. Each site on the polypeptide may bear no group, or one of a variety of carbohydrate groups in accordance with known microheterogeneity. Here, they could be modifiers of activity rather than critical effectors.

In a mucin such as that of ovine submaxillary gland, hundreds of carbohydrate groups exist largely as dimers along the polypeptide. They are charged and hydrophilic, preventing the folding due to hydrophobic association of amino acids within the chain. Sialic acids, linked to *N*-acetylgalactosamine residues along the polypeptide, interact with the amino acids to stiffen the chain. The fixed charges of sialic acids repel each other to stretch the polypeptide, creating rigid, linear molecules that get tangled up with each other, especially at low ionic strength. This is the basis of the viscous, lubricating, and protecting properties characteristic of mucins. When mucins in solution are treated with sialidase, charges are removed leading to a collapse of the extended molecule and a rapid fall in viscosity (Gottschalk and Thomas, 1961). Surface membrane glycoproteins may bear carbohydrate groups on the outside of the cell to stiffen the polypeptide emerging from the lipid membrane so that some active, functional groups may be held away from the tangle of molecules at the immediate cell surface.

Carbohydrate groups, in some instances, are very large and bulky. Since they can be great space occupiers, they are common constituents of intercellular matrices and basement membranes. If

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they bear charged groups in these locations, they may also function as ion exchangers or adsorbents, regulating and stabilizing the composition of intercellular fluid. Sialic acids which are usually highly exposed, can bind cations such as Ca^{2+}. Surprisingly, the glycerol side chain (C7–9), as well as the carboxyl group, is intimately involved in the binding (Jaques *et al.*, 1977).

In the 1980s, the elements of the extracellular matrix have rapidly become known, with new insights into the mechanism of adhesiveness during proliferation and differentiation. It is apparent that there are important, specific, ionic interactions between carbohydrate-rich glycoproteins, such as fibronectin and cadherins, with sulfated and other glycosylaminoglycans, growth factor receptors, and growth factors such as fibroblast growth factor. Some fragments of sulfated glycosylaminoglycans enter the nucleus where they may affect DNA transcription.

The presence of carbohydrate groups along a polypeptide can change its function (Table 2). They may protect it from degradation by proteases, not only because the carbohydrate may induce folding that buries more susceptible regions of the chain but also because it masks the chain. It was observed soon after neuraminidase was discovered that certain glycoproteins rich in sialic acid could be degraded by trypsin, only after sialic acid was removed by sialidase. In a sense, the sialic acid and underlying carbohydrate residues form a charged molecular shield or skin. Since bound carbohydrates can prevent degradation, it is possible that they may be regulators of the rate of protein turnover. The removal of certain sugars by glycosidases, or the programed cessation of glycosylation within the cell or organism, may be a key, permitting a timed and orderly degradation of proteins, which can take place during the mitotic cycle.

The bulk of protein-bound carbohydrate is at the surface of the cell. In the L cell, a mouse fibroblast, at least two thirds of the glycolipid sialic acid is in the plasma membrane. When appropriately stained, a layer of carbohydrate-containing material can be seen extending as much as 15 nm out from the lipid bilayer of some

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