Glycoproteins and Diabetes

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It has been repeatedly postulated for over a decade that some disorder in the metabolism of the "mucosubstances" exists in diabetes mellitus.[1-3] This postulation has been based primarily on the histochemical demonstration of carbohydrate-containing material in the characteristic retinal and glomerular lesions of long-standing human diabetes. At the time that the presence of carbohydrate in these lesions was first observed, it was possible to deduce only that the material was of a high molecular weight and did not represent glycogen. With the recent advances which have occurred in our knowledge concerning both the histochemistry and the structural chemistry of the large number of complex carbohydrate-containing macromolecules present in mammalian organisms, it is now possible to make a more precise identification of the material in the diabetic lesions as glycoprotein in nature. Moreover, rapid progress in the elucidation of the biosynthesis of the monosaccharide constituents of mammalian glycoproteins has indicated that the biosynthesis of these sugars represents a major pathway of glucose metabolism.

In view of the implication of glycoproteins in the diabetic capillary lesions and the quantitative importance of their biosynthesis as a pathway of glucose metabolism, it seems pertinent at this time to review briefly our present knowledge of their structure and metabolism and to attempt to assess their role in the pathogenesis of the diabetic lesions.

CLASSIFICATION OF COMPLEX CARBOHYDRATE-CONTAINING SUBSTANCES

In attempting to understand the glycoproteins, it is first important to differentiate them from the other carbohydrate-containing macromolecules commonly found in mammalian tissues. All of these carbohydrate polymers of higher organisms, with the exception of glycogen and the nucleic acids, contain amino sugars and may be referred to as *mucosubstances*. Used in this context, the prefix "muco" refers not to the viscous properties which only some of these substances have, but rather to the fact they contain amino sugars.

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The glycoproteins should be differentiated primarily from the mucopolysaccharides, with which they have often been confused in the past in literature dealing with diabetes. The *mucopolysaccharides* include well-known compounds of connective tissue, such as hyaluronic acid, the chondroitin sulfates, and heparin. In mammalian organisms the mucopolysaccharides are acidic substances, since they contain hexuronic acids and/or sulfate esters. In the native state these polysaccharides are believed to be associated with protein by ionic linkages or labile covalent bonds. This complex of mucopolysaccharide and protein may be referred to as a *mucoprotein.*[4]

The *glycoproteins,* on the other hand, contain carbohydrate linked by a firm covalent bond to peptide material. While it is possible to dissociate the mucopolysaccharide-protein complex by mild procedures, such as changes in *p*H or salt concentration, it is not possible to separate the carbohydrate and peptide portions of the glycoprotein without also drastically degrading the entire molecule. The glycoproteins further differ from the mucopolysaccharides in that they do not contain hexuronic acids. Their usual sugar constituents are the amino sugars, glucosamine and galactosamine, present in their N-acetyl form; the neutral sugars, mannose, galactose, and fucose; and the N-acetyl or N-glycolyl form of neuraminic acid, the acidic nine-carbon amino sugar whose derivatives are collectively known as the sialic acids. Glucose is seldom a constituent of mammalian glycoproteins. The structure of these sugar constituents is shown in figure 1.

An additional large group of carbohydrate polymers are those containing lipid, which are usually referred to as *glycolipids.*

DISTRIBUTION OF GLYCOPROTEINS

Table 1 gives examples of some of the more important glycoproteins found in mammalian tissues. It should be pointed out that blood contains a large number of glycoproteins, some of which are specified in table 1. Indeed, there is protein-bound carbohydrate in all of the electrophoretic fractions of human serum with the exception of the albumin component. The average value for the total protein-bound carbohydrate in serum of normal humans has been reported to be 273 mg. per 100 ml., with 121 mg. per 100 ml. rep-

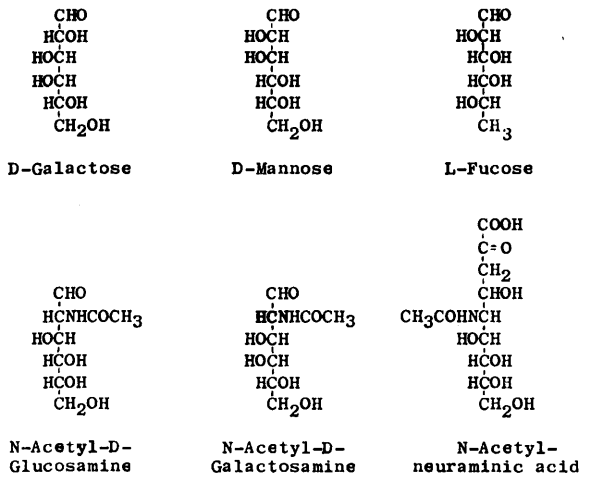

FIG. 1. Configuration of the monosaccharide constituents of mammalian glycoproteins. N-acetylneuraminic acid is shown as a representative of the sialic acids.

TABLE 1

Mammalian glycoproteins

1. *Plasma glycoproteins*
   Orosomucoid, fetuin, haptoglobins, transferrin, ceruloplasmin, fibrinogen, prothrombin, low molecular weight alpha$_2$-glycoproteins, alpha$_2$-macroglobulins, gamma globulins.
2. *Urine*
   Glycoprotein of Tamm and Horsfall.
3. *Blood group substances*
4. *Glycoproteins of mucous secretions*
   Submaxillary, cervical, bronchial, gastric.
5. *Gonadotrophins*
   Interstitial cell-stimulating hormone, follicle stimulating hormone, human chorionic gonadotrophin, pregnant mare's serum gonadotrophin.
6. *Glycoproteins relating to thyroid*
   Thyrotropic hormone, thyroglobulin.
7. *Glycoproteins of connective tissue*
   Reticulin, collagen, basement membranes, lens capsule, Descemet's membrane.
   Soluble glycoproteins.

resented by hexoses, in the form of galactose and mannose; 83 mg. per 100 ml. present as hexosamine, almost entirely in the form of N-acetylglucosamine; 60 mg. per 100 ml. occurring as sialic acid, which is present in the N-acetyl form; and 8.9 mg. per 100 ml. present as the methyl pentose, fucose.[6] It is of interest that the concentration of the protein-bound carbohydrate is approximately three times that of the free glucose in normal blood. Moreover, it has been shown that these glycoproteins are not only abundant, but are also in a rapid state of turnover.[6] In contrast to the large amount of glycoproteins found in blood plasma, there are only very small amounts of mucopolysaccharides present in serum, with an average concentration of the glucuronic acid portion of 0.28 mg. per 100 ml.[7]

In recent years a vast literature has accumulated indicating that the protein bound carbohydrate is increased in a large number of apparently unrelated disease states, including such diverse diseases as neoplasia, tuberculosis, and rheumatoid arthritis.[5] Attempts have also been made by various investigators to determine whether serum glycoproteins are elevated in diabetes mellitus.[8-11] Even though somewhat differing results have been reported, it appears that diabetes mellitus without complications is not associated with a significant increase in serum glycoproteins. It is becoming increasingly evident that the elevation in serum glycoproteins noticed in various disease states is a nonspecific response.

Attention should be directed particularly to the glycoproteins of connective tissue (table 1). Little precise chemical characterization of these substances has as yet been undertaken, and much of the information concerning them rests on histochemical evidence. It is apparent, however, that ground substance contains soluble glycoproteins in addition to its well-known mucopolysaccharide constituents. Moreover, the fibrous proteins reticulin and collagen are also glycoproteins. Many membranous structures, such as the capillary basement membrane, Descemet's membrane of the cornea, and the lens capsule, have also been shown to be glycoprotein in nature.[12]

Knowledge of the distribution of the glycoproteins in tissues, and particularly in pathological deposits such as those of diabetic retinopathy or glomerulosclerosis, has been greatly increased by the use of histochemical technics, and in particular by the application of the periodic acid Schiff (PAS) stain. This stain has been shown to be, under specified conditions, very discriminating in detecting glycoproteins in tissue sections.[13] Figure 2 shows the main features of the PAS reaction. Periodic acid cleaves the carbon-carbon bond in those substances having an unsubstituted vicinal glycol group and oxidizes these two hydroxyl groups to aldehydes. Similarly, periodic acid cleaves the equivalent amino derivative, that is an alpha amino alcohol, with the formation of two aldehydes and ammonia. The aldehydes formed by this reaction, as indicated in figure 2, can be detected by the use of the Schiff reagent (leucofuchsin), but only if they remain nondiffusable throughout the staining process. This in effect limits the reaction to substances of high molecular weight.

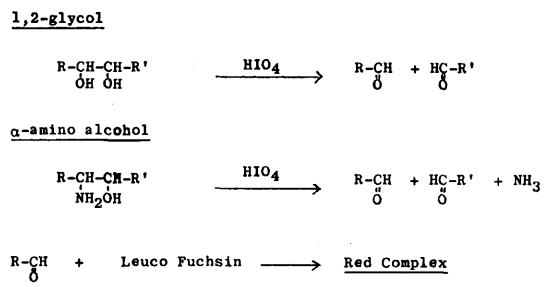

FIG. 2. *Periodic acid Schiff (PAS) reaction. In order to stain in tissue sections, the reactive substances and the aldehydes which they form must be nondiffusible during tissue preparation and staining.*

Table 2 shows several criteria for identifying carbohydrate containing material commonly found in mammalian tissues by histochemical methods. It may be noted that glycogen reacts with the PAS stain, but can be distinguished from other PAS positive substances by its removal by alpha amylase. In recent years it has been shown by several investigators that the acid mucopolysaccharides do not stain with PAS.[14-17] This is not a surprising result in view of the slight to negligible periodate consumption by these compounds in the test tube. The mucopolysaccharides are characterized by their metachromasia and their susceptibility to digestion by hyaluronidase. Glycoproteins, on the other hand, have been shown to stain intensely with PAS,[14-16] which is consistent with their rapid consumption of periodate in the test tube. The glycoproteins are not susceptible to digestion either by amylase or hyaluronidase. Lipids, such as glycolipids, which could be PAS positive, are usually removed by the solvents used in the preparation and staining of the tissue sections; however, if they are still present, they can be located with Sudan black. From table 2 it is evident that in paraffin sections of mammalian tissues from which glycogen has been removed with amylase, glycoproteins are the only known substances which will give a PAS positive reaction.[15]

If one looks at the staining properties of the nodular lesions of diabetic glomeruli in the light of the staining characteristics of these major carbohydrate-containing substances (table 2), it is quite reasonable to conclude that glycoproteins are present and are responsible for the PAS stain. The lack of metachromasia, moreover, indicates that mucopolysaccharides are not present in significant amounts.

It is noteworthy that the PAS stain has been the method of choice for outlining the glomerular capillary basement membrane.[19] In general, the staining properties of the diabetic glomerular lesions and the glomerular capillary basement membrane are very much alike, indicating that both are glycoprotein in nature. The observations that thickening of the capillary basement membrane is the initial occurrence in the development of the diabetic glomerular pathology further relates to the development of the later diabetic glomerular lesions to alterations in the basement membrane.[20,21] Since changes in the basement membrane have been observed very early following the onset of the disease, it is likely that the beginning of renal glomerular pathology coincides with the onset of the metabolic disturbances.[22-26]

STRUCTURE OF GLYCOPROTEINS

Knowledge in regard to the chemical structure of glycoproteins is still in an early stage of progress. Nevertheless, certain generalizations about the structure of these molecules can be made at this time and certain concepts of structure can be proposed, especially for the serum-type of glycoproteins, which have received the most detailed attention. Structural work on mammalian glycoproteins has depended to a great extent on the isolation of well-defined purified chemical entities. For this reason the glycoproteins, orosomucoid[27,28] and fetuin,[29] which have been isolated in highly purified form from serum, have so far received the most detailed study.

Some of the structural problems particularly pertinent to glycoproteins include the determination of the

TABLE 2

Histochemical identification of carbohydrate containing materials in mammalian tissues

	PAS	Metachromasia	Amylase	Hyaluronidase	Sudan black	Lost during tissue preparation
Glycogen	+	—	Labile	Fast	—	—
Mucopolysaccharides	—	+	Fast	Labile	—	—
Glycoproteins	+	±	Fast	Fast	—	—
Lipids	±	±	Fast	Fast	+	+
Nodular lesions of diabetic glomeruli*	+	—	Fast		—	—

*The staining characteristics of these lesions have been summarized.[18]

size, number, and composition of the carbohydrate units; the structure of these units in regard to monosaccharide sequence, linkage, and branching; the chemical nature of the glycopeptide bond, including the nature of the sugar and amino acid involved in this linkage; and the arrangement of the carbohydrate units along the peptide chain or chains.

Some concepts of the structure of mammalian glycoproteins may be derived from recent work on the structure of the serum glycoprotein fetuin.[29-35] At the present state of our knowledge, there is reason to believe that this protein may serve as a model for understanding the structure of the soluble, serum-type of glycoprotein. This alpha$_1$ glycoprotein was isolated from fetal calf serum where it is present in very high concentration, comprising approximately 45 per cent of the total serum proteins.[32] The carbohydrate portion of this protein represents approximately 22 per cent of the total weight of the molecule, the remaining 78 per cent consisting of a peptide portion with an amino acid composition quite typical of other serum proteins.[33] Each molecule of the protein consists of 361 amino acid residues, in addition to fifty-one monosaccharide residues. The sugars which are present are sialic acids (primarily N-acetylneuraminic acid), galactose, mannose, and hexosamines (primarily N-acetylglucosamine).[29] It has been possible to show that the carbohydrate portion of this glycoprotein consists of three heteropolysaccharide units, each of approximately 3,500 molecular weight.[30] Each unit has the same carbohydrate composition, most likely consisting of four residues of sialic acid, four residues of galactose, six of N-acetylhexosamine, and three residues of mannose. Studies have shown that these units are branched structures consisting of oligosaccharide chains in each of which a terminally located sialic acid residue is linked on to the three position of a galactose, which in turn is linked on the four position of an N-acetylglucosamine residue, as depicted in figure 3.[31,34] Each polysaccharide unit would have four such chains, which are in turn linked on to an internal portion consisting of three residues of mannose and two additional residues of N-acetylhexosamine, in the manner shown in figure 3. The details of the structure of this internal portion of fetuin are currently being elucidated.[34]

The three polysaccharide units are attached at three points along the single peptide chain of fetuin. This peptide chain has both free N- and C-terminal amino acids,[35] indicating that the union between the carbohydrate and protein occurs through an available functional group of an amino acid (figure 4). Studies so

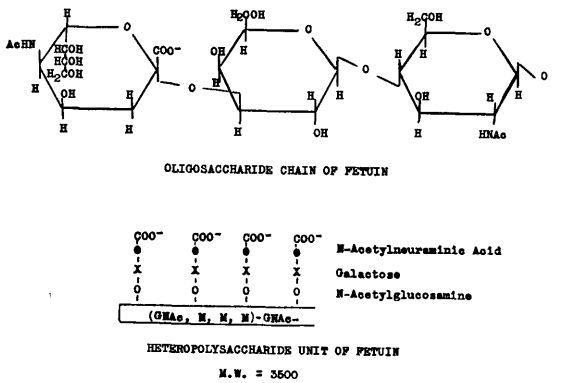

FIG. 3. Structure of the carbohydrate units of fetuin. Upper: Structure of an oligosaccharide chain. Lower: Schematic representation of an entire heteropolysaccharide unit showing the oligosaccharide chains attached to the inner portion. M.W., molecular weight of each heteropolysaccharide unit.

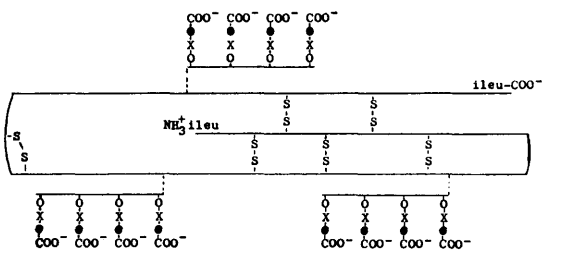

FIG. 4. Schematic representation of the fetuin molecule showing the single peptide chain with its free C- and N-terminal amino acids and the attachment of the three heteropolysaccharide units (figure 3) at three points along the peptide chain. In this representation the six disulfide bridges which are present in fetuin are arbitrarily positioned along the chain.

far indicate that it is likely that the carbohydrate peptide linkage involves aspartic acid and the first carbon of N-acetylglucosamine. Work on ovalbumin[36] and the gamma globulins[37] has also indicated that aspartic acid is involved in the glycopeptide linkage in these proteins. On the basis of work done on ovalbumin[36] it has been suggested that this linkage is through an amide group on the beta-carboxyl group of this amino acid, that is, a beta-aspartyl glycosylamine. This proposed linkage would be consistent with the properties of the glycopeptide bond as observed in many glycoproteins, being a relatively stable covalent bond, resistant to cleavage by alkali and being cleaved under moderately strong acid conditions.

There are indications that the concept of moderately sized, branched heteropolysaccharide units, as proposed for fetuin, may also apply for other soluble, serum-type glycoproteins.[37,38] Since the serum glycoproteins vary considerably in their carbohydrate content, from approximately 3 per cent in the gamma globulins, to 40

per cent in orosomucoid, it is likely that an increased carbohydrate content will be reflected in an increased frequency of occurrence of such polysaccharide units along the peptide chain.

The peripheral location of sialic acid, as in fetuin, is a common finding in glycoproteins. In these proteins, the sialic acid residues can be selectively cleaved from the remainder of the molecule either by very weak acid hydrolysis or by means of an enzyme known as neuraminidase, which is produced by a variety of bacteria and viruses.[39] The sialic acid content of the glycoproteins appears to be responsible for their acid characteristics, and in general those glycoproteins having the highest sialic acid content have the lowest isoelectric point. For example, when the sialic acid residues are selectively removed from fetuin, its isoelectric point is raised from *p*H 3.3 to *p*H 5.2.[20] In those glycoproteins containing fucose, such as the blood group substances, this sugar also can occupy terminal positions.[40]

On the basis of work done on the glycoprotein from submaxillary mucin, it appears that a somewhat different and less complex structural pattern may exist in some glycoproteins.[41]

METABOLISM OF GLYCOPROTEINS

Little information is as yet available about the biosynthesis of these complex carbohydrate-containing proteins. However, in recent years, a great deal of knowledge has been obtained in regard to the biosynthesis of their monosaccharide constituents.[42] Figure 5 shows the metabolic routes involved in biosynthesis of some of these monosaccharides. It is apparent that glucose plays a central role in the biosynthesis of these compounds. Glucose is the main available sugar in the diet of higher animals and is the sole sugar made by the liver for transport to other tissues. The intact glucose skeleton, as shown (figure 5), can be the source of the other monosaccharides through a series of modifying enzymatic reactions. Many of the sugars are represented in figure 5 as their nucleotides. These nucleotides are activated forms of the monosaccharides and have been shown to be intermediates in the synthesis of a large number of oligo- and polysaccharides such as lactose, starch, glycogen, hyaluronic acid, and cellulose, serving as donors of the glycosyl residue.[43] Figure 6 shows the structure of uridine diphosphate N-acetylglucosamine, typical of such nucleotides.[44] In

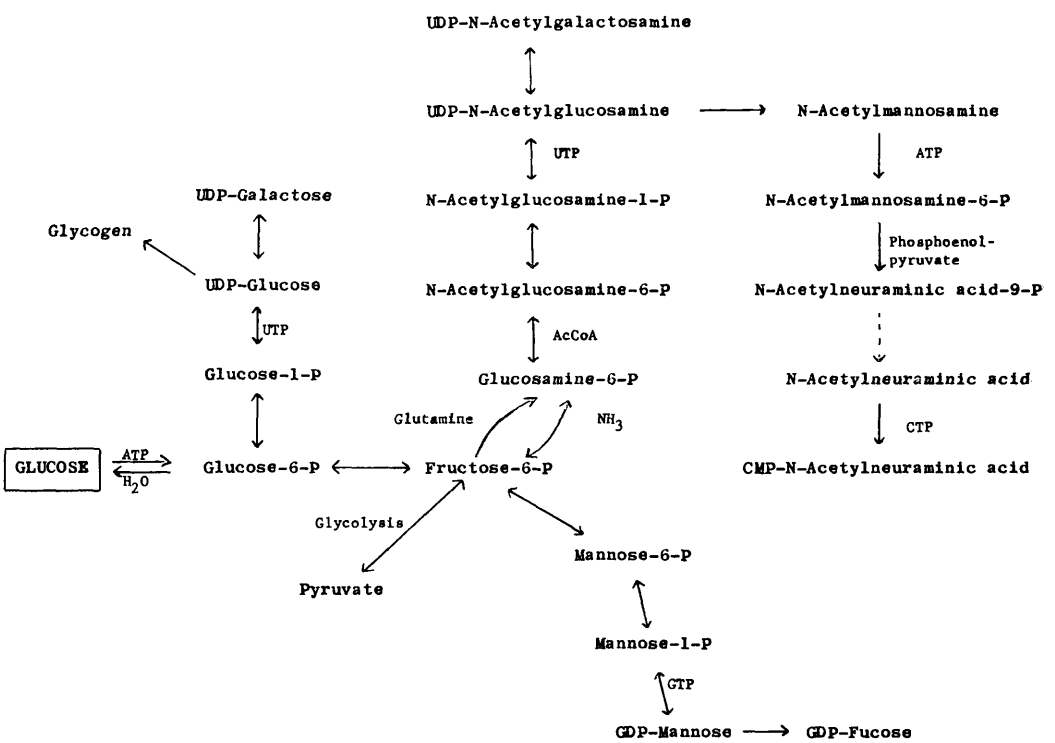

FIG. 5. Pathways for the biosynthesis of the monosaccharide constituents of glycoproteins from glucose. Broken arrow indicates pathway not yet established. UTP, uridine triphosphate; UDP, uridine diphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; CTP, cytidine triphosphate; CMP, cytidine monophosphate; ATP, adenosine triphosphate; AcCoA, acetylcoenzyme A.

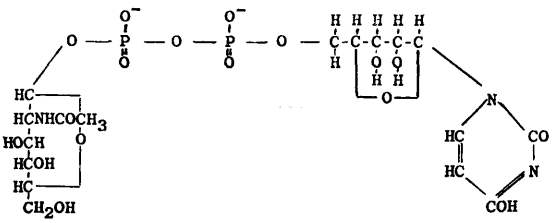

FIG. 6. Structure of a sugar nucleotide, uridine diphosphate N-acetylglucosamine.

other sugar nucleotides, the base which is present may be guanine, thymine, or cytosine, instead of uracil. It has not as yet been shown that these nucleotides are involved in the biosynthesis of glycoproteins. However, the isolation of a uridine nucleotide containing the oligosaccharide chain, sialic acid-galactose-N-acetylglucosamine,[45] in the same sequence as exists in the fetuin polysaccharide units, supports this possibility.[31]

Because of the central role which glucose plays in the biosynthesis of glycoproteins, the biosynthesis of these compounds from glucose in the diabetic state is of particular interest. It has been shown that liver is a mammalian tissue active in glycoprotein synthesis, being primarily responsible for the production of the large number of glycoproteins present in serum.[6] This was demonstrated by measuring the incorporation of tracer doses of C-14 glucose into the glucosamine component of the serum and tissue glycoproteins of the intact rat.[6] This study showed the turnover of the serum glycoproteins to be very rapid, as indicated by a turnover time of approximately two hours which was calculated for the glucosamine component. This rate is considerably faster than any values calculated for the turnover time of the peptide portion of serum proteins.

Because of the active role of the liver in glycoprotein synthesis, it was selected as an appropriate tissue for the evaluation of the role of insulin deficiency on the biosynthesis from glucose of the glucosamine component of the glycoproteins.[46] Moreover, this study permitted a comparison of the synthesis of glycogen from glucose to that of protein-bound glucosamine from glucose in the diabetic state, which is of interest since both routes go through glucose-6-phosphate.

Table 3 presents data from this study, indicating that the glucosamine pools in both liver and serum were essentially the same in normal and alloxan diabetic rats, while the glycogen pool of the diabetic rats was markedly diminished. The radioisotope data obtained confirmed this finding, indicating that while the in vivo incorporation of radioactivity from C-14-labeled glucose into the liver glycogen was reduced to about 1 per cent of the normal, the incorporation of radioactivity into

TABLE 3

Comparison of pool sizes in normal and diabetic rats[46]

Type of animal	Liver		Serum
	Glycogen (glucose equivalents)	Glucosamine	glucosamine
Normal ± s.e.	1,720 ± 191	21.5 ± 0.4	50.0 ± 2.0
Diabetic ± s.e.	206 ± 54	26.9 ± 0.6	46.0 ± 5.2

All values expressed as μmoles per 250 gm. rat.

both liver and serum glucosamine of the glycoproteins was essentially unaffected in the diabetic state at different time intervals. Figure 7 graphically depicts the marked difference in the effect of insulin deficiency on the synthesis of glycogen and glucosamine from glucose by liver. In other tissues of the diabetic animals studied, such as kidney, lung, testis, and spleen, glucosamine synthesis from glucose was also shown to be unimpaired.[46]

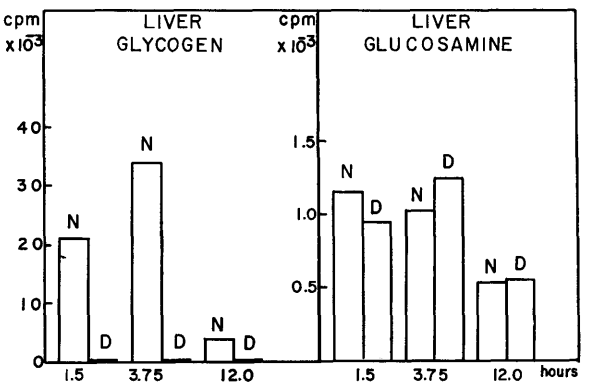

FIG. 7. A comparison of the total activity in the liver glycogen and protein-bound glucosamine of normal and diabetic rats. The values are means for each time and are expressed per 250 gm. rat.[46]

This difference between the effect of insulin deficiency on glycogen and glucosamine synthesis from glucose indicates that insulin does not play the same role in regulating these two pathways of glucose metabolism and that glucosamine is synthesized by an "insulin-independent" pathway despite going through glucose-6-phosphate. It is therefore conceivable that if insulin does not control all pathways of glucose metabolism, the glucose molecule may be shunted from "insulin-dependent" pathways to insulin-independent routes in the insulin deficient state. In the presence of the high diabetic blood glucose level, this could result in an increased synthesis of some products of glucose metabolism, such as some of the sugar components of the glycoproteins. If such a rerouting of the metabolism of glucose should occur in the diabetic state because of

the specific regulatory action of insulin, it is conceivable that cells active in synthesizing glycoproteins would produce increased and/or abnormal molecules of this type. Such cells could be the epithelial cells of the glomerulus which are thought to be responsible for the synthesis of the capillary basement membrane[47] and which also may well be responsible for the synthesis of the pathological glycoprotein deposited in the diabetic state.[26]

SUMMARY AND CONCLUSIONS

It is hoped that this brief review has indicated that important constituents of the diabetic vascular lesions are glycoprotein in nature and may well result from some abnormality in the metabolism of these complicated molecules in the diabetic state. This is not difficult to accept in view of the central role which has been indicated for glucose in the biosynthesis of the monosaccharide constituents of these compounds. Moreover, since the work on the diabetic animal has shown that the biosynthesis of at least the glucosamine component of the glycoproteins is not affected by insulin deficiency, it is conceivable that there is a shunting of glucose from its insulin-dependent pathways to the biosynthesis of glycoproteins, which may, over a long period of time, contribute to the development of the vascular lesions.

The area of glycoprotein biochemistry is still at an early stage of investigation and a considerable amount of work needs to be done in gaining a basic understanding of both the structure and biosynthesis of such complex molecules. However, at this point one may raise the hope that with an increased knowledge in this field it will eventually be possible to map out the metabolic sequence of events leading from an abnormal glucose metabolism to the occurrence of the vascular lesions of diabetes, and thereby to find a rationale for both their therapy and prevention.

SUMMARIO IN INTERLINGUA

Glycoproteinas e Diabete

Es sperate que iste breve revista ha indicate que importante constituentes del diabetic lesiones vasculari es de natura glycoproteinic e pote ben resultar del un o del altere anormalitate in le metabolismo de iste complexe moleculas in le stato diabetic. Iste these non es difficile a acceptar in vista del rolo central que ha essite ascribite a glucosa in le biosynthese del constituentes monosaccharidic de iste compositos. In plus, viste que le travalio con animales diabetic ha monstrate que le biosynthese de al minus le componente glucosaminic del glycoproteinas non es afficite per carentia de insulina, il es ben imaginabile que il existe un shunting de glucosa ab su circuito metabolic dependente de insulina ad le biosynthese de glycoproteinas, lo que—possibilemente—contribue in le curso de prolongate periodos de tempore al disveloppamento del lesiones vascular.

Le dominio del biochimia de glycoproteina es ancora in un precoce stadio de investigation, e un considerabile quantitate de travalio remane a facer ante que nos pote disponer del cognoscentias fundamental in re tanto le structura como etiam le biosynthese de si complexe moleculas. Tamen, a iste puncto il pare justificate exprimer le spero que le augmento de nostre cognoscentias in iste dominio va in le curso del tempore render possibile le delineation del sequentia de evenimentos metabolic que duce ab un anormal metabolismo de glucosa al occurrentia del lesiones vasculari de diabete e assi le elaboration de un theoria pro tanto le therapia como etiam le prevention de ille lesiones.

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