PREPARATION, PROPERTIES, AND APPLICATIONS OF CARBOHYDRATE CONJUGATES OF PROTEINS AND LIPIDS

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I. INTRODUCTION

Modification of proteins by the attachment of sugars was first carried out by coupling with *p*-diazophenyl glycosides as a way of preparing antigens with carbohydrate haptens.[1-4] In more recent years with the rapidly growing awareness of the role that oligosaccharide moieties of glycoproteins and glycolipids play in a wide array of recognition and binding phenomena,[5,6] a need has arisen for more specific and varied methods of modifying proteins by the attachment of carbohydrates. The different methods that have been introduced reflect the varying purposes that investigators have had in mind. In some instances an improvement in thermal stability or resistance to proteolysis was the property sought; in others the need was to introduce into the protein (in a manner causing as little disarray as possible) specific carbohydrate moieties for the investigation of biological phenomena, e.g., plasma clearance.

For the purposes of this review, modification by carbohydrate conjugation* is defined very broadly to include not only methods for the introduction of simple sugars and oligosaccharides, but also methods for coupling polysaccharides such as dextran, hydrophilic polymers such as polyethylene glycol, and glycopeptides. The majority of preparations reported so far utilize chemical methods of modification, the discussion of which (Section II) is followed by a detailed discussion of the rationale for preparing the various modified proteins, and the results achieved in experiments in which they were employed (Section III). Glycoproteins with new or altered carbohydrate prosthetic groups can also be prepared by a biosynthetic approach, however, and this new and expanding field is dealt with in Section IV. Interest is also increasing in the biochemical role of carbohydrate found conjugated to lipid (Section V), and other prospective developments are discussed in Section VI. Specifically excluded are methods for preparing insoluble carbohydrate-substituted materials.[7]

* The term "glycosylation", though sometimes loosely applied to processes involving the attachment of carbohydrate to other species, is more correctly confined to those cases where the linkage established is in fact glycosidic. Few of the procedures described in this review involve the formation of glycosidic bands, therefore the more cumbersome term "carbohydrate conjugation" was used.

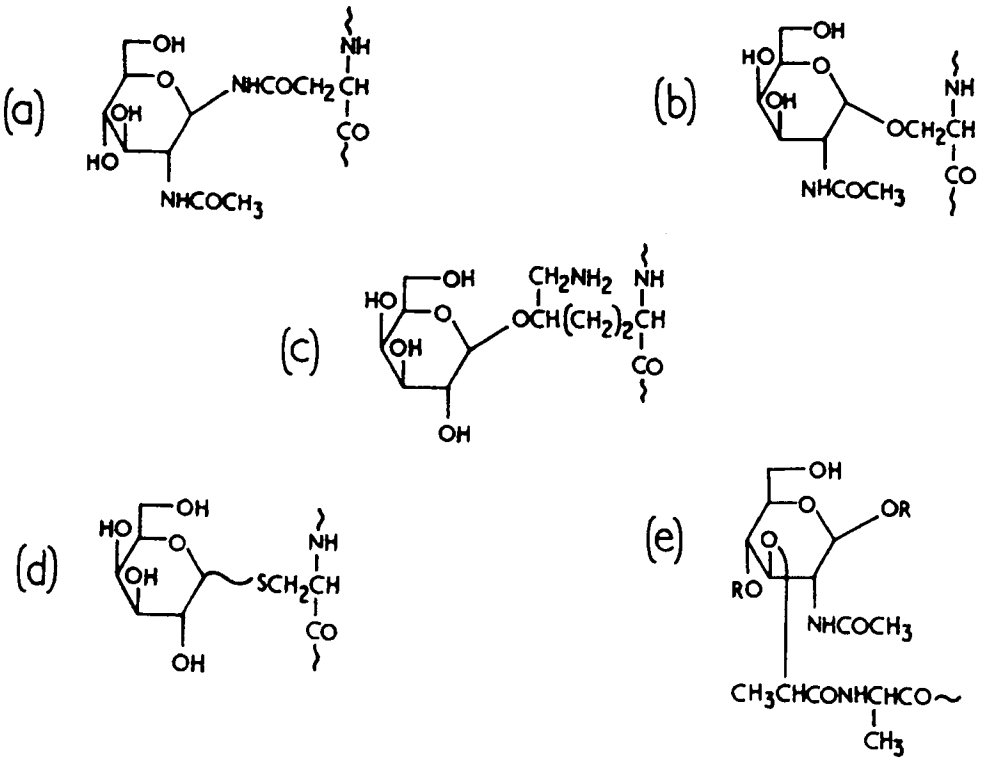


FIGURE 1. Linkages frequently found in glycoproteins: (a) GlcNAc-Asn, (b) GalNAc-Ser(orThr), and (c) Gal-Hyl. Several sugars in addition to GalNAc are found linked to Ser/Thr, e.g., xylose, galactose, and mannose. Less common glycoprotein linkages: (d) Gal-cysteine and (e) *N*-acetylmuramic acid peptide unit.

The kinds of covalent linkages found so far between carbohydrate and protein in naturally occurring glycoproteins and proteoglycans are shown in Figure 1. The structures shown in the upper part of the figure (*N*-acetylglucosamine linked as an *N*-glycoside to asparagine, *N*-acetylgalactosamine linked *O*-glycosidically to serine and threonine, and galactose linked *O*-glycosidically to hydroxylysine) are by far the most common, but others, some shown in the lower part of Figure 1, have also been found. The linkage between an *N*-acetylmuramic acid unit and peptide in bacterial cell peptidoglycans is also included as a special case.

With minor exceptions, then, the term "naturally occurring" glycoprotein linkage refers to structures in which sugar chains of greater or lesser complexity are linked as *N*-glycosides to asparagine, or as *O*-glycosides to serine and threonine. The biosynthesis of glycoproteins like these is under enzymic control, but it has been known for some time that certain other glycoproteins exist, formed by nonenzymic glycosylation in vivo.[8-10] Thus the minor hemoglobin component Hb A_{Ic} has glucose attached by a Schiff's base or aldimine linkage to the valines that are *N*-terminal in the two beta chains.[11,12] Hemoglobin glycosylation is of considerable current interest because it occurs to a greater extent in the erythrocytes of persons with diabetes[13] and also because there is now evidence pointing to an increased nonenzymatic glycosylation of lysine residues in basement membrane proteins and lens proteins in diabetes.[14,15] The idea has been advanced that some of the pathological consequences of diabetes, such as cataract formation, may be due to nonenzymatic protein glycosylation.[16]

In addition, Day et al.,[17] have shown that several human plasma proteins are subject to nonenzymatic glucosylation in vitro and have isolated glucosylated albumin from normal human serum (approximately 8% of the albumin is glucosylated); and these same authors have recently studied the nonenzymatic glucosylation of rat albumin in vitro and in vivo.[18] Additional information on the subject of nonenzymatic glycosylation may be found in recent reviews.[19-21]

In this light, certain points that should be considered in choosing a method for attaching carbohydrate are as follows:

1. Is preliminary chemical modification of the sugar necessary? If so, since most oligosaccharides of interest are not readily available, how involved are the manipulations, and can they be carried out on a small scale?
2. Is the site of attachment to the protein (or lipid) "natural", i.e., one found in naturally occurring glycoproteins? Is the linkage itself natural?
3. To what extent is the sugar (or the innermost or "linkage" sugar, in the case of di- and oligosaccharides) chemically altered? Does the modification involve the introduction of other structures in addition to the carbohydrate (e.g., phenyl groups, or -(CH_2)- "arms")? How specific is the reaction with respect to the sugar? For example, a polysaccharide is likely to be activated at a number of points by cyanogen bromide.)
4. Does the modification alter the charge on the protein?
5. Is it feasible to control the extent of modification, and are methods available to determine the extent of modification?
6. Are the reaction conditions likely to denature proteins, and what has been the experience of other investigators using the method with respect to recovery of activity and efficiency of sugar incorporation?

II. CHEMICAL METHODS OF CARBOHYDRATE CONJUGATION

A. Reaction Types and Systems Studied

In Table 1 chemical methods of linking carbohydrate to protein (reported in the literature up to November 1979) are classified according to the protein functional group modified and the modifying species. In Table 2 some of the more important characteristics of these same reactions are presented, together with a listing of the proteins and ligands used in each study.

B. Natural Linkages

We can point to only two situations where the synthetic connection established between carbohydrate and protein reflects a naturally occurring structure with full accuracy: where glycopeptides are used as ligands and where 1-amino sugars are coupled to protein carboxyl. In the first case, an unnatural linkage will, in general, be formed between the glycopeptide and the protein, although this may not, of course, affect recognition of the carbohydrate in biological systems. In the second case, *N*-glycosides of glutamine (not found so far in nature) as well as of asparagine are formed; in addition, the anomeric orientation will depend on methodology. This latter reaction also suffers from the drawback of rapid hydrolysis of 1-amino sugars in aqueous incubation mixtures, and the need to use a considerable excess of 1-amino sugar as nucleophile in the carbodiimide procedure to obtain a useful degree of modification.

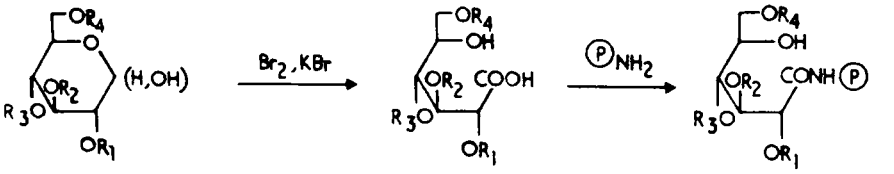
In most cases, then, the ligand-protein bond in neoglycoproteins (i.e., glycoproteins which do not correspond in all ways to a naturally occurring glycoprotein) is not natural, and the results of experiments carried out with such materials must be assessed with this

Table 1
METHODS OF CARBOHYDRATE CONJUGATION

I. Protein amino group (ⓅNH_2)

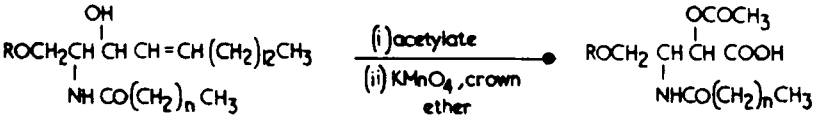
A. Using mono-, di-, or oligosaccharides (R)

1. Sugars converted to aldonates by Br_2 or I_2 oxidation, coupled by mixed anhydride method[22-24] or with water-soluble carbodiimide.[25,26] pH 11, 1 hr at O°C, or 18 hr at room temperature (RT) for mixed anhydride method; pH 4.75, RT, 6 hr for water-soluble carbodiimide.

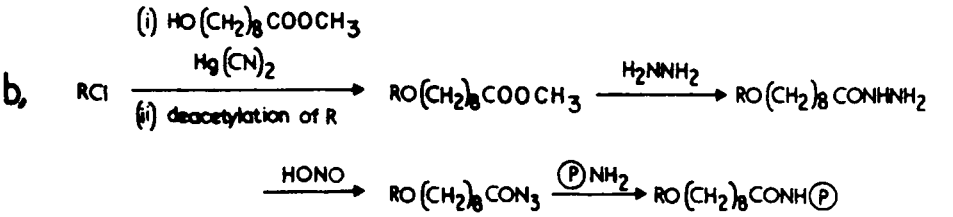


2. Glycosides synthesized with aglycons with terminal carboxyl groups[27-31] (see a and b below); then coupled via acyl azide (formed from ester via hydrazide, see b below);[27,28] *N*-hydroxysuccinimide and ethyl dimethylaminopropyl carbodiimide;[29,31] or mixed anhydride method.[30]

a,



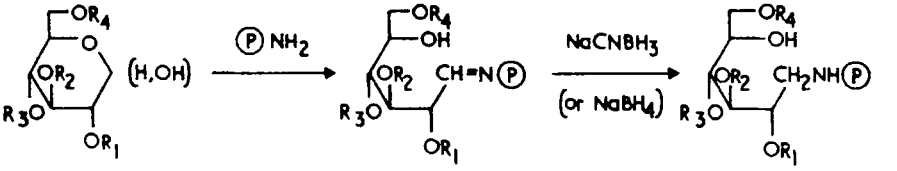
b,



3. Thioglycosides converted to 2-imino-2-methoxyethyl thioglycosides (imidates), coupled to proteins via amidine linkages;[29,32,33] pH 8—9, RT.

4. Sodium cyanoborohydride reduction of Schiff base formed with protein amino groups and (a) C-1 of reducing terminal sugars,[34-41] (b) aldehyde generated at C-6 of galactosyl residues,[42] or (c) synthesis of glycosides with aglycons having ω-aldehyde groups;[43] pH 7, RT, up to several days — less with ligands with free aldehyde functions.

a,



c,

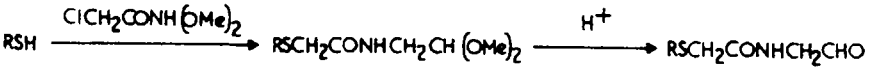
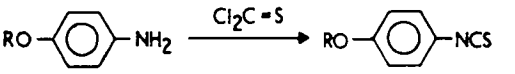
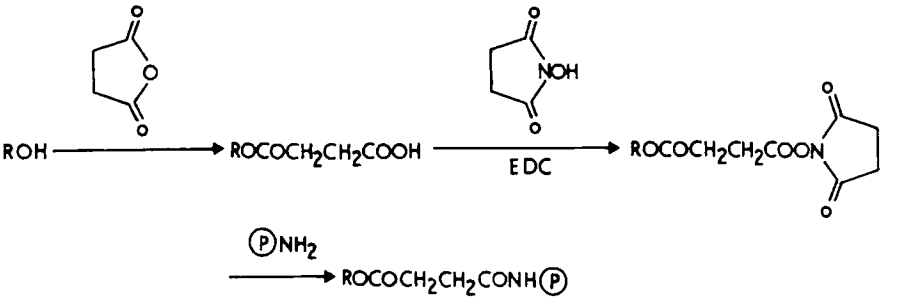


Table 1 (continued)
METHODS OF CARBOHYDRATE CONJUGATION

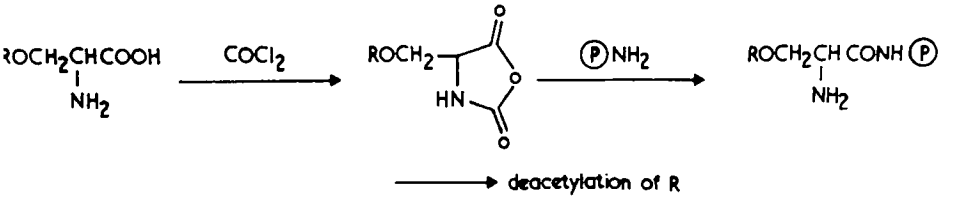
5. *p*-Aminophenylglycosides converted to *p*-isothiocyanato-phenyl glycosides,[44-49] or coupled to proteins with dimethyl suberimidate;[50] pH 9, RT, 6 hr; pH 8.5, RT, 2.5 hr (with dimethyl suberimidate).



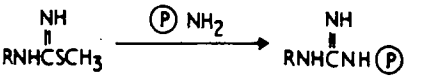
6. Amino derivatives of sugars (e.g., glycosylamines, 2-deoxy-2-amino sugars, or glycosides with aglycons with terminal amino groups) coupled via triazine trichloride;[30,51] or sugars activated with triazine trichloride coupled to proteins;[52,53] pH 7, 45°C, 1 hr for coupling by first method, but deacetylation in anhydrous methanol/NH_3, 0°C;[30] pH 7.2, RT, 1—3 hr[52] (see I.D.1 for chemistry of this coupling reaction, and References 54 and 55 for use of triazine trichloride in coupling proteins to insoluble matrices and properties of the reagent).
7. Succinyl sucrose activated with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide;[56] pH 7.4, 23°C, 16 hr.



8. Serine glycosides linked as *N*-carboxy anhydrides;[57-59] pH 7,4°C, but deacetylation as in I.A.6.



9. *S*-Methylglucosyl isothiourea coupled to protein (Lys, His residues) via guanidino groups;[60] pH 8.4, 0°C, 3 days.



B. Using glycopeptides as ligands
1. Coupled with toluene diisocyanate;[61] pH 9.4, 37°C, 12 hr (in the simplest case, with glycopeptide represented as *N*-glycosyl asparagine).

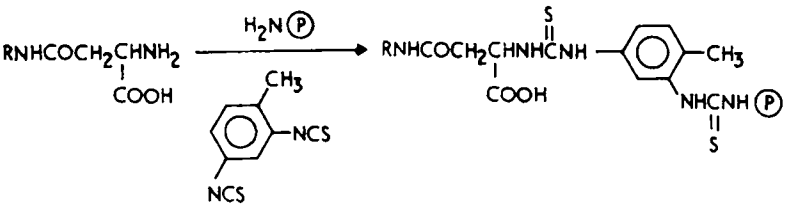
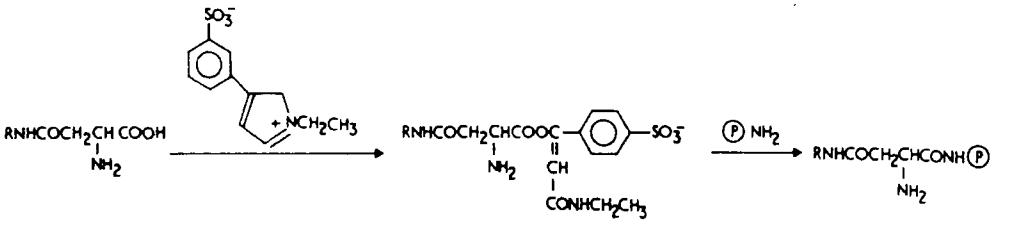


Table 1 (continued)
METHODS OF CARBOHYDRATE CONJUGATION

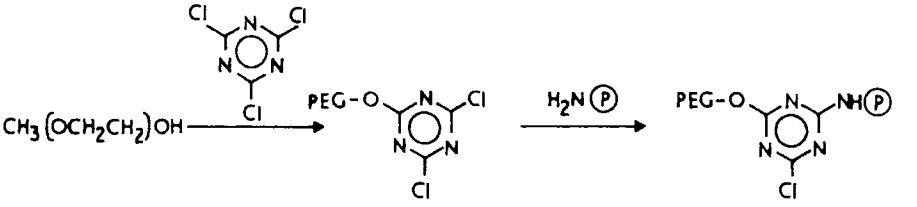
2. Woodward reagent K to activate carboxyl group of glycopeptide;[62] pH 6.5, RT, 6 hr.



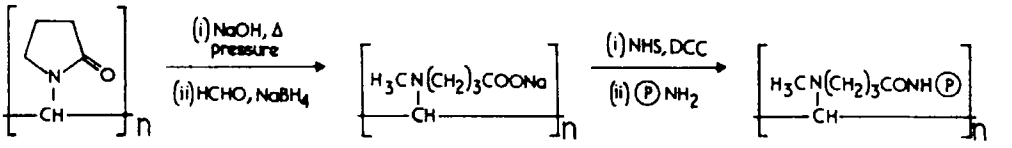
3. Glycopeptide cross-linked to protein with glutaraldehyde.[63] Activated glycopeptides incubated with protein 18 hr, RT, pH not specified. (This is a complex reaction involving several side-chain groups.[64])
4. Glycopeptide coupled by diazotization after attaching a *p*-aminophenyl group;[65] pH 9.3, 0°C.

C. Using dextrans, Ficoll or mannans
1. Cyanogen bromide activation of polysaccharide. (For reviews, see References 66-84. The chemistry is the same as that used in attaching various ligands to insoluble matrices for affinity chromatography (for example, see Porath[85]). Conditions vary with authors and proteins; in general, pH 7.5—9, 4°C, 12 hr.
2. Dextrans converted to dialdehyde by periodate oxidation, coupled by borohydride reduction;[86-87] pH 7.8—9.05, 25°C, 6—24 hr.
3. Galactomannan coupled with triazine trichloride.[88] For chemistry, see I.D.1; 25°C, 1 hr; 5°C, 18 hr.
4. Dextrans linked after activation with triazine trichloride.[89,90] For chemistry, see I.D.1.

D. Using polyethylene glycols (PEG)
1. Coupling with triazine trichloride.[99-102] Activated PEG incubated with protein at pH 9.2, 4°C, 1 hr or less[91-99] or pH 9.05, 25°C, 5 hr.[101] Disubstituted product thought to predominate.



E. Using polyvinylpyrrolidone (PVP)
1. Partial hydrolysis to expose PVP carboxyl groups, and blocking of secondary amine function by reductive methylation; carboxyl activation with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide (see I.A.7).[103-105] Activated PVP incubated with protein at pH 8.5, 4°C, 12 hr.



II. Protein carboxyl groups (Ⓟ COOH)
A. Water-soluble carbodiimide coupling of 1-amino sugars;[106-110] pH 4.75, RT, 1—2 hr in 8 *M* urea or 5 *M* guanidine;[107-109] pH 5.0, RT, 1—2 hr.[110]

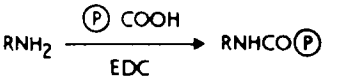
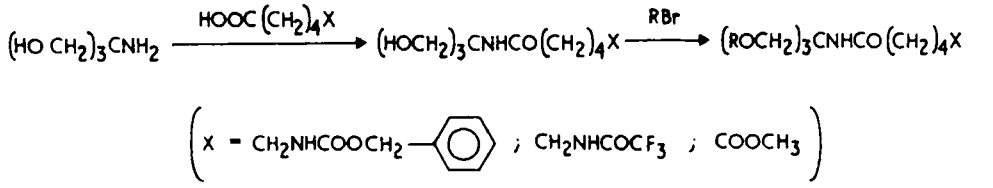


Table 1 (continued)
METHODS OF CARBOHYDRATE CONJUGATION

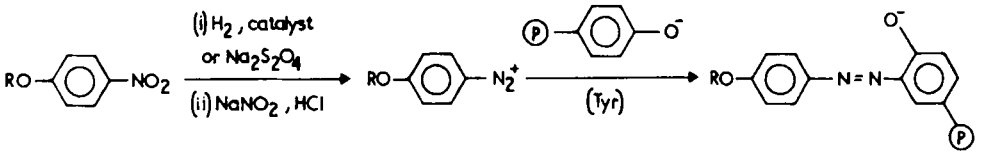
B. Preparation of "cluster" glycosides with aglycons having terminal amino groups suitable for coupling to protein carboxyl. No coupling to protein yet reported, but these glycosides have been immobilized on *N*-hydroxysuccinimide-activated polyacrylamide gels.[111,112]



C. Coupling of *p*-aminophenyl derivative of polyvinyl alcohol with water-soluble carbodiimide.[113]

III. Protein tyrosine, histidine, etc. (diazotization procedure)

A. For citations to the classical work of Goebel and Avery and others and details of procedures, see McBroom et al.[44] and References 1-4, 29, and 65; pH 9, 0° C, 2 hr.



B. Flavazole modification;[114-116] orthophenylenediamine and *m*-nitrophenylhydrazine condensed with sugars to form nitroflavazoles; pH 9, 0° C, 3 hr.

C. Glycosides with aglycons terminating in amino function coupled via diazonium salt,[117-119] pH 7.8, 0° C, 24 hr.

D. Diazo coupling of glycopeptides linked to *p*-aminophenyl group.[65] See also I.B.4; pH 9.3, 0° C.

in mind. From the point of view of divergence from natural structures there is, however, a considerable difference between a sugar hapten linked through a bulky, hydrophobic diazophenyl group to a side-chain tyrosyl residue, and a thioglycoside linked through a short spacer arm to an amino group of lysine. The choice of method for modification with carbohydrate will obviously be influenced by several factors, including any prior knowledge as to the contribution to protein function of certain side-chain groups.

C. Analytical Procedures for Attached Ligands

The classical procedures of carbohydrate chemistry are usually adequate. It must be kept in mind, of course, that when a disaccharide or other low molecular weight sugar is coupled by a procedure that alters the chemical nature of the innermost sugar (e.g., coupling of lactobionic acid), complete hydrolysis will yield only a part (50% for a disaccharide) of the sugar content of the sugar used for conjugation.

Alternative methods that have been used to estimate the degree of modification include automatic ion-exchange chromatographic analysis of borate complexes of released sugars,[29,32,37] analysis for amino sugars with the amino acid analyzer,[38] determination of lysine derivatives by amino acid analysis in the case of coupling procedures that form stable linkages to lysine,[36-38] decrease in the number of free amino groups, and covalent coupling of a radiolabeled ligand. The degree of substitution has not always been determined, especially in modifications involving high molecular weight ligands, and in these latter cases it is useful to estimate the molecular weight of the product.[80]

Table 2
SOME CHARACTERISTICS OF CARBOHYDRATE CONJUGATION

Reaction type (from Table 1)	Preliminary modification of ligand	Modification of innermost sugar	Effect on pI	Proteins applied to and ligands used
I.A.1	Some	Yes	Lower	Polylysine and milk oligosaccharides;[22] BSA and isomaltose, isomaltotriose;[23,24] BSA, ovalbumin, Con A, and several disaccharides[25,26]
I.A.2	Extensive	No, but "arm" present	Lower	BSA, ovalbumin, and several synthetic blood group antigens;[27,28] α-amylase and Gal, Glc;[29] BSA and GlcNAc, chitobiose, cellobiose;[30] BSA and "glycolipid acid" from gangliosides[31]
I.A.3	Extensive	No, except S-glycoside, and "arm" present	No change	α-Amylase, lysozyme and Gal, Glc, GlcNAc, Man[29,32,33]
I.A.4	None	Yes	No change	BSA and lactose, maltose, cellobiose;[14-16,40] *E. coli* asparaginase and lactose, NAN-lactose;[17] RNase A and lactose;[18] BSA, fetuin, asialofetuin, RNase B and raffinose;[42] polylysine and oligomers of GlcNAc;[36] BSA and several thioglycosides with aldehyde function on aglycon;[43] ricin and monophosphopentamannose, maltotriose;[41] diphtheria toxin A chain and LDL, monophosphopentamannose[41]
I.A.5	Extensive (conversion to isothiocyanate)	No, but "arm" present	Lower	BSA and Glc;[44] BSA and synthetic *Salmonella* antigenic determinants;[46-47] BSA and sialyl-oligosaccharides;[45,48] BSA and hemocyanin with α-(1 → 2) mannobiose[49]
	Some (coupling with dimethyl suberimidate)	No, but "arm" present	Lower	Lysozyme and gal[50]
I.A.6	None to extensive, depending on amino derivative used	No, but "arm" present	Lower	BSA and 2-aminoethylglycosides of GlcNAc, chitobiose, cellobiose;[30] glycine ethyl ester and $GlcNH_2$, $GalNH_2$, 1-amino derivatives of Glc, Gal, Man (model reactions)[51]
	None (with sucrose activated with triazine trichloride)	No, but "arm" present	Lower	LDL and asialofetuin, with ^{14}C sucrose[52,53]
I.A.7	Some	No, but "arm" present	Lower	LDL and BSA with sucrose[56]
I.A.8	Extensive	No, but "arm" present	No change	Serine glycosides of Glc, Gal, GlcNAc, rhamnose, cellobiose, lactose, and synthetic polyamino acids[57-59]

I.A.9	Some	No	Lower	Trypsin and Glc[60]
I.B.1	Some	—	Lower	Glycopeptides from fetuin, immunoglobulin, and lysozyme, BSA[61]
I.B.2	Some	—	Lower	Lysozyme, and glycopeptides from fibrinogen[62]
I.B.3	Some	—	Lower	*Acinetobacter* glutaminase-asparaginase and glycopeptides from bovine fibrin, human γ-globulin[63]
I.B.4	Extensive	—	No change	Horseradish peroxidase, ferritin, and glycopeptides from thyroglobulin[65]
I.C.1	None	Yes	Lower	Dextrans and trypsin, α-amylase, β-amylase, catalase;[71,72] α-amylase, catalase;[76,77,79] lysozyme, chymotrypsin, trypsin;[80,81] CPase G and arginase;[82] heparin and Ficoll;[83] chymotrypsin;[32] insulin;[69] Sephadex G-200 and cytochrome c, adduct solubilized with dextranase[84]
I.C.2	Some	Yes	Lower	Lysozyme, BSA, ragweed allergen, and dextran[86]
I.C.3	None	Yes	Lower	Bovine γ-globulin and a galactomannan[88]
I.C.4	None	—	Lower	Chymotrypsin;[90] α-amylase[89]
I.D.1	None	—	Lower	PEG and catalase;[92] BSA;[91] trypsin;[93] arginase;[94] ovalbumin, ragweed allergen;[100] ragweed allergen;[101] *E. coli* asparaginase;[102] amidohydrolase of *Achromobacter*;[95] uricase;[95,99] superoxide dismutase;[97] adenosine deaminase;[98] phenylalanine ammonia lyase[96]
I.E.1	Extensive	—	Lower	PVP and trypsin, chymotrypsin;[103,104] β-D-*N*-acetylhexosaminidase A[105]
II.A	Some (glycosylamine)	None	Higher	Poly-L-asp and chitobiosylamine;[106] chymotrypsinogen, Glc;[110] lysozyme and 1-amino derivatives of Gal, Man, Glc, lactose, GlcNAc[107-109]
II.B	Extensive ("cluster" glycosides)	No, but "arm" present	Higher	6-(Aminohexamido) tris (galactosyloxymethyl- and *N*-acetyl-glucosaminyloxymethyl) methane derivatives linked to polyacrylamide;[108] no applications to proteins to date
II.C	Extensive	No, but "arm" present	Higher	Glucose oxidase, horseradish peroxidase[113]
III.A	Extensive	No, but "arm" present	No change	A wide variety of sugar haptens coupled to such proteins as BSA, γ-globulin, edestin[1-4]
	Extensive	No, but "arm" present	No change	Lactose, chitobiose coupled to horseradish peroxidase and ferritin;[65] α-amylase and *p*-diazophenylthioglycosides of Gal, Glc, Man[29]
III.B	Extensive	Yes	No change	Variety of sugar haptens coupled to proteins like BSA, edestin[114-116]
III.C	Extensive	No, but "arm" present	No change	BSA, edestin with synthetic di-, tri-, and tetrasaccharides[116-118]
III.D	Extensive	—	No change	Horseradish peroxidase, ferritin, and *p*-aminophenyl derivatives or glycopeptides from thyroglobulin[65]

D. Extent of Modification

The feasibility of varying the extent of modification has been examined systematically in only a few cases. When a protein is being used simply as a carrier for sugar haptens in studies involving immunogenicity or cross-reactivity, the goal is usually to attach as many moles of ligand as possible without complete denaturation and the formation of an insoluble product. On the other hand, if the goal is to modify an enzyme so as to extend its plasma clearance time or its resistance to protease destruction, then it is necessary to consider the likelihood that carrying the modification too far will adversely affect the biological activity.

1. Aldonates (I.A.1)

Aldonate coupling using the mixed anhydride method gives low yields (13 to 40% based on starting sugar) and requires high molar ratios of sugar to protein (50-100:1). With a series of milk oligosaccharides, Zopf and Ginsberg[22] showed that high ratios usually gave more highly substituted products, but not in a readily predictable way. Using the water-soluble carbodiimide coupling procedure, Lonngren et al.[25,26] carried out a more extensive study of the effect of varying sugar to protein ratios. They found the highest degrees of substitution (about 30 mol sugar per mole of protein) required ratios of 500:1, but even with a ratio of only 60:1 (1:1 in terms of moles of aldonate to lysyl residues in the BSA used), the product had a 7:1 molar ratio of sugar to protein.

2. Glycosides with Carboxyl-Terminal Aglycons (I.A.2)

Lemieux et al.[27,28] reported that the glycosides synthesized with acyl azide groups at the free end of the aglycon are sufficiently reactive to allow easy variation of the degree of substitution, but the only example described is the incorporation of 30 haptens/mol of BSA at a hapten:protein incubation level of 60:1. Working with acyl azides prepared from tripeptide haptens, Inman et al.[120] reported that the degree of conjugation increased with the amount of azide added up to about 0.08 m*M* hapten azide. This experience is probably transferable to the sugar acyl azide haptens. Krantz et al.[29] reported that an 80:1 ratio of thioglycosides with carboxyterminal "arms" was required for the incorporation by the carbodiimide method (*N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) in dimethylsulfoxide) of 6 to 10 mol/mol of alphaamylase. Under these conditions, enzyme activity was completely lost. Less than 1 mol was incorporated with "modest excesses" of ligand.

Hakomori[31] has used potassium permanganate (solubilized in benzene with a crown ether) to cleave the olefinic bond in glycosphingolipids and generate a "glycolipid acid" which can then be coupled to various materials. In the application relevant here, "glycolipid acid" activated with *N*-hydroxysuccinimide and EDAC was coupled to methylated bovine serum albumin (ratio of ligand to protein approximately 15:1). The extent of coupling was not reported (see Table 1, I.A.2).

3. Thioglycoside Imidate Coupling as Amidines (I.A.3)

Lee et al.[32] carried out an especially thorough study on the effects of several variables on the degree and rate of protein modification by 2-imino-2-methoxy-1-thioglycosides (IME-thioglycosides). The same level of coupling was achieved at 0°C as at room temperature, but completion at the lower temperature required 25 to 30 hr instead of 6. The rate and extent of reaction increased with incresing pH up to about pH 9, with only about 2 mol of galactose being incorporated per mole at pH 7.3 as compared to 11 at pH 8.9 or 9.6.

Lee et al.[32] also found that not only did the extent of modification vary from one protein to another, but the rate of modification varied, with BSA being more rapid than either lysozyme or α-amylase.

Finally, the number of sugars incorporated depended on the ratio of imidate to protein, with 28 to 41 mol of sugar being attached to BSA (with 59 amino groups) with a 30-fold excess of ligand to protein amino groups. The number of amino groups reactive to the IME-thioglycosides was similar to the number reactive to reagents like dinitrobenzenesulfonate.

4. Cyanoborohydride Reduction of Schiff's Base (1.A.4)

The cyanoborohydride reduction method introduced by Gray[34,40] is attractive because it is the only procedure for incorporating di- or oligosaccharides that requires no prior chemical modification of the ligand. Several investigators have examined the effects of pH, temperature, incubation time, and to a lesser extent, ligand to protein ratio on the extent of modification. These results are not in complete agreement, perhaps because different proteins were studied, but they allow several generalizations to be made.

The reaction is slow with reducing sugars because they exist in the free aldehyde form to a very limited extent. Incubations are usually carried out for several days, and the extent of modification can be regulated conveniently in this way. More studies have been done with lactose than other ligands (the rate varies with different oligosaccharide ligand).[37,39] In general, from 5 to 20 mol of lactose can be coupled per mole of protein in 2 to 5 days.[37,38] Exceptions to this generalization — that maximal modification is achieved only after several days — are found in systems containing aldehyde groups not involved in hemiacetal formation.[42,43] Van Zile et al.[42] studied the incorporation of raffinose (Galpα1-6Glcpα1-2βFruf) treated with galactose oxidase. The reaction was complete in 18 to 24 hr at room temperature, and although they obtained incorporations of only 0.3 to 0.4 mol of tritium-labeled ligand per mole of protein, they did so with ratios of ligand to protein lysyl groups of only 1-3:1.

Lee and Lee[43] recently described the preparation of a series of thioglycosides with ω-aldehyde groups in the aglycon and the coupling of one of these to bovine serum albumin by reductive amination. As with the work of Van Zile et al.,[42] this procedure overcomes the need for prolonged incubations. In the example cited 14 mol of galactose were linked to bovine serum albumin in a 22-hr incubation.

5. Coupling of p*-Isothiocyanatophenyl Glycosides (1.A.5)*

There appear to have been no studies on the feasibility of varying the extent of modification by this procedure. In the single example cited by McBroom et al.[44] 90% modification of lysyl residues in BSA was achieved at a ligand:protein ratio of about 200:1, but Lindberg et al.[47] (using the same procedure) reported an average of only 4 mol of disaccharide per mole of BSA. Reichert and Goldstein[49] used this procedure recently in studies designed to generate antibodies with the same sugar-binding specificity and other biological properties as concanavalin A. Thus *p*-phenylisothiocyanato-2-O-α-D-mannopyranosyl-α-D-mannopyranose was coupled to BSA and hemocyanin. They found 22 mol of the ligand conjugated to BSA, and 533 mol/mol of keyhole limpet hemocyanin (molecular weight assumed to be 2×10^6).

6. Coupling of 2-Aminoethyl Glycosides with Triazine Trichloride (1.A.6)

King et al.[30] compared the extent of modification of BSA by two coupling procedures, the triazine trichloride method with 2-aminoethylglycosides and the mixed anhydride procedure with 8-carboxyoctyl glycosides. Under what were judged to be optimum conditions, they reported that the degree of substitution was much the same in each series

(15 to 24 mol/mol of BSA). This is lower than that found by Lemieux et al.[27,28] with acyl azide coupling, higher than with the *p*-isothiocyanato example cited by Lindberg et al.,[47] and about the same as with diazo coupling.[44] No attempts to vary the degree of substitution were reported. Pittman et al.[52,53] have used triazine trichloride activation to couple small amounts of ^{14}C-sucrose to LDL and asialofetuin.

7. Coupling of Succinyl Sucrose (I.A.7)

With 5 to 12 μmol of activated sucrose and 500 mg of LDL* (approximate molar ratio of ligand to protein of 20-50:1 assuming LDL molecular weight of 2×10^6), from 2 to 10 μmol of sucrose were bound.[56]

8. Serine Glycosides Linked as N-*Carboxyanhydrides (I.A.8)*

Rude et al.[57-59] used the *N*-carboxy-α-amino acid anhydride derivatives of *N*- and *O*-acetylated serine *O*-glycosides to make derivatives of basic synthetic polypeptides, so the glycosidic linkage in the conjugate is a naturally occurring one. Glycosides of glucose, galactose, rhamnose, *N*-acetylglucosamine, cellobiose, and lactose were used both singly and in combination. Up to 10% by weight of sugar (or a 1:6 molar ratio of sugar to amino acid) could be incorporated. Efficiencies of incorporation were about 40% after reaction of equimolar quantities of serine glycoside and peptide in anhydrous dioxane. In each case the sugar was de-*O*-acetylated in anhydrous methanolic ammonia after conjugation to the polypeptide.

9. Modification with Glycopeptides (I.B)

All of the methods used for coupling glycopeptides to proteins involve prior activation of the glycopeptide. It should be possible to vary the degree of substitution in the final conjugate by varying the ratio of activated glycopeptide to protein but such studies have not been reported, presumably because of the difficulties inherent in preparing adequate quantities of glycopeptide. Both Rogers and Kornfeld[61] and Moczar and Sepulchre[62] reported the formation of conjugates of lysozyme containing 1.5 to 2.0 mol glycopeptide per mole protein. The degree of substitution was sufficient in the first case to demonstrate the expected recognition phenomenon in the modified protein. Holcenberg et al.[63] did not attempt to vary the degree of substitution and only reported that *Acinetobacter* glutaminase-asparaginase modified with fibrin and gamma globulin glycopeptide had 7.1 and 14%, respectively, by weight of neutral sugars, and free amino residues were decreased 16 to 22% by both succinylation and carbohydrate conjugation.

10. Modification with Dextrans (I.C)

Marshall[66,67] has pointed out that dextran-enzyme conjugates are likely to consist of a heterogeneous mixture of high molecular weight aggregates, with cross-linking attributable to the activation by cyanogen bromide of several monosaccharide units in a single dextran molecule. The degree of substitution becomes, then, a question of the average number of dextran molecules per mole of enzyme (or vice versa) based either on molecular weight estimates[80] or percent carbohydrate on a weight basis.[72,74] Varying the extent of modification appears to be difficult by this method.

11. Modification with Polyethylene Glycol (I.D.1)

Abuchowski et al.[91] showed that increasing the ratio of activated PEG to available amino groups led to increased substitution. With a tenfold excess there is a 90%

* Low density lipoprotein.

disappearance of amino groups in the case of BSA; with a 1:1 ratio about 42% of the amino groups are substituted by PEG-5000. Catalase is less reactive; tenfold excess of PEG causes the modification of about 40% of amino groups.[92] There is no apparent difference in the reactivity of PEG-1900 and PEG-5000. Abuchowski et al.[93] have also shown that the degree of substitution can be changed by changing the incubation time. Ashihara et al.[102] also reported increased substitution of amino groups in *E. coli* asparaginase upon increasing the molar ratio of activated PEG. As the ratios were changed from 0.5:1 to 10:1, the degree of substitution changed from 18 to 73 mol PEG per mole of enzyme (there are 92 lysines), and the enzyme activity declined from 50% retention at the lowest degree of substitution examined to 7 to 15% at the highest.

12. Coupling of 1-Amino Sugars to Protein Carboxyl with Water-Soluble Carbodiimide (II.A)

It should be possible to alter the degree of substitution by changing the incubation time, but since 1-aminoglycosides are unstable in aqueous solution, this is not simple. In the examples reported the degrees of modification have been rather low (up to 3 of 11 carboxyl groups modified in lysozyme,[107-109] and up to 3 of 15 carboxyl groups modified in chymotrypsinogen[110]).

Shier[106] prepared an "artificial antigen" by coupling chitobiosylamine to poly-L-aspartate. When these two materials were incubated with water-soluble carbodiimide for a week at room temperature in an approximately equimolar ratio of 1-amino sugar to aspartyl units in polypeptide, about 13% of the available carboxyl groups were derivatized.

There appears to be no good reason why glycosides with stable aglycons terminating in free amino groups could not be coupled efficiently to protein carboxyl groups. The "cluster" glycosides of Lee[111] are examples of such materials and although they have not (to the best of our knowledge) yet been coupled to proteins, they have been linked to *N*-hydroxysuccinimide-activated polyacrylamide gels.[112] In most such methods where coupling of amine-containing ligands to protein carboxylate is effected through an activated ester (e.g., using carbodiimide or *N*-hydroxysuccinimide), the problem accrues that not all of the activated groups may be coupled to ligand, producing instead unwanted by-products the presence of which may in itself affect protein activity.

13. Diazo Coupling (III)

Most of the applications of this classical procedure have been for the purpose of preparing antigens with carbohydrate haptens in situations where extensive modification was desirable and where the availability of oligosaccharide was not an acute problem. We are aware of only one study on the feasibility of varying the degree of modification using the diazo procedure. Using *p*-diazophenyl thioglycosides to modify α-amylase, Krantz et al.[29] reported that increased ratios of ligand to protein resulted in increased levels of incorporation, but the "extent of modification as a function of the thioglycoside:protein ratio was not highly reproducible." In the case of *p*-diazophenyl β-thiogalactoside, for example, as the ligand to enzyme reaction mixture ratio was changed from 25:1 to 500:1, the number of moles of sugar bound to protein increased from 4 to 79.

III. EFFECT OF CARBOHYDRATE CONJUGATION ON BIOLOGICAL ACTIVITY

A. Introduction

Experiments involving modification with carbohydrate have rarely been carried out for the express purpose of studying the effect on enzyme activity. Originally the purpose

of carbohydrate conjugation was to introduce sugar haptens into proteins devoid of enzyme activity (bovine albumin, edestin, gamma globulin, etc.) to permit studies on the immune response to these determinants. Work of this kind continues,[49] but interest in glycosylation has broadened recently for several reasons. There is much current interest in the feasibility of enzyme chemotherapy for the treatment of lysosomal deficiency diseases[121-125] and leukemia.[126,127] This has led in turn to an interest in extending the clearance time of injected proteins, reducing the antigenicity of such proteins, and improving their resistance to thermal denaturation and inactivation by proteases. Improved thermal stability is also of interest in bioengineering applications. The realization that processes such as cell-cell interaction, cell-substrate binding, specific endocytosis, and the binding of hormones, other plasma proteins, and lectins to surface receptors all involve glycoproteins has been responsible for increased interest in methods of glycosylation. In this section the effects carbohydrate conjugation has on several biological properties are discussed.

B. Effect on Immune Response

No attempt will be made to review the large amount of work that has been done on the nature of the immune response to sugar haptens coupled to protein carriers. Many of the principles of immunology were established with the help of synthetic carbohydrate-protein conjugates, and the subject has been covered in numerous reviews and monographs.[2-4,128] When proteins modified by the attachment of relatively low molecular weight carbohydrate haptens are used as antigens, the conjugates are still antigenic, sometimes more so than the original protein. The resulting antibody population will include molecules reactive towards the native protein molecule if the modification has not been too extensive, since not all of the determinant groups in the original protein have been masked by the hapten. However, when proteins are modified by the attachment of high molecular weight materials such as dextrans or polyethylene glycols that are themselves weakly or even nonimmunogenic, it is often the case that the capacity of the protein to react with antiprotein antibody is reduced or abolished. Its immunogenicity is also often affected in the same way.

1. Modification with Dextrans

Modification of proteins by the attachment of high molecular weight dextrans reduces both the antigenicity of proteins (i.e., capacity to interact with antibody) and the immunogenic or allergenic activity. Using high molecular weight periodate-oxidized dextran, King et al.[86] obtained conjugates with ragweed pollen antigen E (Ag-E) containing from 2 to 5 mol of dextran per mole of Ag-E (approximately 70% by weight of dextran), and found that both antigenic and allergenic activities were seven- to eight-fold less than with Ag-E on a molar basis (skin tests on ragweed-sensitive patients and histamine release assays with leukocytes from ragweed-sensitive donors). Treatment of the conjugates with dextranase restored initial activity. The conjugates were, however, still immunogenic in rabbits, producing antisera with hemagglutination activity against both Ag-E and dextran.

Marshall and Humphreys[78,79] showed that conjugates of high molecular weight dextran with alpha-amylase and catalase were less reactive with antibody to native enzyme than was the native enzyme, and the immunogenicity of the conjugate was reduced. They also reported that the allergenic response towards conjugates in guinea pigs presensitized with alpha-amylase was eliminated. The conjugates still caused antibody formation in guinea pigs, although at a reduced level.

The importance of using relatively high molecular weight dextrans to cause these effects is pointed out by the work of Richter and Kagedal.[70] Using dextrans with a

molecular weight of only 4400, they made dextran conjugates containing up to 30% carbohydrate with several proteins and found that these materials were strongly immunogenic in several species when administered repeatedly in adjuvant. In this case it would appear that the protein macromolecule has increased the immunogenicity of the low molecular weight homopolymer used as ligand.

2. Modification with Polyethylene Glycols

Davis and collaborators at Rutgers have carried out several studies on the effects of modification with polyethylene glycol (PEG) on the antigenicity and immunogenicity of various proteins.[91-99] BSA conjugated with PEG-1900 (i.e., with a molecular weight of 1900) loses its immunogenicity in rabbits by either the i.m. or the i.v. route when there has been a sufficient degree of modification, and the conjugate does not react against anti-BSA.[91] These results were obtained using both immunodiffusion and complement fixation techniques. Antialbumin antisera raised by either route reacted with decreasing effectiveness against PEG-5000-albumin conjugates containing increasing amounts of PEG-5000; there was no reaction at all with 42% modification of amino groups.

In addition, the clearance pattern of PEG-1900-BSA from rabbits immunized with BSA was the same as in nonimmunized animals, in sharp contrast to the clearance of albumin itself; the failure to observe any difference between clearance of PEG-1900-BSA in nonimmunized animals and animals treated with PEG-1900-BSA showed that the conjugates were nonimmunogenic.

Similar results were obtained with PEG conjugates of catalase, although the masking of determinant groups on catalase by PEG seems to be somewhat less effective than with BSA. For example, it was possible to raise antisera that reacted with both conjugate and native catalase using a PEG-1900-catalase conjugate (43% modification of amino groups) via the i.m. route.[92]

More recently Davis et al. have extended the PEG modification technique to trypsin and arginase.[93,94] In general, very similar results were obtained. Trypsin modified by the attachment of PEG-5000 to 24% of its amino groups (corresponding to about 3 PEG strands per mole) did not react with antitrypsin antisera. Arginase modified with PEG-5000 (53% modification) did not react with antisera against native arginase, and an anticonjugate antiserum reacted with neither native enzyme nor conjugate. These experiments were conducted using the i.v. route only for attempts to raise antisera against conjugates, and the conclusions reached were based on immunodiffusion alone, not complement fixation.

Davis's group has also used PEG to modify an amidohydrolase from *Achromobacter*[95] and a phenylalanine ammonia lyase.[96] Results with the former were much the same as with trypsin and arginase, i.e., the conjugate was nonimmunogenic. With the phenylalanine ammonia lyase, however, anomalous results were obtained. The conjugate, while reacting poorly with antibodies formed against it, was nevertheless a good immunogen, and the authors point out that in spite of their success with other enzymes the PEG coupling method cannot be regarded as a completely general method for controlling immunogenicity.

One of the most striking observations in this area was made by Lee and Sehon.[100] Allergic conditions such as hay fever and drug hypersensitivities are known to be mediated by allergen-specific IgE antibodies. Lee and Sehon[100] used modification with PEG to convert a well-defined protein antigen (ovalbumin) and ragweed pollen allergen (RAG) to products that are not only nonallergenic, but also tolerogenic. PEGs with average molecular weights of 6000 and 20,000 (PEG_6 and PEG_{20}) were coupled to ovalbumin (OA) and RAG with triazine trichloride. The extent of modification was not reported. Intravenous administration of OA-PEG_6 to mice 4 hr before immunization

with the sensitizing dose of DNP-OA completely suppressed the primary anti-DNP and anti-OA IgE responses, and a further sensitizing dose of DNP-OA had little effect. The injection of free PEG or triazine trichloride-treated OA did not interfere with the animal's ability to produce an IgE response to DNP-OA. The immune suppression is specific; treatment with OA-PEG_6 did not affect the capacity of the animals to produce IgE antibodies to both components of another DNP conjugate, DNP-Asc, in which DNP is conjugated to protein in an *Ascaris* extract. Similarly, the administration of RAG-PEG_6 or RAG-PEG_{20} to mice which had been sensitized with RAG blocked the ongoing anti-RAG IgE response. The tolerance induced with PEG-antigen conjugates could be maintained after spleen cells from an animal made tolerant were transferred into X-irradiated recipients.

King et al.[101] made similar observations with respect to the sharply reduced allergenicity of RAG-PEG conjugates, but their work did not extend to the question of tolerogenicity. They reported an allergenicity of only 0.024 (compared to 1 for unmodified RAG allergen) in a sample prepared by coupling 7 residues of PEG-2000 per mole of RAG.

Finally, Ashihara et al.[102] modified *E. coli* asparaginase extensively with PEG-5000 (73 amino groups of 92). This extensive modification led to complete loss of activity towards antiasparaginase, together with almost complete loss of enzyme activity. A series of samples with increased substitution showed a steady decrease in antiasparaginase binding.

C. Effects on Thermal Stability and Resistance to Proteolysis

In general, with adducts of high molecular weight dextrans containing a high proportion of carbohydrate (e.g., 89% in the case of a trypsin-dextran conjugate described by Marshall and Rabinowitz[71,72]), thermal stability and resistance to proteolysis are substantially improved. The trypsin-dextran conjugate incubated at 60° C, e.g., showed little loss of activity over a 2-hr period (in either glass or plastic), while the native enzyme lost 25% activity in plastic and 85% in glass. Similarly, the alpha-amylase of *Bacillus amyloliquefaciens* showed a change in $t_{1/2}$ at 65° C from 2.5 min for native enzyme to 60 min for the conjugate; sweet potato beta-amylase had its $t_{1/2}$ at 60° C increased from 5 to 175 min.[79] Wykes et al.[89] and O'Neill et al.[90] reported similar findings for alpha-amylase and chymotrypsin linked to dextran using the triazine trichloride coupling procedure. There was also an increased resistance to denaturation in the presence of 8 *M* urea and mercaptoethanol, or sodium dodecyl sulfate. Also, with alpha-amylase — which is usually inactivated in the presence of EDTA due to chelation of essential calcium followed by unfolding — conjugation to dextran markedly reduced the loss of activity seen in the presence of EDTA over a 2-hr period.[74]

Similar results were found when resistance to proteolysis was examined. In the case of trypsin there was no loss with the conjugate during a 2-hr incubation, whereas native enzyme retained less than 20% activity. Also, inhibition of trypsin by high molecular weight trypsin inhibitors was less effective with the dextran conjugate, sometimes strikingly so. As an expected corollary, trypsin activity against high molecular weight substrates (i.e., its proteolytic activity as opposed to its esterase activity) was also sharply reduced. Similar results were obtained by Vegarud and Christensen[80,81] with dextran conjugates of lysozyme, alpha-chymotrypsin, and trypsin.

In partial contrast to dextran modification, modification with polyethylene glycol seems to have no effect on thermal stability, at least in the case of catalase,[92] but does provide protection against proteolysis in much the same way as dextrans. For example, PEG-5000-catalase still had 90% of the original activity after 150 min incubation with trypsin, while native catalase showed a total loss in 40 min. Also, catalase lost 90% of its

activity with *Streptomyces griseus* pronase at 60 min, whereas the PEG-5000 conjugate lost 20%. Similar results were obtained with PEG-trypsin[93] and PEG-phenylalanine and ammonia lyase.[96] A marked stabilization to autolysis was also found by von Specht et al. with PVP conjugates of trypsin and chymotrypsin,[103,104] and a PVP conjugate of hexosaminidase A (containing approximately 9 mol of PVP, 10,000 mol wt, per mole of enzyme) was more resistant to proteolysis than the free enzyme, although no effect was seen with respect to thermal denaturation or denaturation caused by low pH.[105]

The resistance of these conjugates to autoproteolysis or to effective interaction with high molecular weight inhibitors or substrates is presumably due to the steric interference provided by the large amount of carbohydrate (or other hydrophilic substance) attached in the form of bulky chains. It is worth noting that in general, improved heat stability is only seen with the dextran derivatives and not with proteins modified by the attachment of PEG or PVP. However, further physical studies are required to elucidate the different mechanisms of stabilization.

Modification by the attachment of low molecular weight carbohydrate ligands had less dramatic effects than those seen with dextran, PEG, or PVP. For example, Marsh et al.[37] found that the attachment of 8 galactosyl residues per mole of *E. coli* asparaginase (i.e., lactose coupled by reductive amination) had no effect on thermal stability (10 min at 60°C), but that a sample with 20 such residues per mole retained 63% activity as compared to only 19% for the unmodified enzyme. This modification also provided protection against inactivation by subtilisin. The native enzyme was completely inactivated after 20 min under the conditions used, whereas the lightly modified asparaginase sample retained approximately 40% activity and the extensively modified sample, 70%.

D. Effects on Specific Binding to Receptors: Clearance

Proteins are removed from the circulation as a result of several mechanisms including glomerular filtration (for low molecular weight proteins), the action of plasma proteases, and endocytosis. The term "endocytosis" includes both phagocytosis and pinocytosis, the former generally used to describe the uptake of large aggregates or denatured proteins and pinocytosis ("drinking") referring to the uptake of small particles and solutes of all kinds in microdroplets. Most — perhaps all — eukaryotic cells engage in endocytosis, but aside from circulating leukocytes and macrophages, it is the liver that plays the most important role in protein clearance by this mechanism.

Clearance by endocytosis may be nonspecific (e.g., the phagocytic clearance of heat-denatured protein, or the removal of solute in pinocytotic vesicles formed at a rate characteristic of a particular cell type), but solutes that bind specifically to the plasma membrane are cleared far more rapidly than can be accounted for solely on the basis of their concentration in the imbibed extracellular fluid. There is ample evidence now to show the role of carbohydrate in the specific binding of a number of proteins to plasma membrane receptors, and it is this particular mode of clearance, i.e., clearance by specific or adsorptive endocytosis, that is of interest to us here.[129]

The liver is comprised of three major cell types: hepatocytes (also referred to as parenchymal cells), Kupffer cells, and endothelial cells. The latter two account for about 15% each of the liver cell population and are important constituents of the reticuloendothelial system. It is generally recognized that denatured proteins are phagocytized by cells of this kind.[130] Nevertheless, there is evidence now to suggest that Kupffer and endothelial cells participate in adsorptive pinocytosis as well as in phagocytosis. Hepatocytes make up approximately 70% of the liver cell population and their role in adsorptive endocytosis of plasma glycoproteins has been documented with remarkable thoroughness by Ashwell, Morell, and collaborators in the last 10 years. This

work has been the subject of several reviews,[131-133] and a summary only will be presented here as an example of glycan-receptor interaction, and background for the discussion of the effect of various protein glycosylations on their clearance.

Starting with the initial observation that human asialoceruloplasmin was cleared from the circulation of rabbits in minutes, compared to the known half-life of native ceruloplasmin of 56 hrs Ashwell, Morell, and collaborators have proceeded to show the following:

1. With the apparent exception of human transferrin in rabbits (which can be explained in another way, not relevant to the present discussion[134]), the phenomenon is general for all plasma glycoprotein examined.
2. Rapid clearance of asialoglycoproteins in mammals is galactose-specific; i.e., the penultimate beta-galactosyl residue that is made terminal by neuraminidase treatment must be present in unmodified form.
3. Radioactivity from labeled asialoglycoproteins is located exclusively in the parenchymal cells.
4. The hepatocyte plasma membrane receptor has been isolated from rats; it is a glycoprotein with nonreducing terminal sialic acid, the latter being essential to the recognition of galactosyl-terminated glycoproteins.
5. The rat liver binding protein is not restricted to the external hepatocyte membrane, but is also present on the membranes of Golgi apparatus and smooth microsomes (luminal surface) and lysosomes (cytosolic surface).[135,136]
6. The galactose-specific binding protein is absent from avian and, presumably, reptile liver (as judged in the latter case only by the presence of circulating asialoglycoprotein); but there is present in avian hepatocytes a binding protein specific for terminal *N*-acetylglucosamine residues in glycoproteins. The binding activity of this latter protein is not affected by neuraminidase, but is destroyed by a combination of neuraminidase and beta-galactosidase.[137,138]

Very recently Hubbard et al.[139,140] used electron microscope autoradiography to identify the cell types responsible for binding and removing glycoproteins. Specifically, they assessed the distribution in liver of seven different ^{125}I-labeled glycoproteins. They found that three of these (asialofetuin, asialoorosomucoid, and lactosaminated RNase A dimer) were bound and internalized almost exclusively by hepatocytes. Four others, however, the oligosaccharide chains which terminated in either *N*-acetylglucosamine (agalactoorosomucoid) or mannose (ahexosamino-orosomucoid, preputial beta-glucuronidase and mannobiosaminated RNase A dimer) were internalized only by Kupffer and endothelial cells, with the latter being considerably more active than Kupffer cells.

There is good reason to believe that these recognition processes may be influenced by other features of the glycoprotein, in addition to the exposed sugar,[141] but the crucial involvement of carbohydrate in plasma protein clearance is unequivocally established. In addition to the *N*-acetylglucosamine binding protein of avian liver, the presence of mammalian membrane receptors for mannosyl/*N*-acetylglucosaminyl[139] and fucosyl[142] residues has been proposed.

One of the goals of protein modification by carbohydrate conjugation is to alter the clearance behavior of the protein, either in a relatively nonspecific way so that the injected protein simply remains in the circulation for a longer time, or in a specific way with the objective of specific uptake by a particular organ or tissue ("targeting"). Rogers and Kornfeld[61] were one of the first to recognize the value of carbohydrate conjugation as a tool for studying specific interactions at cell surfaces. They studied the uptake of ^{131}I-labelled lysozyme and albumin, and conjugates of these proteins with glycopeptides and asialoglycopeptides of fetuin into rat liver in vivo. They found that neither lysozyme

nor BSA were removed during the 10-min experimental period. Intact glycopeptide conjugates likewise were not taken up into liver, but the sialidase-treated glycopeptide conjugates were rapidly removed. These results were not unexpected, in view of the work of Ashwell, Morell, and collaborators already discussed, but they represent an early and important extension of that work.

Considerable interest has been shown in asparaginase because of its established antileukemic activity.[127] Its clearance behavior (and that of several amidohydrolases active against both glutamine and asparagine and possessing antileukemic activity) has been studied by several workers using different methods of modification.[37,63,87,95]

Holcenberg et al.[63] used glutaraldehyde to couple glycopeptides to an *Acinetobacter* glutaminase-asparaginase, and found a marked prolongation of the half-life of the enzyme in mice, rats, and rabbits. The $t_{1/2}$ for the unmodified enzyme in normal mice, e.g., is about 1.1 hr. This was increased to a maximum of about 16 hr by extensive coupling of glycopeptides from gamma globulin and fibrin. It has been known for some time that the clearance time of exogenous proteins in mice is greatly extended by infection with lactate dehydrogenase elevating virus (LDH virus).[143,144] The basis for this unusual phenomenon remains unknown, but it is of interest that Holcenberg et al.[63] found that infection of mice with LDH virus increased the $t_{1/2}$ of untreated enzyme to 16 hr., and of succinylated enzyme from 9 hr to 17 hr, but had only a slight effect on the already elevated $t_{1/2}$ of the enzyme modified with glycopeptide.

Abuchowski et al.[95] also studied the effect on clearance of an antileukemic amidohydrolase modified with PEG. Working with a glutaminase-asparaginase from *Achromobacter* in which 71% of the amino groups were modified with PEG-5000, they found a $t_{1/2}$ in mice of about 24 hr for the conjugate compared to about 2 hr for the native enzyme. They also noted that the conjugate reached and maintained a higher level in blood following intraperitoneal injection than did the unmodified enzyme, even though the molecular weight of the conjugate is more than twice that of the native enzyme.

Holcenberg et al.[63] did not estimate the extent of modification of their samples, except indirectly by determination of pI. They reported an increase in $t_{1/2}$ with decreasing pI, and suggested that the effect of glycopeptide attachment on clearance might be mediated through a pI effect on the protein. Similar observations have been made before with *E. coli* and *Erwinia* asparaginases,[145-147] but it would be dangerous to accept this as a generalization. Marsh et al.,[37] for example, have studied the clearance of *E. coli* asparaginase from mice after using cyanoborohydride reduction to couple lactose and NAN-lactose to the enzyme. Clearance was accelerated by the attachment of lactosyl residues (as expected in view of the existence of the galactosyl-specific receptor in mouse liver parenchymal cells[131]), but the $t_{1/2}$ was approximately doubled in the case of asparaginase with 13.6 NAN-lactose residues per mole of enzyme. The introduction of NAN-lactose residues lowered the pI, of course, and this appeared at first to be a likely explanation of the increased $t_{1/2}$ that was observed. The situation is more complex than this, however, because Nickle and Wriston[148] have shown that succinyl-asparaginase, which also has a lower pI than native enzyme, was cleared more rapidly than native enzyme.

Benbough et al.[87] have also studied the effect of several modifications — including attachment of dextran — on the clearance of *Erwinia carotovora* asparaginase. In samples extensively modified by dextran but retaining about 30% of the initial activity, the rate of clearance from the circulation of rabbits was greatly reduced. The time required for the level of enzyme in the circulation to be reduced to one half its initial level was changed from 11 hr for unmodified enzyme to approximately 190 hr for 2 dextran conjugates.

Wilson[38] carried out reductive lactosamination of the dimer of RNase A in an attempt

to change the target organ for clearance. RNase A dimer has a molecular weight of 27,400 and was cleared rapidly, with the bulk of the label from a sample of injected enzyme being found in the kidney after 10 min. With a sample modified by the attachment of eight lactose residues per mole, however, even though the clearance rate from the circulation was more rapid than the native enzyme, most of the radioactivity was now found in the liver.

Pittman et al.,[52,53] Pittman and Steinberg,[56] and Van Zile et al.[42] were seeking a method that would permit them to study the contributions of different tissues to the degradation of plasma proteins with long circulating half-lives. A protein marker that would not be cleaved until entry into the lysosome and would tend to accumulate there in contrast to the behavior of normal metabolites resulting from protein catabolism would provide a direct measure of protein degradation. Also, of course, the marker itself should not alter the normal clearance mechanism. All of the groups of workers recognized that sucrose had the desired properties. It is taken up by fluid pinocytosis, accumulates in cells with little leakage, and is undegraded because of the absence of lysosomal sucrose activity.

Two different approaches were taken to bring about a coupling of sucrose to protein. Van Zile et al.[42] used galactose oxidase to convert the C-6 of the galactose moiety of [^{3}H] raffinose (Galα1-6Gluα1-2Fru) to aldehyde and coupled this to protein by reductive amination with sodium cyanoborohydride. Pittman and Steinberg[56] first prepared succinyl sucrose and coupled it to protein after activation with *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. The procedure is involved and may introduce some negatively charged succinate groups in sucrose that do not[52] become linked directly to protein. Subsequently these authors coupled [^{14}C] sucrose to protein after activation with triazine trichloride and showed that the glycosylated LDL was indistinguishable, in terms of binding, uptake, and intracellular degradation, from [^{125}I]LDL.

The two groups obtained similar results: Pittman et al.[52] working with BSA, LDL and asialofetuin, and normal fibroblasts, and Van Zile et al.[42] with a series of plasma proteins and plasma protein derivatives doubly labeled with [^{3}H]raffinose and ^{125}I in intact rats.

Pittman et al.[52] found that labeled sucrose accumulated in cells, as expected, but the level of ^{125}I in the cells reached a plateau, and degradation products appeared in the medium at a linear rate. Van Zile et al.[42] found that the half-lives of two long-lived plasma proteins, BSA and fetuin, were not altered by attaching raffinose. Raffinose did not alter the tissue or cellular site of protein uptake since [^{3}H] raffinose-asialofetuin was recovered almost exclusively in the liver parenchymal cell fraction, whereas similarly labeled RNase B and heat-denatured albumin were found mostly in nonparenchymal cells. Also, the [^{3}H]RAF label stayed in liver for a long time ($t_{1/2} > 100$ hr) while the ^{125}I label from asialofetuin or RNase B was rapidly lost from liver ($t_{1/2} < 30$ min).

Even more recently, Pittman et al.[53] used the same glycosylation procedure to prepare [^{14}C] sucrose-LDL and study the tissue site of its degradation in vivo in swine. They found (as with the in vitro studies already mentioned) that the method is valid because the fractional catabolic rate of [^{14}C] sucrose-LDL was the same as that of [^{125}I] LDL. Their results suggest that in swine the amount of LDL apoprotein degraded in the liver is approximately the same as that degraded in all of the nonhepatic tissues examined.

Several groups of workers (in addition to Benbough et al.[87] and Abuchowski et al.[95] mentioned above) have studied the effect on clearance of attaching high molecular weight ligands. Sherwood et al.[82] studied dextran conjugates of carboxypeptidase G (CPaseG) and arginase; Marshall et al.,[77] dextran conjugates of alpha-amylase and catalase; Abuchowski et al.,[91,92,94,96] PEG adducts of catalase, bovine serum albumin, arginase, and phenylalanine ammonia lyase; Geiger et al.,[105] a PVP conjugate of human hexosaminidase A; and Teien et al.,[83] a heparin-Ficoll conjugate. With but a single exception, in all these studies the rate of removal of conjugated protein from the

circulation was slower than that of native enzyme, sometimes strikingly so.

CPase G and arginase — the enzymes that Sherwood et al.[82] worked with — are of therapeutic interest. The CPase G used is a folic acid-hydrolyzing enzyme from a *Pseudomonas* mutant, the arginase is from *Fusarium solani,* and it is conceivable that depletion of either plasma folate or arginine would impede tumor growth. Gel filtration indicated that 1 and 2 mol of dextran (40,000 mol wt) was bound per mole of CPase G and arginase, respectively. The clearance rate in both normal and tumor-bearing mice was two- to threefold slower for the dextran-CPase G conjugate, and six- to eightfold slower for the dextran-arginase conjugate as compared to tumor-bearing animals given native enzyme. The difference between the clearance rate of native enzyme and dextran-arginase was even more pronounced in normal animals.

Marshall et al.,[77] using enzyme preparations with relative amounts of carbohydrate and protein that "on a weight basis were nominally 10:1," found markedly lower rates of clearance for both *B. amyloliquefaciens* alpha-amylase and bovine liver catalase in rats and acatalasemic mice, respectively. In the case of alpha-amylase given intravenously to rats, 75% of the dextran-conjugated enzyme was still present in the circulation after 2 hr, compared to only 16% for the native enzyme. With catalase, 93% of the unmodified enzyme was removed in 60 min, but only 30% of the conjugate. When native catalase was given i.p. to acatalasemic mice, it appeared in the blood rapidly and reached its maximum level in 2 hr, while the conjugate required 4.5 hr.

Abuchowski et al.[92] also used acatalasemic mice to study the effect of PEG-coupling on bovine liver catalase clearance. With unmodified catalase, endogenous levels of activity in the circulation were reached in 12 hr; but PEG-1900-catalase remained active for 48 hr, and the PEG-5000-catalase appeared to have a slightly longer half-life.

Abuchowski et al.[91] also studied the clearance of ^{125}I-labelled PEG-albumin in rabbits. In contrast to their results in mice with catalase, they found that the clearance pattern of native BSA and the PEG-1900 and PEG-5000 conjugates were quite similar. There is no ready explanation for this finding, which appears to be the sole exception to the general pattern of the extension of protein clearance times by modification with high molecular weight hydrophilic ligands.

The properties of PEG-5000 conjugates of arginase and phenylalanine ammonia lyase have also been reported recently by the same laboratory.[94,96] If mice were given unmodified arginase intravenously, the activity fell within 12 hr to 10% of the initial level, but with PEG-arginase, 52% of the activity was still present at 12 hr, and 16% at 72 hr. In the case of phenylalanine ammonia lyase, the $t_{1/2}$ in mice for the unmodified enzyme is 6 hr after the first injection, and about 1 hr after the fifth. On the other hand, the conjugate (with 44% of the amino groups modified) initially had a $t_{1/2}$ of 20 hr, although this was reduced to 4 hr and less than 1 hr after the 7th and 13th (30 days) injection, respectively, showing the failure in this case to suppress the immunogenicity completely by modification.

The term "lysosomal storage disease" refers to a group of conditions (metabolic or enzyme deficiency diseases) each of which is characterized by the absence of a particular lysosomal hydrolase. These enzymes are generally glycoproteins, and it has been recognized for some time that in several cases the defect is due not to an inability to synthesize the apoprotein, but to improper glycosylation.[149-151] "High uptake" and "low uptake" forms have been identified for several of these enzymes, the terms referring to the extent to which normal human fibroblasts can take up the enzyme. A high uptake form would be isolated from a normal individual (e.g., in urine or cultures of fibroblasts), whereas a low uptake form would refer to preparations with the same kind of enzymic activity, but isolated, for example, from fibroblast cultures from a person suffering from one of these diseases.

Enzyme deficiency diseases could, in theory, be treated by enzyme replacement therapy, given solutions to several practical difficulties. In addition to enzyme availability, these include the rapid inactivation and clearance of infused enzymes, immunogenicity, and inability of the infused enzyme to gain access to various organs or intracellular spaces.

Little work has been done so far in applying carbohydrate conjugation to these problems. Enzyme replacement therapy has been attempted in several of these conditions (Gaucher, Fabry, Tay-Sachs) by the intravenous injection of purified human enzymes.[124,125,152] No significant therapeutic effect has yet been observed, due, presumably, to inability of injected enzyme to cross the blood-brain barrier in the case of Tay-Sachs disease, and to rapid clearance and inactivation of injected enzyme in general. Geiger et al.[105] attempted to deal with the latter of these problems by modifying the enzyme with PVP. With a conjugate containing on the average 9.2 mol PVP per mole of enzyme, they found a significantly slower clearance rate in rabbits. The free human enzyme was cleared rapidly (activity virtually absent at 60 min), whereas with the conjugate there was 40% activity after 1 hr, and activity was still present at 48 hr.

Also, Desnick et al.[153] have recently reported a development relevant to protein modification with carbohydrate. They found that the plasma and splenic isozymic forms of alpha-galactosidase (the enzyme whose activity is defective in Fabry's disease) were cleared at quite different rates. The splenic enzyme had a $t_{1/2}$ of about 10 min, whereas the circulating half-life for the plasma enzyme was approximately 70 min. The plasma enzyme differed in its isoelectric point and sensitivity to neuraminidase treatment, suggesting that it was more extensively sialylated than the splenic enzyme. The plasma enzyme decreased the circulating levels of the substrate glycolipid more than did the splenic enzyme, and the decrease persisted for a longer period of time.

In some of the examples cited above, it is not clear why a particular modification should increase clearance time. In other cases, however, reasonable suggestions can be made. Alpha-amylase, for example, has a molecular weight of about 45,000, and there is substantial excretion of the enzyme in urine in rats.[77] The large increase in molecular weight in the dextran conjugate will undoubtedly reduce, if not eliminate, clearance by glomerular filtration and extend the clearance time accordingly. This explanation is supported by Wilson's work already cited.[38] Wilson was able to shift the main path for the clearance of low molecular weight RNase dimer from kidney to liver, not by an increase in molecular weight, but by attaching a ligand (lactose), the galactosyl moiety of which interacts specifically with a surface receptor of liver. There was no increase in clearance time in this instance. With larger proteins not subject to renal clearance in the native form, however, there is no simple explanation for increased clearance time upon carbohydrate conjugation since an increase in molecular weight, taken by itself, will increase the susceptibility of conjugates to reticuloendothelial clearance.

E. Effect on Specific Binding to Receptors: Uptake, Binding

Obviously, many of the interactions described in the previous section are excellent examples of specific binding to receptors. The examples considered in this section, however, are those where the objective was to establish specific binding to a cell-surface receptor, not by clearance studies, but by following uptake into the target tissue, or by binding studies with receptors. Some of this work was done to extend the utility of cytochemical techniques. In other cases it was done to explore details of the specificity of the binding interaction between carbohydrate and receptor.

One of the first experiments involving neoglycoproteins was carried out by Shier[106] who coupled chitobiosylamine to poly-(L-Asp) with a water-soluble carbodiimide (see Table 1, II.A). Wheat germ agglutinin (WGA) is a lectin with specificity for *N*-

acetylglucosamine capable of agglutinating tumor cells. Shier found that mice immunized with his "synthetic antigen" were able to reject five times as many myeloma cells as controls.

Cederberg and Gray[39] also used neoglycoproteins to study lectin binding specificity. They coupled di-*N*-acetylchitobiose and tetra-*N*-acetylchitotetraose to poly-L-lysine by reductive amination, and found that the conjugates were effective precipitating agents for wheat germ agglutinin and a lectin from *Bandeiraea simplicifolia II.*

Ghosh et al.[50] used carbohydrate conjugation as the basis for a sensitive assay for lectins. First they used dimethylsuberimidate to couple the *p*-aminophenylglycoside of galactose to lysozyme, and found that the conjugate (with 6 mol of galactose per mole of enzyme) retained full activity. They then showed that binding of galactose-specific lectin led to inhibition of lysozyme activity, presumably for steric reasons, and further, that the inhibition increased nearly linearly with the amount of lectin present.

In one of the most recent examples of the use of neoglycoproteins to study cell-surface events at the molecular level, Kieda et al.[65] prepared "glycosylated cytochemical markers" as a way of detecting lectins. They noted that the cytochemical markers that have found greatest application are either not glycosylated (ferritin) or are glycosylated with ligands recognized only by glucose- or mannose-binding lectins (horseradish peroxidase, hemocyanin, dextrose-iron complex, mannan-iron). Kieda et al.[65] coupled lactose, chitobiose, and glycopeptides from porcine thyroglobulin to horseradish peroxidase and ferritin after making the *p*-diazophenyl derivatives of the sugars and the glycopeptides (see Table 1, III. D). Porcine thyroglobulin was chosen as a source of glycopeptide because it reacts with a large number of lectins. They found that the various glycosylated markers with conjugated carbohydrate bound lectin specifically according to the nature of the ligand introduced, and that the binding interactions were strong enough to cause a precipitation reaction.

The most detailed studies in this area to date have come from Lee's laboratory at Johns Hopkins. Krantz et al.[29] studied the binding to rabbit liver plasma membranes of neoglycoproteins prepared from alpha-amylase, lysozyme and BSA and IME-thioglycosides of several sugars (see Table 1). Efficiency of binding was measured by determining the ability of the neoglycoproteins to inhibit ^{125}I-labeled asialoorosomucoid (AOM) binding to the membranes. The relative inhibitory power (RIP) was defined as the ratio of AOM producing 50% in inhibition to the amount of neoglycoprotein doing the same. Thus RIP values of inhibitors weaker than AOM are less than 1, and better inhibitors have values greater than 1. Krantz et al.[29] found that increasing the number of D-galactosyl residues bound to alpha-amylase increased its inhibitory power. With 10 to 11 mol of D-Gal per mole of enzyme, the RIP value was 10^3-fold higher than unmodified alpha-amylase, although still only 1/20 as effective an inhibitor as AOM itself. The attachment of mannosyl and *N*-acetylglucosaminyl residues had little effect on the RIP values, although the alpha-amylase modified with glucose did show a modest increase in RIP relative to unmodified alpha-amylase.

The same specificity pattern was seen with lysozyme neoglycoproteins, but here even at the highest levels of modification (3 to 4 mol of D-Gal per mole of enzyme), lysozyme was a much weaker inhibitor (1/500) than AOM. Similar results were obtained with amidinated BSA, except that in this case the neoglycoprotein with 34 galactosyl residues is actually a 20-fold better inhibitor than AOM. The D-mannosyl and *N*-acetyl-D-glucosaminyl derivatives are very poor inhibitors, but surprisingly enough, at high levels of incorporation the D-glycosyl derivatives of BSA are even better inhibitors than the D-galactosyl, for reasons that are not clear.

Another unexplained observation arises in connection with differences in binding that seem to depend on the coupling method. The ability of the D-glucosyl derivatives to bind

(not as strongly as D-galactosyl with alpha-amylase, but as good or better with lysozyme and BSA) was different, depending on linkage type, with the neoglycoproteins made by amide bond formation or diazo coupling being better inhibitors than the amidino derivatives. Also, the linkage type seemed to affect the galactosyl and glucosyl derivatives differently.

D-glucose is a rare component of glycoproteins, and there is no obvious explanation for the binding of D-glucosyl neoglycoproteins. Krantz et al.[29] suggested that the very scarcity of D-glucose may explain why hepatocytes do not need to discriminate with respect to the configuration at C-4 where glucose and galactose differ, and pointed out that several enzymes are known that also lack this particular specificity, e.g., almond beta-D-glucosidase and human beta-*N*-acetylhexosaminidase.

More recently, Stowell and Lee[33] examined this question in more detail and established that both neoglycoproteins bind to the same rabbit hepatic asialoglycoprotein receptor. They reached this conclusion partly by showing that the binding of both types of proteins (a D-glucosyl-BSA neoglycoprotein and a D-Gal-terminated glycoprotein, namely, asialoorosomucoid) showed the same dependence on calcium concentration, sensitivity to modification of the receptor by neuraminidase, and inhibition by various carbohydrate derivatives, but even more convincingly by using affinity chromatography with D-Gal-BSA or D-Glu-BSA immobilized on Sepharose, and showing that the receptors isolated from rabbit liver in these two separate procedures were identical.

Youle et al.[41] have recently extended this work to another receptor system in an elegant way: by altering the cell-type specificity of the toxin ricin. Several proteins (e.g., ricin, diphtheria toxin, chorionic gonadotropin, and other protein hormones) are known to enter cells by receptor-mediated transport processes and then proceed to specific intracellular locations.[154] It is clear with several of these proteins that one chain carries the receptor binding activity, while a second is responsible for the biological activity after internalization. A number of "hybrid" proteins have been prepared with novel combinations of receptor specificity and intracellular function.[155-157] The coupling of an oligosaccharide moiety known to function in receptor binding is, in a sense, an extension of this idea. Monophosphopentamannose, obtained from a yeast phosphomannan, is an analogue of the receptor recognition factor for fibroblast lysosomal hydrolases.[158,159] Youle et al.[41] coupled this material to ricin and showed by inhibition studies that it bound to the phosphomannosyl receptor on fibroblasts and inhibited protein synthesis. The studies had to be done with intact ricin since the A chain, lacking the binding subunit B, was unstable to the reductive amination coupling procedure; thus the fibroblast incubations were carried out in the presence of lactose, a competitive inhibitor of ricin binding. Under these conditions, the toxicity of ricin and a maltotriose-ricin adduct were sharply reduced, while that of the phosphopentamannosyl derivative was not appreciably affected. Two other noteworthy observations emerged from this work. The phosphomannosyl derivative of ricin was no more toxic to human amnion and HeLa cells than ricin itself, suggesting that these cells lack the fibroblast receptor. Also, although it proved possible to couple monophosphopentamannose to the toxic subunit of diphtheria toxin (with retention of much of the NAD ribosylation activity characteristic of that protein), and although binding to the phosphomannosyl receptor of fibroblasts did occur as judged by competitive inhibition experiments, the adduct had virtually no cytotoxicity. The reason for the different results obtained with this material and the ricin conjugate are not known.

F. Effect on Enzyme Activity

With the exception of an early paper by Maekawa and Liener[60] on the use of

S-methylglucosyl isothiourea, we are unaware of any studies on modification by carbohydrate conjugation carried out for the express purpose of studying the effects on enzyme activity: retention of activity, changes in K_m values, etc. Indeed a number of investigators with other purposes in mind have not even reported the extent to which such modifications affected the activity, even though the retention of native enzyme activity and specificity during a modification provide one measure of its general practicality. Most modifications have been carried out on a few familiar enzymes, but a total of 19 different enzymes have been modified (Table 3).

IV. ENZYMATIC APPROACHES TO GLYCOCONJUGATE PREPARATION

The participation of sugar nucleotides in glycoprotein biosynthesis has been known for a long time. More recently another class of activated sugar derivatives has been discovered, and these compounds also are now known to participate in the assembly of glycoproteins and complex glycans in a wide variety of living systems. In bacteria the compounds are derivatives of sugars linked *O*-glycosidically via phosphate and pyrophosphate bridges to the polyisoprenoid alcohol, undecaprenol. In eukaryotes the alcohol is usually referred to as dolichol, this term representing a family of polyisoprenols ranging from C_{90} to C_{100} in chain length, with the terminal hydroxyl-bearing isoprene unit (i.e., the unit which is phosphorylated) being saturated (in contrast to the situation with the C_{55} undecaprenol).

Many glycoproteins in eukaryotes are either membrane-associated or secreted, and considerable attention has focused in recent years on their biosynthesis. Two types of reactions are known to be involved. In the first single sugar residues are transferred from the nucleotide derivative to the growing oligosaccharide chain by the action of glycosyl transferases. The second kind of reaction involves the transfer of mono- or oligosaccharide from glycolipid carriers under the influence of membrane-associated glycosyl transferases. Sometimes single sugar residues are transferred, but there are also enzyme-catalyzed transfers of an entire oligosaccharide block. Many details remain to be worked out, but much has already been established as indicated in Figure 2.[160-162]

Glycoprotein biosynthesis is of interest here because of the use of glycosyl transferases for protein glycosylation. Although it proved possible in earlier investigations to use enzymes to replace single residues removed by the action of glycosidases (e.g., neuraminidase), little success was achieved at first in attempts to glycosylate proteins *de novo* or use synthetic peptides or isolated glycopeptides as acceptor substrates. Until recently most of the work done in connection with elucidating the glycoprotein biosynthetic pathway was carried out with impure preparations of endogenous acceptors. This situation has now changed, however, and recent success in achieving glycosylation with relatively simple synthetic peptides would seem to make available a new indirect route to protein glycosylation.

One of the first examples of the use of glycosyl transferases for protein glycosylation was contributed by Paulson et al.[163] They incubated purified desialylated rabbit liver binding protein with CMP-NAN and pure β-D-galactoside α2-6 sialyl transferase isolated from bovine colostrum, and obtained a restoration of more than 95% of the missing sialyl residues and more than 80% of the binding activity. With the asialoagalactolectin much the same kind of result was obtained with a mixture of UDP-Gal, CMP-NAN, and the two purified transferases.

Hill's group[164] also used purified sialyl transferases to restore M and N blood group antigenic activity to neuraminidase-treated glycophorin, and reconstitute functional viral receptors on erythrocytes.[165]

Table 3
ENZYMES MODIFIED BY CARBOHYDRATE CONJUGATION

Enzyme	Method of conjugation (Table 1 entry)	Retention of Activity	Ref.
Alpha-amylase *(Aspergillus oryzae)*	Amidination with thioglycosides (I.A.3)	100% or more at low levels; slight loss at higher levels	32
	Amide formation (I.A.2)	Complete loss of activity	29
	Diazo coupling (III.A)	High losses except in presence of maltose	29
Alpha-amylase *(Bacillus amyloliquefaciens)*	Dextran/CNBr (I.C.1)	In most cases >50%	71
	Dextran/CNBr (I.C.1)	Specific activity in conjugate 42% of native	77
	Dextran/CNBr (I.C.1)	Specific activity of conjugate 20—30% of native	74,79
Alpha-amylase (variety not specified)	Dextran/triazine trichloride (I.C.4)	Up to 67%	78,89
Beta-amylase (sweet potato)	Dextran/CNBr (I.C.1)	In most cases >50%	71
Glucoamylase	Dextran/CNBr (I.C.1)	90—100%	67
Adenosine deaminase	PEG-triazine trichloride (I.D.1)	—	95,98
Arginase *(Fusarium solani)*	Dextran/CNBr (I.C.1)	45%	82
Arginase (beef liver)	PEG/triazine trichloride (I.D.1)	65%	94
Asparaginase *(E. coli)*	Reductive amination (I.A.4)	Moderate substitution, no loss; at 20 mol lactose per mole, 75% retention	37
	PEG/triazine trichloride (I.D.1)	7%	102
Asparaginase *(Erwinia carotovora)*	Dextran dialdehyde/borohydride (I.C.2)	30%	87
Glutaminase-asparaginase *(Acinetobacter)*	Glycopeptides/glutaraldehyde (I.B.3)	50%	63
Glutaminase-asparaginase *(Achromobacter)*	PEG/triazine trichloride (I.D.1)	12% of the asparaginase activity	95
Carboxypeptidase G *(Pseudomonas)*	Dextran/CNBr (I.C.1)	52%	82
Catalase (beef liver)	Dextran/CNBr (I.C.1)	70%	67,77
	PEG/triazine trichloride (I.D.1)	95% at 40% modification of amino groups	92

Alpha-chymotrypsin	Dextran/CNBr (I.C.1)	—	80
	PVP (I.E.1)	—	104
	Dextran/triazine trichloride (I.C.4)	81% against low molecular weight substrate, 73% against casein	90
	Dextran/CNBr (I.C.1)	100%	68
Chymotrypsinogen	Carbodiimide/1-amino sugar (II.A)	40% against low molecular weight substrate	110
Glucose oxidase (E.C. 1.1.3.4)	Polyvinyl alcohol (II.C)	—	113
Hexosaminidase A (human placenta)	PVP (I.E.1)	No loss in 9:1 conjugate against low molecular weight substrate; same Km value	105
Horseradish peroxidase	Diazo coupling/*p*-aminophenyl-glycoside derivatives (I.B.4)	95%	65
Lysozyme	Amidination with thioglycosides (I.A.3)	62—112%	32
	Glycopeptides/toluene diisocyanate (I.B.1)	40%	61
	Woodward (I.B.2)	—	102
	Dextran/CNBr (I.C.1)	—	80
	Dextran dialdehyde/borohydride (I.C.2)	—	86
	Carbodiimide/1-amino sugars (II.A)	—	107,109
	p-Aminophenyl glycosides/suberimidate (I.A.5)	100% with 6 amino groups substituted	50
	Dextran/CNBr (I.C.1)	Inactive	67
Phenylalanine ammonia lyase *(Rhodotorula glutinis)*	PEG/triazine trichloride (I.D.1)	60% active with 23% modification, 15% with 52%; elevated apparent Km; competitive inhibitor cinnamate offers some protection	96
RNase dimer (bovine pancreatic)	Reductive amination (I.A.4)	100% against cCMP, but 40—80% loss against poly (A) · poly (U)	38
RNase (bovine pancreatic)	Dextran/CNBr (I.C.1)	90—100%	67
RNase B	Reductive amination (I.A.4)	No change	42
Superoxide dismutase	PEG/triazine trichloride (I.D.1)	—	95

Table 3 (continued)
ENZYMES MODIFIED BY CARBOHYDRATE CONJUGATION

Enzyme	Method of conjugation (Table 1 entry)	Retention of Activity	Ref.
Trypsin	Dextran/CNBr (I.C.1)	53% against low molecular weight substrate, 7% against casein	71,72
	Dextran/CNBr (I.C.1)	—	80
	S-methylglucosyl isothiourea (I.A.9)	Only slight loss against low or high molecular weight substrates, with 6 Glc residues per mole	60
	PEG/triazine trichloride (I.D.1)	95—150% for low molecular weight substrates; conjugates hydrolyze angiotension II more slowly than native	93
	PVP (I.E.1)	Higher against low molecular weight substrate, but only 18% with azocasein	103, 104
Uricase (hog liver and *C. utilis)*	PEG/triazine trichloride (I.D.1)	—	95,99

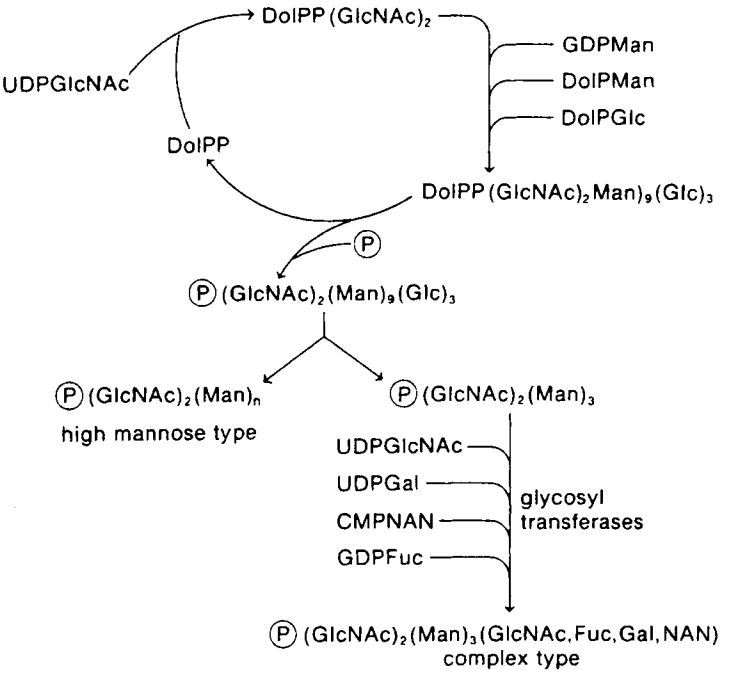


FIGURE 2. A simplified representation of glycoprotein biosynthesis in the eukaryotic cell.

As additional examples, Schindler et al.[166] were able to establish conditions for the quantitative glycosylation of the terminal *N*-acetylglucosamine moieties in the peptidoglycans of *Micrococcus luteus* and *E. coli* by UDP-[^{14}C] Gal and human milk galactosyl transferase, as indicated by a linear relationship between the amount of [^{14}C] Gal introduced and the peptidoglycan concentration. Shaper and Stryer[167] used the same system to attach galactose residues from UDP-Gal *in situ* to rhodopsin in retinal disk membranes, and Denis and Wriston[168] used galactosyl transferase to catalyze the transfer of galactose from UDP-Gal to asparaginase to which a mixture of $(GlcNAc)_5$ and $(GlcNAc)_6$ had been attached by reductive amination.

The two most common linkages between sugar and protein in mammalian glycoproteins are the *N*-glycosidic bond between *N*-acetylglucosamine and asparagine, and the *O*-glycosidic bond between *N*-acetyl-galactosamine and serine/threonine. An enzyme catalyzing formation of the latter, UDP-GalNAc:mucin polypeptide transferase was first characterized by McGuire and Roseman[169] and Hagopian and Eylar.[170] These investigators tested a number of proteins and small substrates, but found that only apomucin (deglycosylated submaxillary mucin) and basic myelin protein would serve as transferase substrates. More recently Hill et al.[171] found that a 74-residue apomucin peptide was readily glycosylated by a porcine submaxillary transferase, but that shorter peptides were poorly glycosylated, and they suggested that the enzyme exhibited a minimum size requirement. Now, however, Young et al.[172] have prepared several peptides with sequences identical to those in the vicinity of a glycosylated threonine in bovine basic myelin protein. They found that the tetrapeptide Thr-Pro-Pro-Pro and larger peptides containing this sequence — especially those with residues *N*-terminal to the threonine residue — were glycosylated with [^{14}C] UDP-GalNAc and a crude detergent-solubilized preparation of UDP-GalNAc to mucin polypeptide *N*-acetylgalactosaminyl transferase.

Recently Hart et al.[173] have also been successful in demonstrating the *N*-glycosylation of asparagine in low molecular weight peptides. It is known that only asparagine residues which are part of the sequence -Asn-X-Thr/Ser- are glycosylated, and it was suggested that this is a "necessary but not sufficient condition" since not all asparagines in such sequences in glycoproteins are in fact glycosylated. Lennarz's group showed in 1977[174] that RNase A and alpha-lactalbumin served as acceptors of the $(Man)_n(GlcNAc)_2$ chain from oligosaccharide-P-P-dolichol in their hen oviduct enzyme system only after denaturation, but that this treatment did not convert several other proteins with the requisite tripeptide sequence into active acceptors. A year latter Kronquist and Lennarz[175] reported that of an additional seven proteins tested (proteins containing the appropriate asparagine glycosylation sites, but not usually found in the glycosylated form), two were converted to acceptors by converting them to denatured, *S*-derivatized forms, but five others were not. Peptides obtained from two of the latter group by cyanogen bromide treatment, however, were acceptors. Struck et al.[176] also showed that several peptides obtained from *S*-carboxymethylated or *S*-aminoethylated alpha-lactalbumin could serve as in vitro glycosylation substrates, and concluded that the critical factor needed for in vitro glycosylation by membrane-linked transferases is adequate exposure of the critical tripeptide linkages. It was suggested that failure of a second asparagine in alpha-lactalbumin to be glycosylated (present as $-Asn_{74}-Ile_{75}-Ser_{76}-$) might be due to steric or charge interference from cysteine residues present at both ends of the tripeptide sequence. Very recently this same laboratory[173] synthesized several peptides containing the -Asn-X-Thr/Ser- sequence, and showed that they served as efficient acceptors if both termini are blocked. Ronin et al.[177] also have reported that a thyroid microsomal system will glycosylate small peptides containing the tripeptide sequence.

The impressive results of this recent work contribute a great deal to our understanding of glycoprotein biosynthesis, and while they do not represent glycosylation methods that can always be applied to native proteins, the likelihood that peptides prepared in this way could be coupled to proteins in the same way that Rogers and Kornfeld coupled sialoglycopeptides of fetuin to bovine serum albumin some years ago[60] does indicate an indirect way of preparing a new series of neoglycoproteins, albeit with larger "spacers" than one might prefer.

A second approach to the study of glycosylation in living systems has developed based on the drug-mediated inhibition of glycoprotein biosynthesis. This method is limited by its lack of specificity (it is assumed in the absence of evidence to the contrary that all components within a treated cell which bear particular types of prosthetic groups are affected similarly), but can have the advantage of being reversible. For example, tunicamycin, which inhibits transfer of nucleotide sugar (UDP-GlcNAc) to the dolichol intermediate and hence the formation of asparagine glycosides,[178] has been used to show that glycosylation is not necessary for membrane insertion and proteolytic processing of Semliki Forest virus proteins in infected hamster cells.[179] *N*-glycan chains do, however, seem to be required at the cell surface for normal adhesion and spreading of hamster cells on substrata coated with the serum glycoprotein fibronectin.[180] Inhibition of formation of lipid-linked saccharide intermediates also occurs in the presence of 2-deoxy-D-glucose, 2-deoxy-2-amino-D-glucose, 2-deoxy-2-fluoro-D-glucose, and 2-deoxy-2-fluoro-D-mannose,[181] as well as with the bacterial products streptovirudin, antibiotic 24010,[182] amphomycin,[183] and bacitracin.[184] Enhanced glycosylation appears to be caused by adriamycin.[185]

Carbohydrate components of glycoconjugates can also be altered using purified glycosidases to remove one or more nonreducing terminal saccharides and expose new

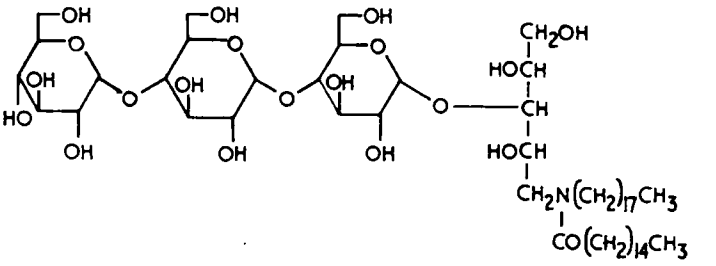


FIGURE 3. Model glycolipid: glycosyl-α1 → 4 1-deoxy-1-palmitamido-octadecylaminosorbitol.

determinants. The use of these enzymes to study structure-function relationships has recently been reviewed.[186]

V. MODEL GLYCOLIPIDS

One of the objectives of preparing protein glycoconjugates is to provide materials useful for studying the role of carbohydrate in binding phenomena. Oligosaccharides are present on cell surfaces as components of glycolipids as well as glycoproteins. The two best-characterized examples of membrane glycolipids serving specific biological functions are the ABO blood group antigens[187-189] and ganglioside G_{M1} as a receptor for cholera toxin.[190] Others have been proposed.[191-194] It is for this reason and because of recent reports that exogenous glycolipids can lead to the restoration of functional receptors in deficient cells[195,196] that examples of the preparation of model glycolipids with defined oligosaccharide moieties are presented here. (For a review of glycolipid synthesis, see Reference 197.)

Perhaps the simplest type of model glycolipid is one in which alkyl chains are joined directly to a sugar ring functionality — either amine[198] or hydroxyl[199] — via ether, ester, amine, or amide links. The crystal structure of one such derivative, 1-decyl-alpha-D-glucopyranoside, has been determined,[200] and consists of alternating bilayers of polar and nonpolar groups; the former hydrogen-bonded, the latter fully extended and close-packed. Given sufficiently long hydrophobic segments, micelles are generally found to form in aqueous solution. The use of 1-octylglycoside as a mild, dialysable, nonionic detergent in solubilization of membrane proteins is increasing.[201]

Reductive amination has been applied to the synthesis of model glycolipids. Wiegandt and Ziegler[202] used ammonium acetate and sodium cyanoborohydride to reduce oligosaccharides obtained from glycosphingolipids to 1-deoxy-1-amino-sorbitol derivatives which could then be *N*-stearoylated. Read et al.[203] reductively aminated maltotriose and maltotetraose with octadecylamine and sodium cyanoborohydride. Palmitic acid *p*-nitrophenyl ester was then used to convert the initial secondary amine products to *N*-substituted amides, as shown in Figure 3. These and other glycolipids were then used in lipid monolayers to study binding of the lectin concanavalin A as a function of surface pressure.

Williams et al.[204,205] oxidized a series of disaccharides to the corresponding aldobionic acids, formed the lactones, and then reacted these with 1-alkylamines of varying chain lengths to give amides. The synthetic glycolipids formed micelles which were precipitable with lectins.

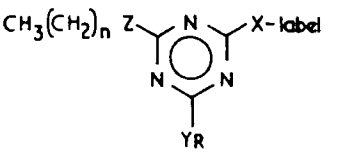


FIGURE 4. Model glycolipid: trisubstituted conjugate formed from triazine trichloride.

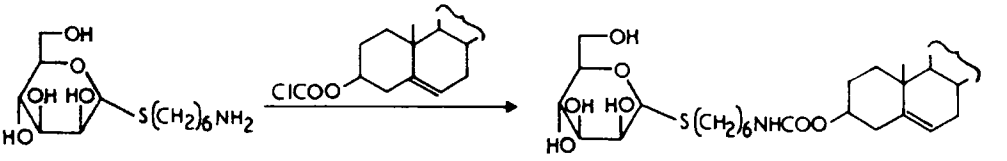


FIGURE 5. Model glycolipid: thiomannoside derivative of cholesterol.

Triazine trichloride, used in a number of instances to create conjugates of protein with sugar (see Tables 1 and 2, I.D), has also been used in the synthesis of model glycolipids, by displacement of the first chloride by long chain alkylamines; the second by amine, thiol, or hydroxyl groups at one of several positions on monosaccharide rings; and the third by a similar nucleophile on a spectroscopic probe thus resulting in a glycolipid analogue of the form shown in Figure 4, where R is a saccharide and X, Y, and Z may be S, O, or NH.[206] The behavior of the lipid analogue may be monitored spectroscopically, and the s-triazine ring thus provides a trivalent synthetic "nucleus" of great flexibility.

Two groups have reported the synthesis of model glycolipid substances based on cholesterol. Orr et al.[207] coupled 6-aminohexyl-1-thio-α-D-mannopyranoside (as well as a similar derivative with a longer spacer arm) to cholesteryl-3-chloroformate, as shown in Figure 5. The products were incorporated into small unilamellar liposomes which were subsequently shown to aggregate in the presence of the alpha-mannoside binding lectin, concanavalin A (see Section VI, B).

Chabala and Shen[208] used an approach based on nucleophilic displacement of iodide from 3-cholesteryl-6-iodohexyl ether by acetylated 1-thiosugars, followed by deprotection of the sugar. This procedure, used to obtain β-D-glucose, β-D-galactose, and α-D-mannose derivatives, was also modified to give the corresponding 6-amino-6-deoxyl-1-thio-α-D-mannopyranoside. All four compounds were again incorporated into liposomes with a view to investigating in vivo distribution.

A considerable literature is developing on polysaccharides modified with lipophilic moieties. More often than not modification is not confined to specific sugar residues or substituent positions. Thus, for example, dextrans have been esterified with increasing amounts of palmitoyl chloride to enhance antitumor and adjuvant activities.[209] Similar derivatives of PEG and dextran are finding application in two-phase polymer chromatography of macromolecules and cells,[210] and PEG palmitate has been used to modulate the motility and other membrane properties of polymorphonuclear leukocytes.[211] *N*- and *O*-acyl chitosans have also been prepared.[212] Derivatives of dextran stearate containing both the antigenic hapten trinitrophenyl and fluorescent probes have been used in studying diffusion and distribution in planar lipid bilayers and the effect of

antibodies and lectins on these processes.[213] The nonionic detergent Triton X-100 has been coupled via ether bonds to inulin, dextran, amylose, and cellulose in an attempt to find membrane-disrupting agents sufficiently mild to insure the retention of enzymic activities and other conformation-dependent properties in membrane-bound proteins.[214] Further developments in this rapidly developing area can be expected, as, for example, in the application of glycosyl transferases to purified endogenous lipid or glycolipid acceptors.

VI. FUTURE PROSPECTS

Work designed to explore the effects of carbohydrate conjugation on enzyme stability and clearance will remain an active area, but it seems likely that the greatest effort in the next few years will be directed towards studies of the role of carbohydrate in binding phenomena. It is likely that more complex glycoconjugates will be used in model experiments, and advances will occur in the use of glycosidases and glycosyl transferases to modify naturally occurring processes involving carbohydrate recognition. We also think it likely that increased attention will be paid to ways of modifying membranes with model glycoconjugates so as to be able to study their location and environment.

We have chosen three specific areas for further discussion: (1) improved preparation of glycoconjugates, (2) direct modification of cell surface glycoconjugates, and (3) modification to study location and environment of membrane glycoconjugates.

A. Improved Preparation of Glycoconjugates

Advances in stereoselective synthetic methods have led to the preparation in good yield of a large number of disaccharides and several trisaccharides in recent years, many with functional groups suitable for coupling to proteins.[215,216] Also, Jeanloz and collaborators have developed methods for the synthesis of glycopeptides containing *N*-asparaginyl and *O*-seryl linkages,[217,218] and Lee[111] has reported the synthesis of "cluster" glycosides, triglycosides with a terminal amino or hydrazido group for attachment to protein (see Table 1, II.B). This last development appears to open up the possibility of preparing neoglycoproteins analogous to natural glycoproteins which contain bi- or triantennary glycans. Such derivatives would presumably be of considerable value in studying the possible role of "clustering" of sugar residues in recognition processes. Regoeczi et al.,[134] for example, have recently reported that three human asialotransferrins (differing with respect to their bi- and triantennary glycans) exhibit different binding affinities for the rat hepatic lectin.

In spite of these developments, however, it seems likely that only a limited number of chemists will be in a position to synthesize the necessary oligosaccharides. We anticipate that more conjugation work in the future will utilize oligosaccharides found in nature (coupled by chemical methods already described), and include the use of glycosyl transferases to both extend relatively simple initiating glycan chains introduced onto proteins and prepare millimolar amounts of di- and oligosaccharides not readily available at present. Much work has already been done on the identification and small-scale isolation of plant and animal oligosaccharides (e.g., the yeast mannans[219] and fucose- and sialic acid-containing oligosaccharides in human milk and urine[220-222]). Complex bacterial extracellular polysaccharides seem to be a relatively neglected source at present,[223,224] and we would expect increased interest in such materials (especially where enantiomers of the configuration opposite to that of the mammalian glycoproteins are of interest) and a scaling up of isolation procedures from plant and animal sources.

With respect to the use of glycosyl transferases for protein glycosylation, several examples have already been described (see Section IV). Increased activity in this area is

expected for several reasons. First, there are the recent demonstrations that relatively simple synthetic peptides (a blocked tripeptide in the case of *N*-asparagine glycosylation) can serve as acceptors (see Section IV). Second, there is the increased availability of di- and oligosaccharides with a variety of sugars and linkages suitable for coupling chemically to proteins, to serve either as initiating glycans or as recognition signals in their own right. Finally, recent improvements in purification of the membrane-associated transferases will assist work of this kind.

B. Direct Modification of Cell Surface Glycoconjugates

Cell surface modification can in principle be achieved by either introducing exogenous glycoconjugates (purified from natural sources or prepared by modification or synthesis) or the direct modification of cell membrane structures *in situ*. The first approach allows the introduction at one stroke of complex naturally occurring glycoconjugates, but suffers from the disadvantage of leaving unresolved, at least in most cases, the question of whether incorporation leads to a natural orientation within the bilayer. The second approach obviates this difficulty, but its success depends on the range of sufficiently mild modification procedures that can be developed, and there is also the problem of assessing the extent of penetration of the material into the interior of the cell.

Two striking examples of cell surface modification by the incorporation of exogenous glycoconjugates have been described recently. Mouse L-cells, a tissue culture cell line, do not contain the hepatocyte glycoprotein receptor responsible for binding, internalizing, and bringing about the degradation of plasma asialoglycoproteins. Doyle et al.[225] prepared "right side out" membrane vesicles from rat liver, enriched eight- to tenfold over crude homogenate in their ability to bind asialoorosomucoid but incapable of degrading this protein. They then found that these vesicles interacted with L-cells in the presence of polyethyleneglycol to yield modified L-cells that had taken on the property of the hepatocyte receptor, i.e., the capacity to bind, internalize, and degrade asialoorosomucoid.

In the second example Stern and Bretscher[226] showed that several cell types were able to incorporate Forssman antigen isolated from sheep erythrocytes as shown by the ability of the modified cells to bind monoclonal anti-Forssman antibody. The uniform distribution of ferritin-conjugated anti-Forssman antibody suggested that exogenous glycolipid had inserted into the lipid bilayer. Not all of the cell types examined took up the same amount of material.

The recent report by Gal et al.[227] of the synthesis of L-glucosylceramide is also relevant. This compound differs from the naturally occurring D-isomer only in its total resistance to in vitro hydrolysis by glucocerebrosidase. The D-analogue accumulates in liver and spleen in persons with Gaucher's disease, and it is anticipated that availability of the resistant, unnatural stereoisomer will be useful in studies on the biochemical basis of the disease pathology.

Hu and Wisnieski[228] have shown that their photoreactive glycolipid probe, 12-(4-azido-2-nitrophenoxy) stearoyl 1-[^{14}C] glucosamine, is restricted in its reaction in a model system (M13 coat protein in dimyristoyl lecithin vesicles) to the part of the protein that would be buried in the bilayer, thus confirming its normal incorporation into the plasma membrane.

Hughes and Gardas[196] have demonstrated the phenotypic reversion of ricin-resistant hamster fibroblasts to a ricin-sensitive state after coating the cells by incubation with a ricin-binding glycolipid fraction isolated from human blood group O erythrocytes. Also of interest here (although it involves inserting a synthetic glycoconjugate into a liposome instead of a plasma membrane) is the recent work of Orr et al.[207] who synthesized mannose-containing derivatives of cholesterol (see Section V), incorporated them into

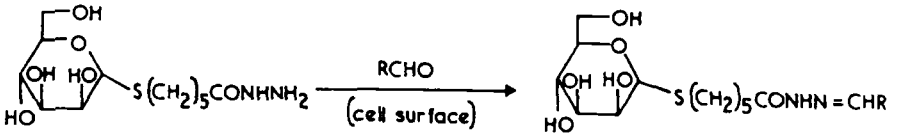


FIGURE 6. Derivatization of periodate-generated aldehyde groups (RCHO) on erythrocyte surfaces.

small unilamellar liposomes, and showed a concanavalin A-mediated aggregation that was reversed with α-methyl-D-mannoside.

Another paper from the same laboratory provides one of the first examples of direct cell surface modification. Orr and Rando[229] prepared a hydrazide derivative of a thioglycoside of α-D-mannose which was then attached to aldehyde groups generated on a cell surface (in this case erythrocytes) by mild periodate oxidation, (Figure 6). The cells were now agglutinable by concanavalin A. Aplin and Wriston[230] have also used reductive amination to couple lactose to fibroblast monolayers (BHK cells).

In other examples, Shaper and Stryer[167] used galactosyl transferase to attach galactose to terminal *N*-acetylglucosamine residues of rhodopsin in retinal disk membranes *in situ* (see Section IV); Pitha[231] prepared dextran derivatives that react specifically with protein thiol (mercury-dextran) or protein phenol/imidazole (diazonium dextran) and used these impermeant conjugates to cause specific modification of the cell surface; and Brossmer et al.[232] modified mouse ascites tumor cells with sialic acid by incubation with the 4′-diazobenzyl-α-ketoside of *N*-acetylneuraminic acid.

C. Modification to Study Location and Environment of Membrane Glycoconjugates

The introduction of radioactive labels into cell surface glycans is commonplace and can be achieved either by incubating the appropriate in vitro system with labeled substrates, or by reducing with tritiated borohydride after oxidation with periodate or neuraminidase and galactose oxidase. Since carbohydrates do not, however, provide a good "target" for the spectroscopist or ultramicroscopist, workers wishing to probe the environment of carbohydrate membrane components must introduce extrinsic probes. Several methods already discussed have been adapted for this purpose. Stryer, for example,[167] introduced a fluorescent label into the retinal disk membrane surface by reacting dansyl hydrazine with an aldehyde function generated with galactose oxidase. Nitroxide spin labels have been introduced into isolated glycoproteins, as well as erythrocyte surface glycoconjugates by periodate or galactose oxidase oxidation, followed by reductive amination. Greater mobility was observed than was expected for the whole glycoprotein, indicating that the oligosaccharide chains are relatively flexible.[233] The same methodology applied to immunoglobulins gave conflicting results, although it seems likely that carbohydrate is not directly affected by antigen binding.[235-237]

Lee and Grant have recently reported[238] the incorporation into model membranes of glycophorin spin-labeled by reductive amination. They found that the label continued to reorient rather rapidly, though less so than with the isolated molecule. The same laboratory earlier reported nonspecific spin labeling of sugar hydroxyl residues in gangliosides by reaction with a sulfonyl chloride nitroxide derivative.[239,240] Adam and Hall[206] (see Section V) have also synthesized glycolipid analogues containing spin labels. Aplin et al.[241] spin labeled sialic acid in glycoproteins and erythrocyte membranes at the C-1 position by coupling with a water-soluble carbodiimide, but the method was found to be relatively nonspecific.

Two interesting examples of the biosynthetic incorporation of spectroscopic probes into complex carbohydrates have been reported. Winterbourne et al.[242] showed that 2-deoxy-2-fluoro-L-fucose, a potential NMR probe, is incorporated into glycoproteins of cultured mouse fibroblasts; Johnston and Neuhaus[243] incubated spin-labeled UDP-GlcNAc with membrane preparations from *Gaffkya homari* to obtain a soluble spin-labeled peptidoglycan whose conformational properties could then be studied by ESR spectroscopy.

An elegant means of ultrastructural localization of carbohydrate has been demonstrated in several recent publications from Skutelsky's laboratory.[244-246] They attached biotinyl hydrazide to periodate-modified cell surfaces. Visualization may subsequently be achieved with ferritin-conjugated avidin. A similar type of experiment has been carried out by Spiegel et al.[247] who conjugated dinitrophenyl-2,4-diamino-butyric acid hydrazide to periodate-oxidized ganglioside, incorporated it into native thymocytes, and showed that these were agglutinated by high concentrations of anti-DNP antibody in the presence of protein A. Such experiments also permit the use of fluorescent antibody (or avidin) visualization, and provide interesting alternatives to the use of fluorescein-conjugated lectins or antibodies to cell surface sugar components.[49,248,249]

ACKNOWLEDGMENTS

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ADDENDUM

Several relevant articles have appeared since this review was first submitted. These are discussed briefly below, and bring the literature coverage through approximately March 1980. This material is not included in the tables.

Lee and Lee[250] have presented a detailed account of the coupling by reductive amination of thioglycosides containing an ω-aldehydoaglycon. (See Tables 1 and 2, I.A.4.) As expected and reported earlier[43] (see Section II, D) coupling by this method proceeds rapidly as compared to the direct coupling of reducing sugars because of the presence of an unmasked aldehyde function. Much of the incorporation occurs within the first 6 hr although it continues to increase slowly over 2 days. Maximal incorporation occurred at a ratio of $NaCNBH_3$/aldehyde of 1-15:1, and a $NaCNBH_3$ concentration of 0.1 *M*. Preincubation of protein with aldehyde before adding $NaCNBH_3$ did not alter coupling efficiency. It is also clear from evidence presented in this paper that disubstitution of lysine residues occurs. At low levels of incorporation, BSA modified by the incorporation of thiogalactoside with a 9-atom aglycon ($-[CH_2]_5CONHCH_2CHO$) is a more effective inhibitor of the binding of asialo-orosomucoid to rabbit liver plasma membrane than a homolog with a 5-atom aglycon ($-CH_2CONHCH_2CHO$).

One of the biological effects of lipopolysaccharides (LPS) from Gram-negative organisms is adjuvanticity for simultaneously administered protein antigens. In order to assess the role of the *O*-antigen component of LPS in this effect, Scibienski[251] recently coupled LPS from *Salmonella typhimurium* to lysozyme by first oxidizing the LPS with periodate, then incubating for 24 hr with protein, and finally either simply dialyzing ("direct" coupling, presumably due to Schiff base formation) or reducing with $NaBH_4$. The results suggest but do not prove conclusively that the "carbohydrate component of

LPS, and the 'O'-antigen elements in particular, play a central role in enhancement of primary antibody responsiveness to a protein antigen coupled to that LPS".

Muramyl dipeptide (MDP, or *N*-acetylmuramyl-L-alanyl-D-isoglutamine) is the minimal structure required to exhibit the adjuvant properties of dead mycobacterial cells in Freund's adjuvant. In experiments designed to examine the mechanism of this effect, two reports have appeared describing the properties of MDP-conjugates. Chedid et al.[252] used *N*-hydroxybenzotriazole and *N*-ethyl-*N'*-(dimethylaminopropyl)- carbodiimide to couple MDP and several related materials to a multipoly (D,L-Ala)-poly(L-Lys) carrier, and Reichert et al.[253] used two methods — carbodiimide coupling and coupling of the *p*-isothiocyanatophenyl glycoside of MDP with several protein carriers. The conjugates prepared by Chedid et al.[252] contained approximately 20% MDP. Perhaps the most notable conclusion was that an inactive stereoisomer of MDP — namely, *N*-acetylmuramyl-D-Ala-D-GluNH_2 — became capable of enhancing non-specific immunity after conjugation to the polymer, although not acquiring other activities characteristic of the natural isomer. Reichert et al.[253] used methylated carrier proteins (human serum albumin and BSA) to minimize side reactions in the carbodiimide procedure, and also prepared conjugates with keyhole limpet hemocyanin and bovine gamma globulin. Using an analytical procedure for muramic acid based on the degradation of released lactic acid to acetaldehyde, they estimated that 15 to 25 residues of MDP per albumin carrier were attached in the carbodiimide method. With the *p*-isothiocyanatophenyl method, 1100 residues of MDP were bound to hemocyanin, and 8 per molecule of albumin. Reichert et al.[253] point out that although the second method gives in general a more highly substituted product, it is less convenient than the carbodiimide method and also has the disadvantage of introducing an unnatural chemical bridge. The conjugates were used to prepare antibodies specific for the MDP hapten; it was also found that conjugation did not abolish the adjuvant potential of MDP.

Smith and Ginsburg[254] have described in detail the preparation of conjugates of sialic acid-containing milk oligosaccharides with BSA and keyhole limpet hemocyanin.[48] Phenethylamine derivatives of the sialyloligosaccharides (five different ones were used) are coupled by way of the isothiocyanate derivative[44,255] (see Table 1 and 2, I.A.5). Synthetic glycoproteins containing 10 to 40 mol oligosaccharide per mole of protein with albumin and 1100 mol/mol with keyhole limpet hemocyanin, were obtained, in yields of 6 to 42% of coupled oligosaccharide based on starting derivative. The ratio of sialyloligosaccharide to protein used to obtain these conjugates was approximately 200:1.

Eby and Schuerch[256] have described the synthesis of several mannose-containing disaccharides and a branched trisaccharide with the structure α-D-Man (1 → 2) -[α-D-Man(1 → 6)] -α-D-Man in the form of their phenethylamine glycosides, including the coupling of these compounds to BSA via the isothiocyanate derivatives for use as synthetic antigens[44] (see Tables 1 and 2, I.A.5). Higher yields of coupled oligosaccharide (from 22 to 35 mol of oligosaccharide per mole of BSA) were obtained by this method than by the diazotization coupling procedure previously used by these workers[117] (see Tables 1 and 2, III.C).

Bernstein and Hall[257] have described a novel method for preparing glycosides containing an ω-aldehydeaglycon. The method involves formation of an alkenyl glycoside using an alcohol such as allyl alcohol, and is followed by reductive ozonolysis with dimethyl sulfoxide in methanol to generate the ω-aldehyde function on the aglycon. These compounds can then be coupled to proteins with $NaCNBH_3$ in the usual way (see References 43 and 250 and Tables 1 and 2, I.A.4). With BSA the authors report incorporations of from 20 to 45 mol of glycoside per mole; with a longer spacer arm (made using 10-undecenyl alcohol in the first step) incorporation was reduced.

Incubations of the ω-aldehydoaglycon with protein were carried out for 48 hr or longer, although the authors note that one of the advantages of the method is the rapidity of the reaction.

Stahl et al.[258] have used a mannosyl-BSA derivative synthesized using the 2-imino-2-methoxyethyl-1-thioglycoside procedure of Lee et al.[32] (see Table 1, I.A.3) to study receptor-mediated endocytosis in macrophages. Binding to receptors was reduced in the presence of yeast mannan. Their studies of binding and uptake kinetics suggest that receptors are probably recycled to the cell surface after internalization.

Bause and Lehle[259] have described the use of synthetic peptide acceptors in enzyme-catalyzed *N* and *O*-glycosylation reactions. The results of these workers, obtained using membrane preparations of *Saccharomyces cerevisiae,* are in general agreement with those of Hart et al.[173] and Ronin et al.[177] on the formation of asparaginyl *N*-glycosides. Bause and Lehle also report that glycosyl transfer from dolichol-P-mannose to serine/threonine requires at least a tripeptide, although a "marker" sequence analogous to Asn-X-Ser/Thr for *N*-glycosylation could not be deduced.

Ponpipom et al.[260] have extended a previous report[208] dealing with the synthesis of a series of glycolipids with 6-(cholesten-3 β-yloxy)-1-thiohexane as a lipid tail and various carbohydrates as the surface determinant.

Two reports have recently appeared which describe extensions of current methodology to fluorescent labeling of cell surface carbohydrates. Abraham and Low[261] described the treatment of erythrocytes with periodate and galactose oxidase, followed by reductive amination using fluorescein amine and sodium borohydride; labeling coincides with PAS-sensitive bands on polyacrylamide gels. The procedure is applicable to other fluorophores containing amine nucleophiles. Wilchek et al.[262] also utilized galactose oxidase or periodate oxidation, reacting the resultant aldehydes with hydrazide derivatives of fluorescein and rhodamine. The method is applied to thymocytes and nematodes, as well as glycoproteins and gangliosides in solution.

Brownlee and Cerami[263] have described an ingenious system for the controlled release of insulin that could find practical application. The system is based on the principle that the release of insulin, modified by the attachment of oligosaccharides complementary to binding site of concanavalin A, will be proportional to the concentration of free glucose in the environment. In the example described, maltose derivatives of insulin were prepared by reductive amination with $NaCNBH_3$ (see Tables 1 and 2, I.A.4). A 5-day incubation led to the introduction of 1.76 mol of maltose per mole of insulin (there are only 3 primary amino groups in insulin) with the retention of 78 to 95% of the biological activity of unmodified insulin. It was then shown that whereas unmodified insulin did not bind at all to immobilized concanavalin A, all of the maltose-insulin conjugate introduced into the same column system was bound, and this derivative could then be displaced by pulses of glucose in the eluant, the amount of insulin released being proportional to the concentration of glucose in the pulse.

Support for the suggestion (see Section VI) that there will be an increased use of glycosyl transferases to prepare oligosaccharides for coupling is found in an impressive, recent paper by Nunez and Barker.[264] These workers used partially purified UDP galactosyl transferase from bovine milk to prepare millimolar amounts of such compounds as Gal β(1 → 4)Glc, Gal β(1 → 4) GlcNAc-β-hexanolamine, and Gal β (1 → 4)GlcNAc β(1 → 4) GlcNAc. They used the same methods to prepare similar compounds with ^{13}C-enriched galactosyl residues in the nonreducing terminal position, thus facilitating studies on solution conformation about the glycosidic linkage. In addition, they demonstrated the feasibility of an enzyme-catalyzed solid phase synthesis of oligosaccharide by transferring the galactosyl moiety to a GlcNAc linked via a hexanolamine spacer arm to agarose beads. Yields of 90% galactosyl saccharide were

achieved under the reaction conditions described with only a 10% excess of UDPGal donor.

Mullins and Langdon[265] have used a carbohydrate-containing affinity reagent to identify a transport protein. They showed that maltosyl isothiocyanate (MITC) meets the kinetic requirements for an affinity label of the glucose transporter of human erythrocytes. ^{14}C-MITC is incorporated into band 3 of the erythrocyte membrane, and this reaction was antagonized both by transportable sugars and by competitive inhibition of transport. Mullins and Langdon present an estimate of the number of glucose transporter molecules present in the erythrocyte membrane; they also suggest that the native glucose transport protein is a component of band 3, and that membrane proteases may be responsible for the conversion of this material to band 4.5 during certain extraction procedures.

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