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Studies on the synthesis and processing of the asparagine-linked carbohydrate units of glycoproteins

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It has become apparent in recent years from the work of a number of laboratories that the *N*-glycosylation of both membrane and secretory glycoproteins is effected by the transfer *en bloc* to nascent polypeptides of a glucose-containing oligosaccharide ($Glc_3Man_9GlcNAc_2$) from a dolichyl pyrophosphoryl carrier; this is followed by a series of modifying reactions to yield the mature polymannose and complex asparagine-linked carbohydrate units.

The enzymic steps involved in the assembly of the precursor oligosaccharide, its transfer to protein and its subsequent processing represent potential sites for the regulation of glycoprotein synthesis. Studies performed in our laboratory have dealt primarily with thyroid slices and particulate enzymes with special regard to the role of glucose in these events. Thyroglobulin, the major secretory glycoprotein of this tissue, has well defined complex and polymannose saccharide units, and indeed the most complete form of the latter ($Man_9GlcNAc_2$) has the same structure as the lipid-linked oligosaccharide without the glucose.

Our studies indicate that effective *N*-glycosylation requires a complete glucose chain (Glc_3) and that the glucose sequence is assembled from dolichol-P-glucose in a stepwise manner through the concerted action of at least two transferases in a fashion complementary to the subsequent excision of this sugar by glucosidases. Pulse–chase studies indicate that, after the transfer to protein, the removal of all three glucose residues as well as of the first mannose takes place in the endoplasmic reticulum and three additional mannoses are excised in the Golgi complex, because in the presence of an inhibitor of intracellular transport, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), there is a pronounced accumulation of protein-linked $Man_8GlcNAc_2$.

Studies with metabolic inhibitors (CCCP, antimycin, N_2) indicate that, under conditions of energy depletion, glucosylation of oligosaccharide-lipid is selectively impaired, resulting in an accumulation, as measured chemically or metabolically, of high-mannose-containing ($Man_9GlcNAc_2$ and $Man_8GlcNAc_2$) lipid-linked saccharides. Further evidence that the glucosylation reaction is very sensitive to the metabolic state is suggested by the observation that tissues not rapidly frozen after removal from the animal show a similar depletion of the glucose-containing oligosaccharide lipids.

Another important aspect for the regulation of *N*-glycosylation of proteins is the availability of dolichyl phosphate for the formation of the lipid-linked mono- and oligosaccharides. Our studies with puromycin suggest that there is a limited supply of the lipid carrier, because in the presence of this inhibitor there is no accumulation of any of the oligosaccharide-lipid species.

Introduction

The biosynthesis of the asparagine-linked carbohydrate units of glycoproteins has been a subject of active investigation in recent years and constitutes one of the most interesting chapters in the field of conjugated saccharides. The *N*-linked carbohydrate units were initially

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recognized through the work on ovalbumin by Neuberger and his colleagues (Johansen *et al.* 1961), and our work on thyroglobulin first indicated that two quite distinct types of asparagine-linked units occur (Spiro 1965). These two forms, which were originally designated unit A (simple) and unit B (complex) and are now frequently referred to as polymannose and complex (figure 1), have been found to be widely distributed among glycoproteins of diverse function

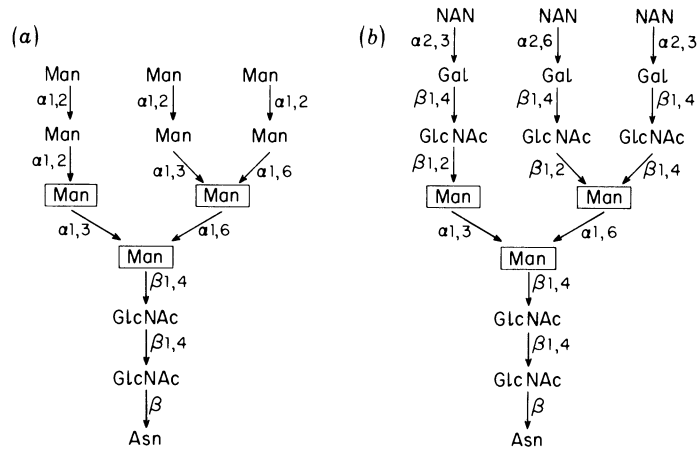

FIGURE 1. Structure of two major forms of asparagine-linked carbohydrate units of glycoproteins. The examples shown are (*a*) the polymannose unit (unit A) of calf thyroglobulin and (*b*) the complex unit from fetuin. The three internal mannose residues common to both types of saccharide unit are boxed.

and origin (Spiro 1973). A comparison of the two units indicates an interesting structural identity of their pentasaccharide ($Man_3GlcNAc_2$) core portions (figure 1). Complex carbohydrate units vary in the number and completion of the sialyl-*N*-acetyllactosamine chains and the presence or absence of fucose substituents; even the simplest variant, the pentasaccharide itself, has been observed (Dunn & Spiro 1967). More recently complex units containing repeating *N*-acetyllactosamine sequences (Järnefelt *et al.* 1978) and chains with sulphated sugars (Bedi *et al.* 1982) have been reported. Although the variations of the complex saccharide units are believed to be a function of differences in glycosylation of a common core, the several forms of the polymannose unit observed ($Man_9GlcNAc_2$ to $Man_5GlcNAc_2$) probably represent degrees of processing. The phosphorylated polymannose units of lysosomal enzymes, however, are the product of a specific transfer reaction (Reitman & Kornfeld 1981), and the 'hybrid' units, such as are seen in ovalbumin, appear to be the result of the addition of peripheral sugars characteristic of complex carbohydrate units to an incompletely processed polymannose oligosaccharide (Harpaz & Schachter 1980).

The work of a number of laboratories has now provided an outline of the elaborate pathways (figure 2) that lead to the synthesis of the asparagine-linked saccharide units of glycoproteins (For a recent review see Hubbard & Ivatt (1981).) The early investigations of Leloir and his coworkers (Behrens & Leloir 1970), which demonstrated the presence of dolichol-linked saccharide intermediates in eukaryotic cells, paved the way for an understanding of the mechanism of assembly of this type of unit. Subsequent studies indicated that *N*-glycosylation is accomplished through the transfer *en bloc* of an oligosaccharide from a dolichyl pyrophosphate carrier to nascent polypeptide and that both dolichyl phosphate sugars and nucleoside diphosphate sugars participate in the stepwise assembly of the lipid-linked saccharide unit (figure 2).

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Structure and function of glucose-containing oligosaccharide-lipids

Perhaps the most fascinating aspect of asparagine-linked carbohydrate biosynthesis is the occurrence on the transferred oligosaccharide of glucose that is subsequently removed and does not appear on the mature carbohydrate unit. The first clear indication that glucose was part of a dolichol-linked oligosaccharide containing the polymannose-di-*N*-acetylchitobiose

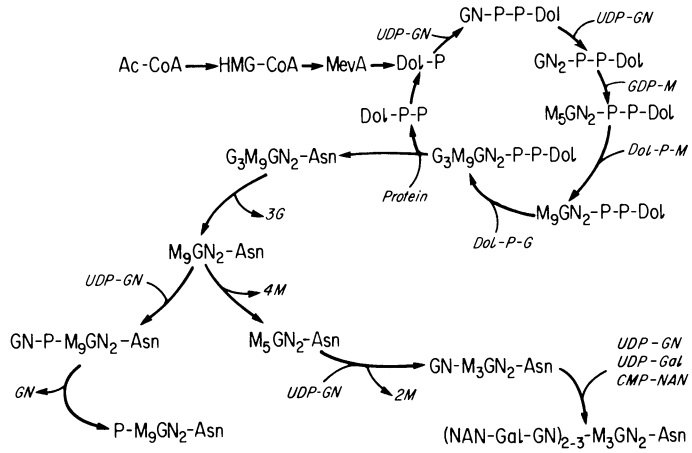

Figure 2. Scheme of the assembly and processing of asparagine-linked carbohydrate units of glycoproteins. The pathways leading to the synthesis of the complex, polymannose and phosphorylated polymannose units are shown. Non-standard abbreviations used are: G, glucose; M, mannose; GN, *N*-acetylglucosamine; NAN, *N*-acetylneuraminic acid; Dol, dolichol; MevA, mevalonic acid; HMG-CoA, hydromethylglutaryl coenzyme A.

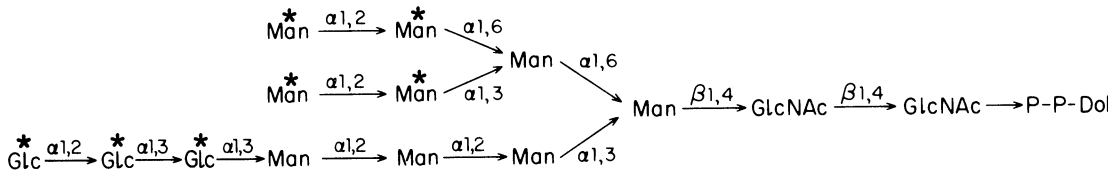

Figure 3. Structure of the dolichol-linked oligosaccharide that is believed to be the physiological *N*-glycosylating agent. The sugars marked with asterisks are derived by transfer from dolichol monosaccharide donors; the others are transferred from nucleoside diphosphate sugars.

unit was provided by our studies on thyroid (Spiro *et al.* 1974; M. J. Spiro *et al.* 1976). The peripheral location of this sugar in the oligosaccharide was indicated by the fact that it prevented the release by α-mannosidase of over half of the mannose residues (R. G. Spiro *et al.* 1976). The lipid-linked oligosaccharide was larger than even the most complete polymannose (unit A) variant of thyroglobulin and we postulated that glucose removal and modification by mannosidases would have to take place after transfer to protein to yield the mature *N*-linked carbohydrate units. A number of processing enzymes, both glucosidases and mannosidases, have now been described that do indeed carry out this remodelling function.

Studies on lipid-linked oligosaccharides from CHO cells (Li *et al.* 1978) led to a complete structural formulation (figure 3), which has proved to be consistent with observations made in a number of laboratories on eukaryotic cells ranging from yeast to higher vertebrates (Hubbard & Ivatt 1981). The structure of this precursor oligosaccharide appears to be identical

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to that of the most complete form ($Man_9GlcNAc_2$) of the unit A from calf thyroglobulin (Arima & Spiro 1972; Ito *et al.* 1977) with the exception that a triglucosyl sequence is attached to the polymannose chain, which is linked by an $\alpha(1 \rightarrow 3)$ bond to the most internal mannose residue. On the basis of chromium trioxide oxidation, α-anomeric configurations have been assigned to the glucose residues (R. G. Spiro *et al.* 1979). Acetolysis, which cleaves 1,6 linkages

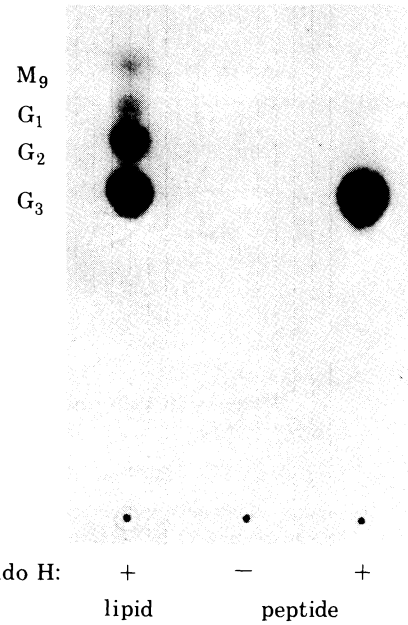

FIGURE 4. Specificity of thyroid microsomal oligosaccharyltransferase for fully glucosylated oligosaccharide-lipid. The enzyme was incubated with ^{14}C-labelled oligosaccharide-lipid from thyroid slices in the presence of an exogenous peptide acceptor. Thin-layer chromatographic separation on silica gel plates (1-propanol–acetic acid–water, 3:3:2) of the oligosaccharides released from the glycosylated peptide by digestion with endo-β-*N*-acetylglucosaminidase (endo H) is shown and compared with the endo H-treated oligosaccharides released by mild acid hydrolysis from the lipid donor. (R. J. Chalifour & R. G. Spiro, unpublished data.) The components were revealed by autoradiography. The abbreviations used are: G_3, $Glc_3Man_9GlcNAc$; G_2, $Glc_2Man_9GlcNAc$; G_1, $Glc_1Man_9GlcNAc$; M_9, $M_9GlcNAc$.

preferentially, has proved to be a valuable tool in deciphering the structure of the oligosaccharide by yielding a mannobiose and mannotriose from the two non-glucosylated chains and leaving intact the mannose chain bearing the glucose residues (R. G. Spiro *et al.* 1976). Although all three glucose residues appear to be transferred to the lipid-linked oligosaccharide from dolichol-P-glucose (Dol-P-Glc), the attachment of mannose seems to proceed from Dol-P-Man as well as directly from GDP-Man. Studies with inhibitors and cell mutants (Kang *et al.* 1978; Chapman *et al.* 1980) have shown that the five mannose residues present in the $Man_3GlcNAc_2$ core and in the chain to which the glucoses are attached are transferred from the nucleoside diphosphate sugar whereas the others are derived from Dol-P-Man (figure 3).

We have observed in studies with thyroid microsomal preparations that the glucose-containing oligosaccharide-lipid is a highly effective *N*-glycosylating agent for endogenous protein (M. J. Spiro *et al.* 1979). Removal of the glucose essentially abolished transfer, but α-mannosidase treatment to release peripheral mannose residues did not reduce glycosylation. Subsequent studies have indicated that the oligosaccharyltransferase requires the intact triglycosyl sequence

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because the oligosaccharide-lipid containing the $Glc_2Man_9GlcNAc_2$ unit was found to have minimal transfer activity (Murphy & Spiro 1981). The selectivity of the transferase can be illustrated by noting that only $Glc_3Man_9GlcNAc_2$ becomes attached to peptide acceptor when the enzyme is incubated with a mixture of oligosaccharide-lipids (figure 4). The glucose sequence represents an intriguing molecular determinant for *N*-glycosylation that is discarded after fulfilling its function. Because lipid-linked $Glc_3Man_9GlcNAc_2$ appears to be the physiological saccharide donor, it is evident that the enzymatic glucosylations of the oligosaccharide-lipid provide potential sites for the regulation of the attachment of carbohydrate units to asparagine residues.

The assembly of oligosaccharide-lipid

The formation of the lipid-linked oligosaccharide is initiated by the transfer of GlcNAc-1-P to Dol-P and is believed to proceed then by an ordered stepwise addition of mannose and glucose residues (figure 2). Various intermediates have been observed (Chapman *et al.* 1979) but little is known about the individual enyzmes involved in this biosynthetic process. Because the complete oligosaccharide consists of 14 sugar residues, that many distinct transferases could potentially be involved.

Our laboratory has recently undertaken a study of the enzymes responsible for the assembly of the glucose sequence, and our findings so far indicate the involvement of Dol-P-Glc as the sole glycosyl donor and the concerted action of at least two enzymes, with the transferase that adds the terminal $(1 \rightarrow 2)$-linked glucose being distinct from the enzyme(s) that attach the two internal $(1 \rightarrow 3)$-linked residues (Murphy & Spiro 1981). When thyroid microsomes are incubated with radiolabelled Dol-P-Glc there is a rapid transfer of glucose to endogenous oligosaccharide-lipid acceptor, with $Glc_3Man_9GlcNAc_2$ being the major saccharide product of all times (figure 5). Inclusion of sodium chloride in the incubation results in a pronounced decrease in $Glc_3Man_9GlcNAc_2$ formation with a proportional molar increase in the less completely glucosylated lipid-linked oligosaccharides, so that $Glc_2Man_9GlcNAc_2$ becomes the major component (figure 6). Methylation of the $Glc_3Man_9GlcNAc_2$ product indicated extensive radiolabelling of internal glucose residues, which was consistent with the biosynthesis *de novo* of the glucose chain beginning with an endogenous glucose-free, lipid-linked acceptor (Murphy & Spiro 1981).

The nature of endogenous oligosaccharide-lipids and the effect of energy state on their biosynthesis

To characterize the endogenous oligosaccharide-lipid present in thyroid we have chemically radiolabelled the oligosaccharides by $[^3H]NaBH_4$ reduction after their release from the lipid by mild acid hydrolysis. The separation of the reduced oligosaccharides was accomplished by thin-layer chromatography under conditions previously described (Murphy & Spiro 1981) and the resolution of the various species was enhanced by performing the reduction after a digestion of the oligosaccharides with endo-β-*N*-acetylglucosaminidase (endo H). Chromatography of the lipid-derived oligosaccharides from unincubated thyroid brought to the laboratory on ice revealed a substantial amount of unglucosylated material, which was primarily present as $Man_9GlcNac_2$ (figure 7). However, after incubation in an oxygenated medium the oligosaccharides became depleted in $Man_9GlcNAc_2$ so that $Glc_3Man_9GlcNAc_2$ became the

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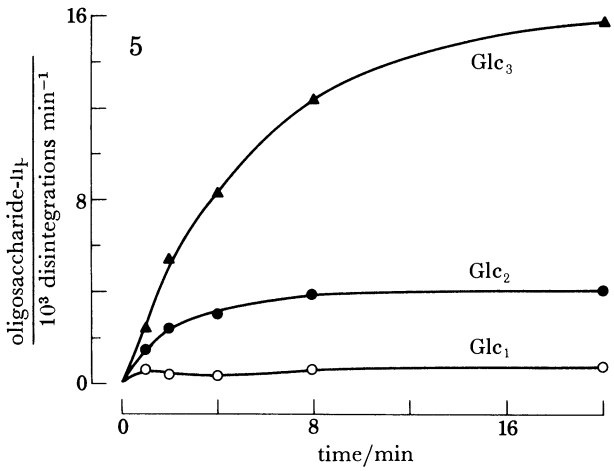
predominant species (figure 7), although on a molar basis the total oligosaccharide-lipid pool remained unchanged (about 2 nmol g^{-1} tissue). The molar distribution of lipid-linked oligosaccharides in thyroid microsomes was similar to that seen in the total thyroid slices (table 1); the substantial amount of $Man_9GlcNAc_2$ probably represents the unglucosylated endogenous oligosaccharide-lipid which serves as acceptor for the Dol-P-Glc:oligosaccharide-lipid glucosyltransferase.



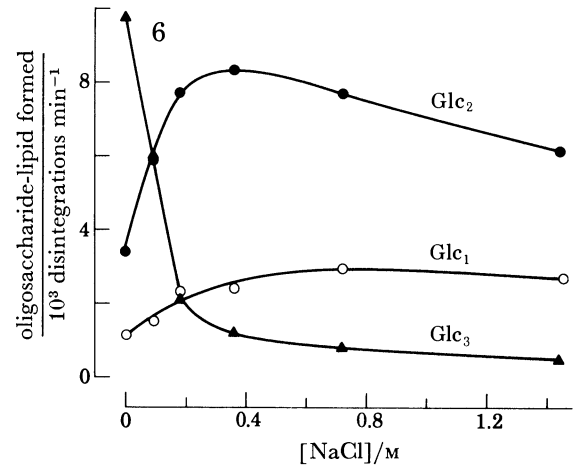


FIGURE 5. Transfer of glucose from Dol-P-[^{14}C]glucose to endogenous oligosaccharide-lipid acceptor by thyroid microsomal enzyme as a function of time. The lipid-linked oligosaccharides of the product were released by mild acid hydrolysis and separated by paper chromatography. (From Murphy & Spiro (1981).) The abbreviations are as follows: Glc_3, $Glc_3Man_9GlcNAc_2$; Glc_2, $Glc_2Man_9GlcNAc_2$; Glc_1, $Glc_1Man_9GlcNAc_2$.

FIGURE 6. Effect of sodium chloride concentration on the nature of the lipid-linked oligosaccharides formed by thyroid microsomal glucosyltransferases. The transfer of glucose from Dol-P-[^{14}C]glucose to endogenous oligosaccharide-lipid acceptor was measured. The oligosaccharides were released by mild acid hydrolysis and separated by paper chromatography. (From Murphy & Spiro (1981).) Abbreviations are the same as in figure 5.

Rapidly frozen tissue, unlike tissue kept on ice, retains its lipid-linked oligosaccharides primarily in the fully glucosylated form ($Glc_3Man_9GlcNAc_2$) and has a similar oligosaccharide pattern to that of incubated slices (table 1). Studies that we have carried out with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, suggest that glucosylation of oligosaccharide-lipid is extremely sensitive to the energy state of the tissue and that this may account for the reversible pile-up of $Man_9GlcNAc_2$ in poorly oxygenated tissue. In the presence of CCCP there is an inhibition of glucosylation with an accumulation of $Man_9GlcNAc_2$ and to a lesser extent $Man_8GlcNAc_2$ (figure 7); a similar situation is observed when slices are incubated under anaerobic conditions (table 1) or in the presence of antimycin (data not shown). In all these cases the total oligosaccharide-lipid pool remains unchanged but there is a pronounced depletion of the glucosylated components (table 1), which suggests that, while $Glc_3Man_9GlcNAc_2$-lipid is still available, transfer to protein remains relatively unaffected in these energy-deprived states. A calculation of the average number of moles of glucose per mole of oligosaccharide-lipid under various conditions is quite revealing (table 1). The unincubated tissue contains oligosaccharide-lipids with an average of 1.3 glucose residues, which is similar to the value previously observed on the basis of sugar analyses performed on

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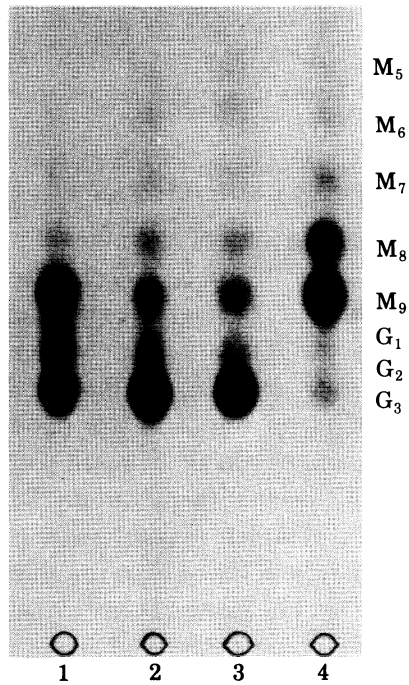

FIGURE 7. Effect of energy status on lipid-linked oligosaccharides of thyroid. Thyroids were brought to the laboratory on ice and sliced. Incubations were carried out in an oxygenated medium containing unlabelled pyruvate (5 mM) for 60 min (sample 2) and 150 min (sample 3). Sample 1 represents unincubated slices whereas the slices in sample 4 were incubated for 90 min in the presence of CCCP (100 μM) after a pre-incubation of 60 min. The lipid-linked oligosaccharides were separated by thin-layer chromatography on silica gel plates (1-propanol–acetic acid–water, 3:3:2) after release by mild acid hydrolysis, endo H digestion and reduction with [^{3}H]$NaBH_4$. The components were revealed by autoradiography. The abbreviations G_3, G_2, G_1 and M_9 are defined in figure 4; additional abbreviations are: M_8, $Man_8GlcNAc$; M_7, $Man_7GlcNAc$; M_6, $Man_6GlcNAc$; M_5, $Man_5GlcNAc$.

TABLE 1. NATURE OF LIPID-LINKED OLIGOSACCHARIDES OF THYROID IN VARIOUS CONDITIONS

(Oligosaccharide-lipids were measured by a chemical radiolabelling method. In this procedure oligosaccharides were released by mild acid hydrolysis, digested with endo H, reduced with [^{3}H]$NaBH_4$ and then separated by thin-layer chromatography (see figure 7) before scintillation counting.)

	oligosaccharide-lipid (mol %)†					
condition	G_3	G_2	G_1	M_9	M_8	Glc/oligo‡
frozen tissue	59	21	7	7	6	2.3
slices, unincubated	30	16	11	33	10	1.3
slices, incubated§	59	11	7	15	8	2.1
slices, incubated + CCCP	13	6	8	48	25	0.6
slices, incubated + N_2	13	5	6	52	24	0.6
microsomes	33	13	7	33	14	1.3

† Small amounts of M_7–M_5 that were present are not included in the calculation of molar distribution (see figure 7 for abbreviations).

‡ Expressed as average number of glucose residues per oligosaccharide.

§ When thyroid slices were metabolically radiolabelled with [^{3}H]mannose the molar distribution of the oligosaccharide-lipids was: G_3, 63%; G_2, 20%; G_1, 6%; M_9, 8%; M_8, 3%.

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such tissue (R. G. Spiro *et al.* 1976), whereas incubated or rapidly frozen thyroid has about 2.3 glucoses per molecule, which is consistent with the pattern observed in thyroid after metabolic labelling (Murphy & Spiro 1981). Less than one glucose residue is found on average during energy depletion induced by inhibitors or oxygen deprivation (table 1).

Processing of asparagine-linked carbohydrate units

It has become apparent through the work of a number of investigators that after the transfer of the oligosaccharide from lipid carrier to protein there is a removal of all three glucose residues, and this is followed by further processing through the action of α-mannosidases to yield variants of the polymannose unit and the core portion of the complex carbohydrate unit. Studies from our laboratory have observed the processing of the carbohydrate of thyroglobulin from $Glc_3Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ in thyroid slices (Godelaine *et al.* 1981) and enzymes that carry out these trimming reactions can be detected with the help of radiolabelled oligosaccharides or glycopeptides in microsomal preparations. The processing of the thyroglobulin carbohydrate units is, however, not complete as can be seen from an examination by thin-layer chromatography of the oligosaccharides released from the mature protein after endo H digestion and [^{3}H]$NaBH_4$ reduction (figure 8). The heterogeneity of the polymannose (unit A), previously observed from an examination of glycopeptides fractionated by ion-exchange chromatography (Arima *et al.* 1972), is clearly evident and a molar distribution of the various oligosaccharide species can readily be calculated from the radiolabelling pattern (figure 8). A substantial portion of the unit A is untrimmed ($Man_9GlcNAc_2$) or minimally digested by mannosidase, suggesting that steric factors or the rapidity of movement of the protein through the cellular compartments or both, may determine the final structure of these carbohydrate units.

Although the excision of the glucose from the transferred saccharide unit is believed to take place in the endoplasmic reticulum (Grinna & Robbins 1979) a number of studies have indicated that mannose removal by two distinct mannosidases is carried out in the Golgi apparatus (Tabas & Kornfeld 1979; Tulsiani *et al.* 1982). Recent pulse–chase studies carried out in our laboratory to follow the processing of the thyroglobulin carbohydrate units (Godelaine *et al.* 1981) have, however, suggested that the first mannose residue may, along with the three glucose residues, be released in the endoplasmic reticulum closer to the site of the initial carbohydrate attachment. When the chase was carried out in the presence of CCCP, which is known to block the transfer of proteins from the rough endoplasmic reticulum to the Golgi complex (Tartakoff & Vassalli 1977), there was a pronounced accumulation of thyroglobulin-bound $Man_8GlcNAc_2$ rather than $Man_9GlcNAc_2$ as might have been expected if all mannosidases were located in the Golgi (figure 9). The altered distribution of radioactivity in the endo H-releasable carbohydrate units in the presence of the CCCP is shown in figure 10.

The regulation of asparagine-linked carbohydrate unit synthesis

From the scheme depicting oligosaccharide-lipid assembly (figure 2) it is apparent that there are a number of potential regulatory points, and perhaps one of the most important is availability of the dolichyl phosphate carrier. Indeed it has been shown that dolichyl phosphate can be a limiting factor in asparagine-linked carbohydrate synthesis (Carson *et al.* 1981), and inhibition of hydroxymethylglutaryl coenzyme A reductase can block lipid-oligosaccharide

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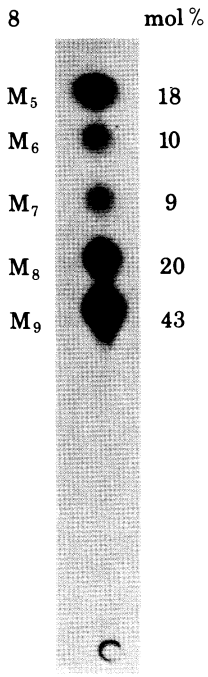
synthesis (Mills & Adamany 1978). Some time ago we observed that in the presence of puromycin transfer of carbohydrate to protein from oligosaccharide-lipid was blocked and that an inhibition of sugar incorporation into lipid-saccharide donor also occurred (M. J. Spiro *et al.* 1976). While the first observation was consistent with the known action of puromycin, the latter finding suggested to us that the available dolichyl phosphate in thyroid was limited. We have now extended these studies by comparing the levels of the individual prelabelled



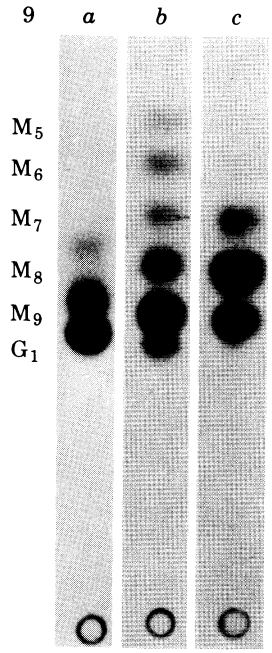


FIGURE 8. Nature and molar distribution of polymannose units of calf thyroglobulin as determined by thin-layer chromatography. The glycopeptide fraction obtained after pronase digestion of calf thyroglobulin was treated with endo H, and the released oligosaccharides were then reduced with [^{3}H]$NaBH_4$. Chromatography of the reduced, radiolabelled oligosaccharides was carried out on silica gel plates in 1-propanol–acetic acid–water, 3:3:2. The components were revealed by autoradiography and their radioactivity determined by scintillation counting after removal from the plate. The abbreviations are the same as in figures 4 and 7.

FIGURE 9. Nature of the radiolabelled oligosaccharides released by endo H treatment of thyroglobulin isolated from thyroid membranes, after a 60 min pulse with [^{14}C]glucose (*a*) and 180 min chase (*b*, *c*) of thyroid slices. The chase incubations were carried out in the absence (*b*) and presence (*c*) of CCCP (80 μM). Separation of the oligosaccharides was carried out by thin-layer chromatography on silica gel plates (1-propanol–acetic acid–water, 3:3:2) and the components were revealed by autoradiography. (Data taken from Godelaine *et al.* (1981).) The abbreviations are the same as in figures 4 and 7.

lipid-linked oligosaccharides after a chase in the presence or absence of puromycin (figure 11). Although puromycin inhibited transfer of $Glc_3Man_9GlcNAc_2$ to protein, no significant increase in this oligosaccharide-lipid was observed (figure 11). Chemical analyses of the lipid-linked oligosaccharides by the [^{3}H]$NaBH_4$ procedure gave similar results. Moreover, because there was no accumulation of smaller species of lipid-linked oligosaccharides, as determined by thin-layer chromatography, a limitation of the dolichyl phosphate available for glycosylation can again be inferred, although the possibility of feedback inhibition in the initial sugar attachment must also be considered. Dolichyl phosphate suitable for participation in lipid-oligosaccharide

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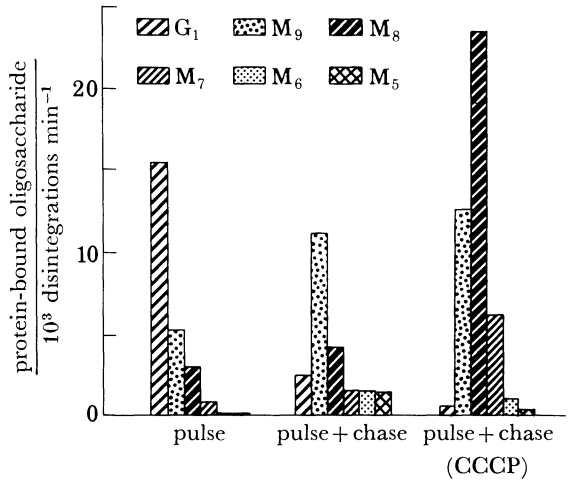

FIGURE 10. Distribution of radioactivity in oligosaccharides released by endo H from thyroglobulin isolated from membranes after incubations of thyroid slices with [^{14}C]glucose. The pulse incubation was 60 min; the chase (180 min) was carried out in the absence and presence of CCCP. The oligosaccharides were separated by thin-layer chromatography (see figure 9) and the radioactivity in each component was determined by scintillation counting after removal from the plate. (Data taken from Godelaine *et al.* (1981).) The abbreviations are the same as in figures 4 and 7.

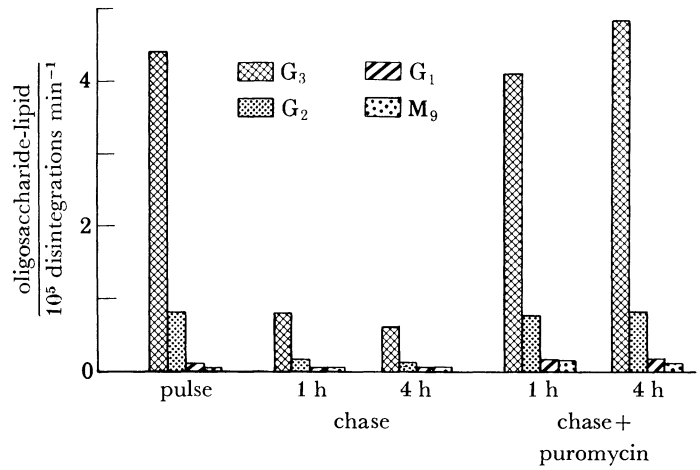

FIGURE 11. Effect of puromycin on the oligosaccharide-lipids of thyroid slices. Incubations with [^{14}C]glucose were carried out under conditions previously described (M. J. Spiro *et al.* 1976). After a 1 h pulse the chase incubations were performed in the absence and presence of puromycin (0.6 mM). The lipid-linked oligosaccharides were released by mild acid hydrolysis and separated by thin-layer chromatography after endo H digestion. The radioactivity of each oligosaccharide was determined by scintillation counting after removal from the chromatographic plate. The abbreviations are the same as in figure 4.

synthesis could be generated through *de novo* synthesis, recycling or phosphorylation of the free dolichol present in tissues.

It is evident that the addition of the glucose residues to the lipid-oligosaccharide represents another important regulating factor in asparagine-linked carbohydrate unit synthesis. Because the triglucosyl sequence seems to be essential for physiological *N*-glycosylation, any interference with its assembly, as in our energy-deprived thyroid slices, would be reflected in an inhibition of saccharide unit attachment.

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The processing steps (figure 2) afford further opportunities for regulation: some carbohydrate units are not reduced in size beyond $Man_9GlcNAc_2$ whereas others are trimmed further and after reaching the level of $Man_5GlcNAc_2$ they may give rise to complex oligosaccharides. Furthermore the asparagine-linked carbohydrate of lysosomal enzymes undergoes a distinctive modification involving the phosphorylation of mannose residues. The amino acid sequence of the glycosylated protein and its speed of migration to the cell surface may play a role in determining the nature and extent of the processing reactions.

Finally it must be appreciated that the assembly and processing steps take place at several intracellular sites and require translocations across membranes and movements from one compartment to another, which provides additional possibilities for the regulation of glycoprotein synthesis.

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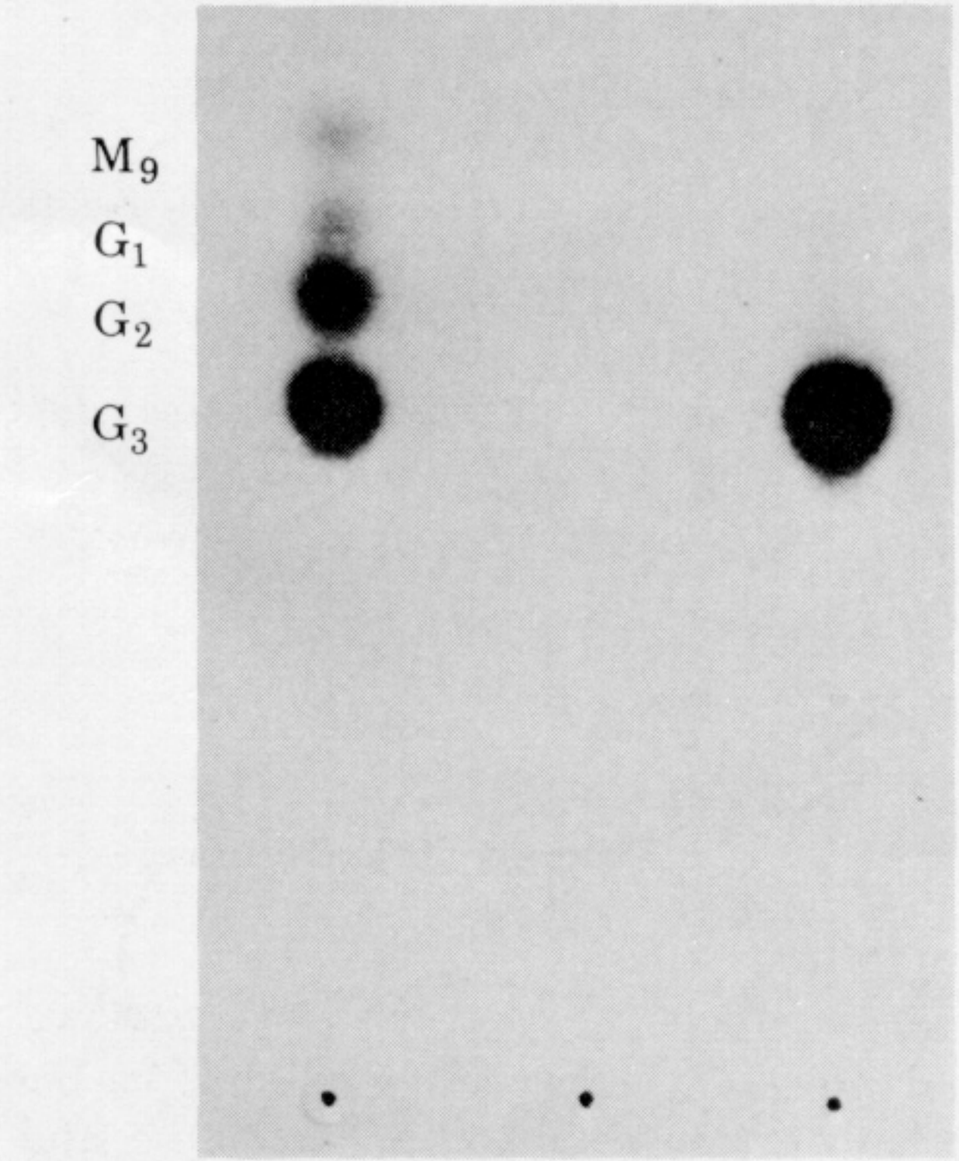

FIGURE 4. Specificity of thyroid microsomal oligosaccharyltransferase for fully glucosylated oligosaccharide-lipid. The enzyme was incubated with ^{14}C-labelled oligosaccharide-lipid from thyroid slices in the presence of an exogenous peptide acceptor. Thin-layer chromatographic separation on silica gel plates (1-propanol–acetic acid–water, 3:3:2) of the oligosaccharides released from the glycosylated peptide by digestion with endo-β-*N*-acetylglucosaminidase (endo H) is shown and compared with the endo H-treated oligosaccharides released by mild acid hydrolysis from the lipid donor. (R. J. Chalifour & R. G. Spiro, unpublished data.) The components were revealed by autoradiography. The abbreviations used are: G_3, $Glc_3Man_9GlcNAc$; G_2, $Glc_2Man_9GlcNAc$; G_1, $Glc_1Man_9GlcNAc$; M_9, $M_9GlcNAc$.

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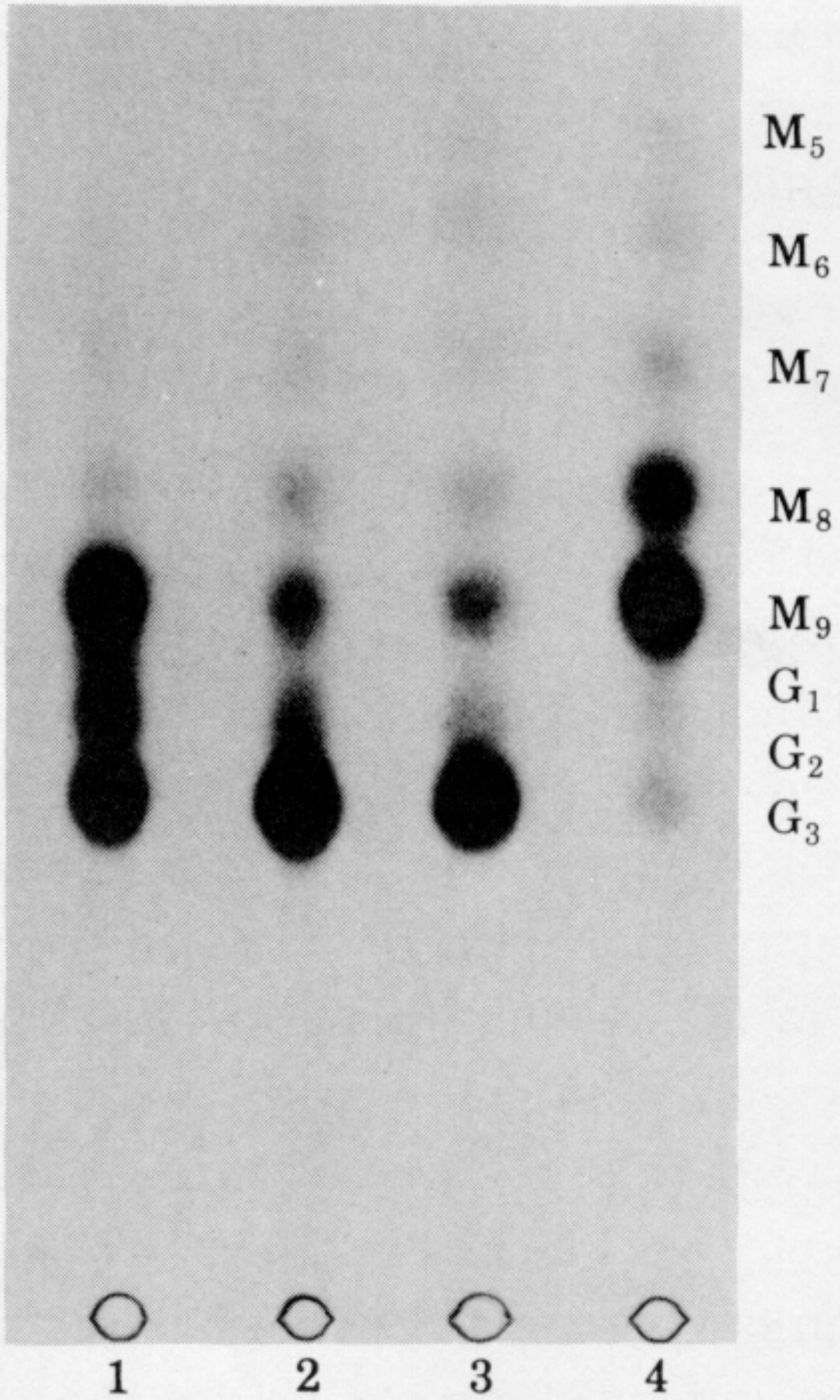

FIGURE 7. Effect of energy status on lipid-linked oligosaccharides of thyroid. Thyroids were brought to the laboratory on ice and sliced. Incubations were carried out in an oxygenated medium containing unlabelled pyruvate (5 mM) for 60 min (sample 2) and 150 min (sample 3). Sample 1 represents unincubated slices whereas the slices in sample 4 were incubated for 90 min in the presence of CCCP (100 μM) after a pre-incubation of 60 min. The lipid-linked oligosaccharides were separated by thin-layer chromatography on silica gel plates (1-propanol–acetic acid–water, 3:3:2) after release by mild acid hydrolysis, endo H digestion and reduction with [^{3}H]$NaBH_4$. The components were revealed by autoradiography. The abbreviations G_3, G_2, G_1 and M_9 are defined in figure 4; additional abbreviations are: M_8, $Man_8GlcNAc$; M_7, $Man_7GlcNAc$; M_6, $Man_6GlcNAc$; M_5, $Man_5GlcNAc$.

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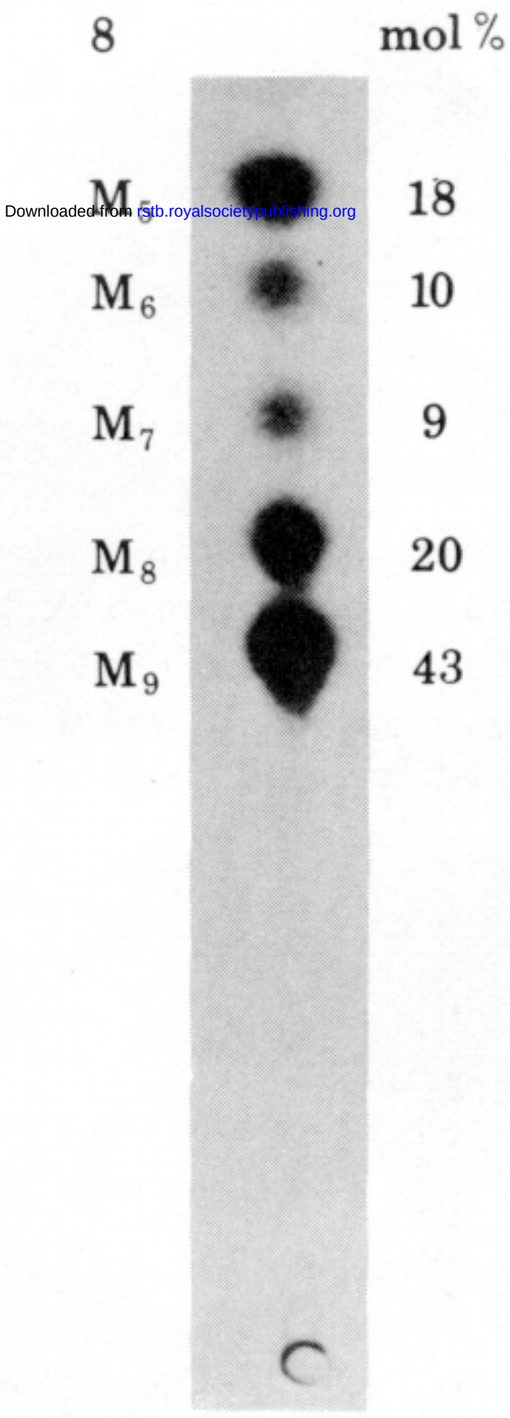

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FIGURE 8. Nature and molar distribution of polymannose units of calf thyroglobulin as determined by thin-layer chromatography. The glycopeptide fraction obtained after pronase digestion of calf thyroglobulin was treated with endo H, and the released oligosaccharides were then reduced with $[^3H]NaBH_4$. Chromatography of the reduced, radiolabelled oligosaccharides was carried out on silica gel plates in 1-propanol–acetic acid–water, 3:3:2. The components were revealed by autoradiography and their radioactivity determined by scintillation counting after removal from the plate. The abbreviations are the same as in figures 4 and 7.

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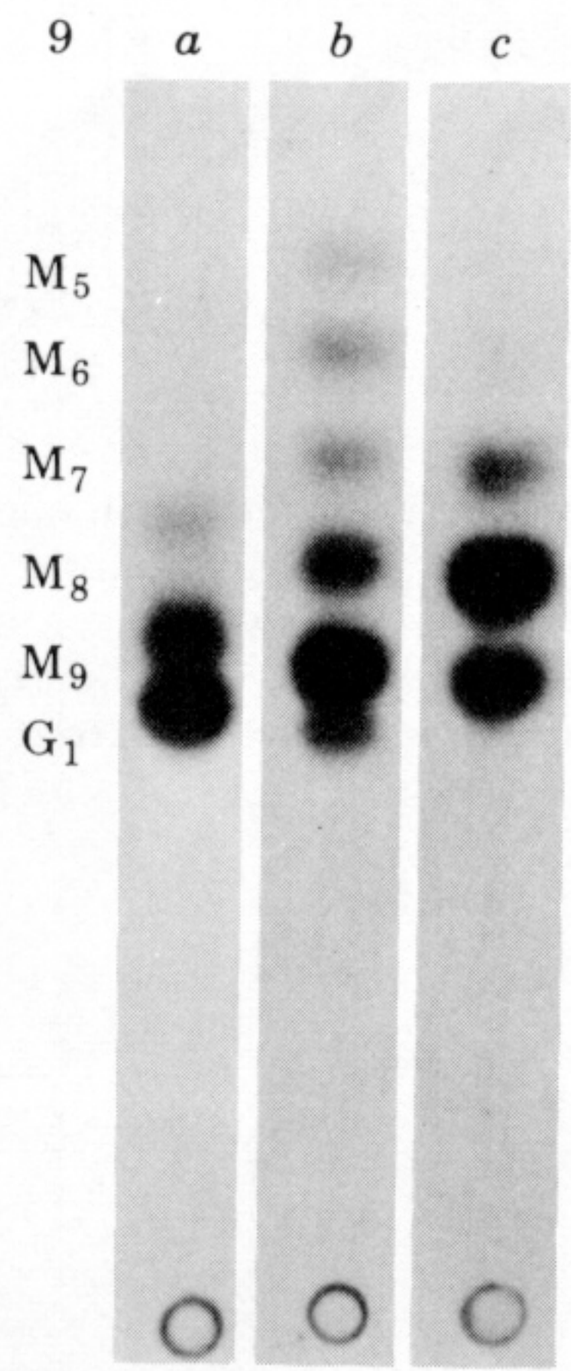

FIGURE 9. Nature of the radiolabelled oligosaccharides released by endo H treatment of thyroglobulin isolated from thyroid membranes, after a 60 min pulse with [^{14}C]glucose (*a*) and 180 min chase (*b*, *c*) of thyroid slices. The chase incubations were carried out in the absence (*b*) and presence (*c*) of CCCP (80 μM). Separation of the oligosaccharides was carried out by thin-layer chromatography on silica gel plates (1-propanol–acetic acid–water, 3:3:2) and the components were revealed by autoradiography. (Data taken from Godelaine *et al.* (1981).) The abbreviations are the same as in figures 4 and 7.

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