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Oligosaccharides containing glucose and mannose in glycoproteins of the thyroid gland

(glycopeptides/dolichol/glycolipid/polyprenols)

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ABSTRACT The glucose-containing oligosaccharides formed by calf thyroid slices incubated with radioactive glucose were studied. A compound soluble in chloroform/methanol/water, 1:1:0.3 (vol/vol), was found that was indistinguishable from the previously described glucose-containing dolichyl diphosphate oligosaccharide formed by liver microsomes. Glycopeptides were prepared by treating the glycoproteins with pronase, the amino acids were removed with alkaline borohydride, and the products were examined by paper electrophoresis and chromatography. A saccharide equal to that which occurs in the glucose-containing dolichyl diphosphate oligosaccharide could not be detected but glucose was found in oligosaccharides that seemed to be smaller by about three to five monosaccharide residues. The same results were obtained by direct treatment of the glycoproteins with alkaline borohydride.

Previous work (1–3) has shown that animal tissues contain enzymes that lead to the formation of a series of saccharides linked to dolichyl diphosphate. The saccharide moieties of these compounds may be *N*-acetylglucosamine, *N*,*N*′-diacetylchitobiose, or oligosaccharides that, in addition to the latter disaccharide, contain mannose and sometimes glucose. The glucose-containing dolichyl diphosphate oligosaccharide was first detected by incubation of liver microsomes with dolichyl monophosphate [^{14}C]glucose (4). The dolichyl monophosphate glucose may be added as such or may be formed in the reaction mixture from UDPG and endogenous dolichyl monophosphate. The reaction product could be separated from other lipids by making use of the fact that it is insoluble in chloroform/methanol/water, 3:2:1 (vol/vol), but soluble in a solvent containing the same components in the proportions 1:1:0.3. Indirect evidence was presented indicating that the oligosaccharide, which will be referred to as the G-oligosaccharide, contains two *N*-acetylhexosamine residues and that it is formed in all or nearly all animal tissues (5) and yeast (6).

Estimates of the composition of the oligosaccharide gave mannose/*N*-acetylglucosamine/glucose ratios of 11:2:1.5 (7) and 12:2:4 (8) for the compounds of thyroid and liver, respectively. Only about one-half of the glucose residues were external as judged by the amount of radioactive formic acid released by periodate (5). The G-oligosaccharide was found to be transferred to an endogenous protein when the dolichyl diphosphate G-oligosaccharide was incubated with liver microsomes (9). Furthermore, the molecular size of the saccharide moiety did not seem to change in the course of the transfer reaction. Glycoproteins containing mannose, glucose, or acetylglucosamine have not been studied thoroughly, and only a few have been described. The reason is that glucose is of such widespread occurrence in biological material and in reagents that, in many cases, it cannot be established whether it is a contaminant or really forms part of the compound.

Experiments have now been carried out in order to find out the form in which the G-oligosaccharide occurs in glycoproteins *in vivo*. Three possibilities were considered: (*i*) that the unmodified G-oligosaccharide occurs in glycoproteins; (*ii*) that some hexose residues are lost after the transfer reaction; and (*iii*) that the G-oligosaccharide joined to protein is enlarged by addition of various sugar residues. The experiments were carried out with tissue slices that had been incubated with radioactive glucose. The mannose- and glucose-containing oligosaccharides were liberated from the glycoproteins and compared with the G-oligosaccharide.

METHODS

Preparation of Radioactive Glycopeptides. Calf thyroid slices (4.5 g) were incubated as described by Spiro *et al.* (10) with 900 μCi of [^{14}C]glucose (268 Ci/mol) for 3 hr at 37° in a 95% O_2/5% CO_2/atmosphere. The slices were washed, homogenized in 0.15 M Tris·acetate, pH 7.4/4 mM $MgCl_2$ and centrifuged at 160.000 x *g* for 2 hr. The pellet was then extracted four times with chloroform/methanol/water, 3:2:1 (vol/vol), and then three times with the same components in the proportion 1:1:0.3 (4). The latter extract was saved for the preparation of the labeled thyroid oligosaccharide. The protein residue was digested with Pronase for 144 hr at 37° (11) for the isolation of the glycopeptides. These were then separated by passage through a gel filtration column.

Treatment with Borohydride and Alkali. Removal of the asparagine residues from the glycopeptides was carried out as described by Chen *et al.* (12). The samples (10,000–40,000 cpm) plus 8 mg of sodium borohydride and 40 μl of 10 M NaOH (total volume, 0.2 ml) were heated in stoppered polypropylene tubes for 6 hr at 100°. They were then deionized by passage through a column of Bio-Gel P-2 (1 × 15 cm). For the direct treatment of the delipidized protein, the amount of alkali added was increased proportionally to compensate for the carboxyl groups that become liberated. For 10 mg of protein, 37 mg of sodium borohydride and 1 ml of 2.3 M NaOH were used.

***N*-Acetylation.** Under the conditions described by Wheat (13), *N*-acetylation was not complete; therefore, an additional amount of acetic anhydride (3 μl) was added after 15 min and after another 15 min the procedure was continued as described. In every case, the samples were submitted to electrophoresis in 5% formic acid in order to remove any charged compounds.

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Abbreviation: G-oligosaccharide, glucose-containing oligosaccharide that occurs combined with dolichyl diphosphate and becomes labeled on incubation of microsomes with radioactive UDPG or dolichyl monophosphate glucose (in previous papers, the whole compound was called GEA for glucosylated endogenous acceptor).

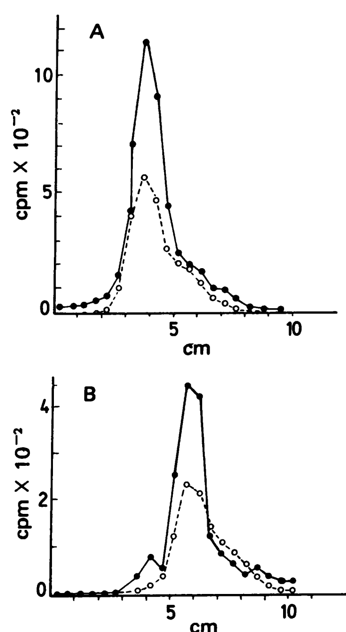

FIG. 1. Paper chromatography of the G-oligosaccharide and the corresponding compound obtained from thyroid slices. Both substances were spotted together on paper and then chromatographed with the following solvents: (*A*) butanol/pyridine/water, 4:3:4; (*B*) *n*-propanol/nitromethane/water, 5:2:3. O, ^{3}H-Labeled G-oligosaccharide obtained with liver microsomes. ●, ^{14}C-Labeled thyroid oligosaccharide obtained by mild acid hydrolysis of the chloroform/methanol/water, 1:1:0.3, extract. Radioactivity was measured in 0.5-cm strips in a scintillation counter.

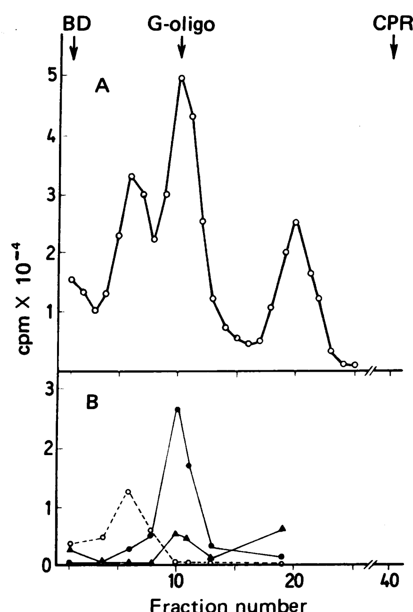

FIG. 2. Gel filtration chromatography of the Pronase glycopeptides. The glycoproteins (2×10^{6} cpm) were treated with Pronase and poured into a column of Bio-Gel P-6 (1.8 × 80 cm). The solvent was 0.1 M pyridine acetate, pH 5. DB, dextran blue; CPR, chlorophenol red; G-oligo, G-oligosaccharide. Each fraction was 3.8 ml. (*A*) Total radioactivity in 0.5 ml. (*B*) Radioactivity in hexoses. O, Galactose; ▲, glucose; ●, mannose.

Some *O*-acetylation occurs under the conditions described as judged by the formation, from glucosamine, of a substance that runs faster than acetylglucosamine in paper chromatography. Therefore, the samples were treated with sodium methoxide as described (14) for removal of *O*-acetyl groups.

Detection of Hexoses. The samples (0.5 ml) plus 0.1 ml of 6 M HCl were heated at 100° for 4 hr in a tube closed with a screwcap. The contents were then passed through a column containing 0.5 ml of an anion exchange resin in the acetate form (Bio-Rad AG, 3 × 4, 300–400 mesh). The column was washed twice with 0.5 ml of water, the collected effluents were dried in a glass planchet, and the radioactivity was measured in a flow counter. For more precise measurements, aluminum planchets were used. The contents of the planchets were then transferred to Whatman no. 1 paper in 2-cm-wide spots and carrier glucose, mannose, and galactose (5 μl of a solution containing 2.5 mg of each per ml) were added to each spot.

Chromatography was carried out with butanol/pyridine/water, 6:4:3 (vol/vol). After 40 hr, the papers were dried and then wetted with aniline phthalate reagent (15) diluted to 1:10 with water-saturated butanol in order to avoid excessive quenching in the measurement of radioactivity. The papers were heated for about 10 min at 120°–130°. The sugars became faintly but clearly visible. The strips were then scanned in an automatic scanner or cut into 0.5-cm segments and assayed for radioactivity, in a toluene-based scintillation fluid, in a scintillation counter. The latter procedure was used for quantitative measurements. There was always some overlapping of the radioactive zones but no great error was introduced by it.

Preparation of G-Oligosaccharide. Labeled G-oligosaccharide was prepared by incubation of liver microsomes with UDP-[^{14}C]glucose or UDP-[^{3}H]glucose as described (16). After extraction with chloroform/methanol/water, 3:2:1, the dolichyl diphosphate G-oligosaccharide was extracted with the same components in the proportions 1:1:0.3. The extract was dried, and thymol blue and 0.2 ml of water were added. The mixture was acidified with HCl (the amount just necessary to give a permanent red color) and heated for 10 min at 100°. After cooling, the mixture was partitioned with chloroform/methanol, and the upper phase containing the G-oligosaccharide was taken.

RESULTS

Radioactive Products. The product extracted from the thyroid slices in the chloroform/methanol/water, 1:1:0.3, fraction was submitted to mild acid hydrolysis and compared with G-oligosaccharide. The two oligosaccharides behaved the same when chromatographed on paper (Fig. 1). In the first (Fig. 1*A*) and second (Fig. 1*B*) solvents, the G-oligosaccharide runs like a malto-oligosaccharide of about 15–16 or 11–12 glucose residues, respectively. Furthermore, both the oligosaccharide obtained from the thyroid and the G-oligosaccharide gave two positively charged compounds when treated with alkali, that are believed to correspond to the deacetylation of one or two hexosamine residues (see below). The ratio of the radioactivity of mannose to that of glucose of the thyroid oligosaccharide was 2.5:1.

Gel Filtration of the Glycopeptide. The glycopeptides obtained by Pronase digestion of the delipidized protein were passed through a gel filtration column (Bio-Gel P-6) and the monosaccharides were estimated in the effluent fractions (Fig. 2). When run under the same conditions, the G-oligosaccharide emerged in tubes 9 and 10, at about the same position as a high peak of radioactivity. Mannose content was maximal in the same region which also corresponded to a peak in the glucose curve. Glucose content was also high in the last peak (tube 19) but mannose was very low. The region in which both glucose and mannose were present was therefore selected for further investigation.

Paper electrophoresis (in 5% formic acid) of aliquots of

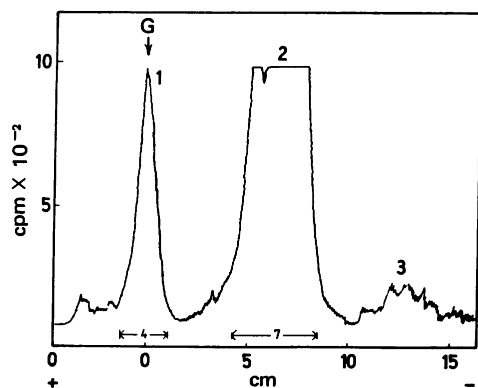

FIG. 3. Paper electrophoresis of a sample of pooled tubes 9–11 of Fig. 2. Electrophoresis was performed in 5% formic acid, 25 V/cm for 6 hr. G, position of glucose. The numbers between the arrows represent the ratio of radioactivities of mannose to glucose.

pooled tubes 9–11 of Fig. 2 gave three peaks of radioactivity (Fig. 3). Estimation of the ratio of mannose to glucose gave a value of 4 for the neutral substance (peak 1) and of 7 for peak 2. No sugar was present in peak 3. The substance in peak 2 corresponded to a glycopeptide because it moved to the negative pole in acidic medium and to the positive pole in alkaline buffer (0.25 M Na carbonate/Na bicarbonate, pH 9.8).

Alkaline Treatment of the Glycopeptides. It was known from previous work (5) that treatment of the methylglycoside of G-oligosaccharide with alkali gives rise to the formation of two substances with different electrophoretic mobilities in 5% formic acid. One of them moves slowly and the other faster toward the anode, and they correspond to the removal of one or two acetyl groups from *N,N'*-diacetylchitobiose residues.

As shown in Fig. 4*B*, treatment of the glycopeptide from peak 2 in Fig. 3 with borohydride and alkali under the conditions necessary for removing the asparagine residues resulted in the formation of slow- and fast-migrating substances. The

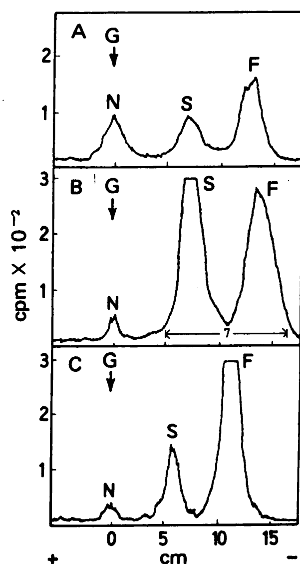

FIG. 4. Paper electrophoresis of the products obtained by borohydride and alkali treatment of the substances from the peaks in Fig. 3. Paper electrophoresis was performed in 5% formic acid, 25 V/cm for 3 hr. *A* and *B* correspond to peaks 1 and 2 of Fig. 3, respectively. *C* corresponds to G-oligosaccharide control treated in the same manner. Distance run by glucosamine, 25 cm. G, positions of glucose. The number under the peaks in *B* is the ratio of radioactivities of mannose to glucose. S, slow; F, fast; N, neutral.

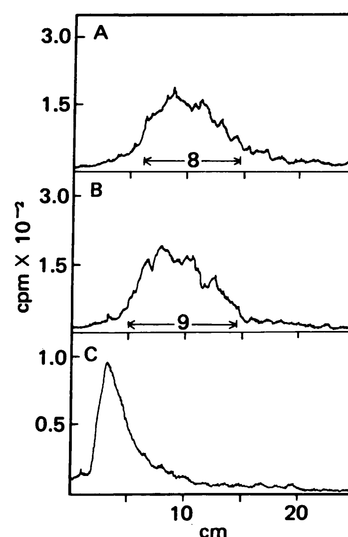

FIG. 5. Paper chromatography of the substances of the peaks of Fig. 4. *A* and *B* correspond to the oligosaccharides S and F obtained from the glycopeptides shown in Fig. 4*B*. *C* corresponds to G-oligosaccharide (peaks S and F in Fig. 4*C*). After elution from the paper, the substances were *N*-acetylated and chromatographed on paper with butanol/pyridine/water, 4:3:4, for 40 hr. The numbers under the peaks represent the ratio of radioactivities of mannose to glucose.

same treatment applied to the oligosaccharides of peak 1 in Fig. 3 gave a similar pattern (Fig. 4*A*). Table 1 shows the electrophoretic mobility of the substances relative to glucosamine. In both cases, the ex-glycopeptide (Fig. 4*B*) moved distinctly faster than the G-oligosaccharide subjected to the same treatment (Fig. 4*C*).

Paper Chromatography of the Ex-Glycopeptide. The substances corresponding to peak 2 and G-oligosaccharide (Fig. 4 *B* and *C*) were eluted from the paper, reacetylated, and treated with sodium methoxide in order to remove *O*-acetyl groups. The products were then chromatographed on paper with butanol/pyridine/water, 4:3:4, as solvent. The two ex-glycopeptide products that corresponded to the slow and fast peaks in Fig. 4*B* had the same mobility whereas the ex-G-oligosaccharide moved slower (Fig. 5). By comparison with the mobility of malto-oligosaccharides, this difference would correspond to three to five hexose residues. Estimation of the radioactivity in the hexoses after acid hydrolysis of the ex-glycopeptide gave a ratio of mannose to glucose of 8–9.

Direct Treatment of the Proteins with Borohydride and Alkali. In order to exclude the possibility that glycosidases contaminating the Pronase preparation could degrade the oligosaccharides, two types of experiments were carried out. One of them consisted of treating the G-oligosaccharide with Pronase under the same conditions used for digesting the glycoproteins. After incubation the product was compared by paper chromatography (butanol/pyridine/water, 4:3:4) with an untreated sample. Practically no difference in mobility was observed. The other type of experiment consisted of treating the delipidized protein directly with alkali and borohydride. The reaction product was run in the Bio-Gel P-6 column. The pattern of radioactivity shown in Fig. 6 is similar to that of the glycopeptides (Fig. 2) but the main peak was slightly displaced as if the components were smaller.

A pool of the fractions corresponding to the major peak (tubes 11–13) was submitted to paper electrophoresis in 5% formic acid (Fig. 7). The pattern was similar to that obtained by treatment of the glycopeptide, with alkali and borohydride. The substances formed (peaks S and F) showed an increased mobility

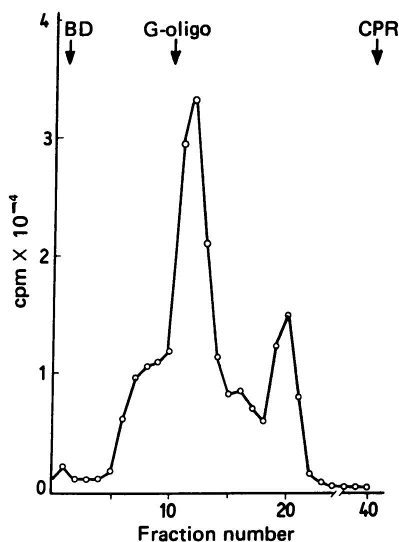

FIG. 6. Gel filtration chromatography of the products of direct borohydride and alkali treatment of delipidized proteins. The glycoproteins (10^6 cpm) were treated with alkali and borohydride, and separation was carried out with a Bio-Gel P-6 column as described in Fig. 2.

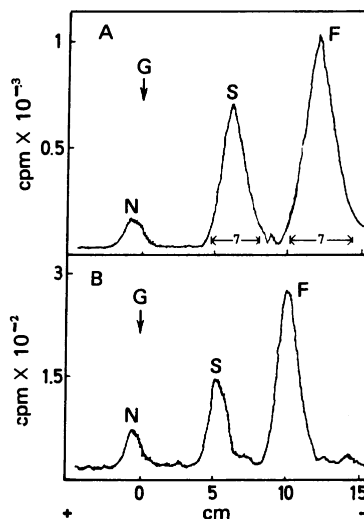

FIG. 7. Paper electrophoresis (5% formic acid, 25 V/cm for 3 hr) of the substances of the major peak of Fig. 6. (*A*) Aliquot of a pool of tubes 11–13 of Fig. 6. (*B*) G-oligosaccharide treated with borohydride and alkali. Glucosamine moved 22 cm. G, position of glucose. The numbers under the peaks represent the ratio of radioactivities of mannose to glucose.

compared to those corresponding to the G-oligosaccharide (Table 1). Measurements of the mannose/glucose ratio gave a value of 7 for both the S and F peaks (Fig. 7).

Paper Chromatography of the Product of Direct Treatment with Borohydride and Alkali. The three peaks obtained by electrophoresis as in Fig. 7 were eluted from the paper *N*-acetylated, treated with sodium methoxide, and chromatographed on paper with butanol/pyridine/water, 4:3:4; a G-oligosaccharide control was run at the same time (Fig. 8). The substances in Fig. 8 *B* and *C* had a greater mobility than the G-oligosaccharide (Fig. 8*D*) and were somewhat more polydisperse. The substance in Fig. 8*A* (the neutral compound) gave a wide peak as if it were a mixture of several substances. The slower and faster parts of the peaks in Fig. 8 *B* and *C* were eluted in two halves, as indicated by the arrows. The ratio of radioactivities of mannose to glucose was the same (8–9) in the slower and faster halves, showing that if compounds of different sizes were present their composition was the same.

The Product of Transfer by Microsomal Enzymes. Experiments designed to detect changes in the G-oligosaccharide occurring in the process of transfer from dolichyl diphosphate to endogenous protein were carried out previously (9). When the glycopeptide obtained from the glycoprotein by Pronase treatment was compared with G-oligosaccharide in a gel filtration column, it was found that both products were similar. These experiments have now been repeated with some changes. The transfer reaction with dolichyl G-oligosaccharide was carried out as before (9).

Samples of the glycoprotein were freed from dolichyl diphosphate G-oligosaccharide in two manners: (*i*) by washing thoroughly with chloroform/methanol/water, 1:1:0.3, before Pronase treatment, and (*ii*) by suspension in 5% trichloroacetic

Table 1. Electrophoretic mobility of oligosaccharides released from glycoproteins

Substance	Slow peak (S)	Fast peak (F)
Ex-glycopeptide (Fig. 4*B*)*	0.27	0.50
Ex-uncharged component (Fig. 4*A*)*	0.26	0.49
G-oligosaccharide (Fig. 4*C*)	0.22	0.43
Ex-glycoprotein (Fig. 7*A*)†	0.27	0.52
G-oligosaccharide (Fig. 7*B*)	0.23	0.43

The data correspond to the experiments shown in Figs. 4 and 7. The numbers represent the mobility relative to glucosamine.

* Ex-glycopeptide and Ex-uncharged component refer to the substances obtained by treating the glycopeptides from peaks 2 and 1 (Fig. 3), respectively, with alkaline borohydride.

† Ex-glycoprotein refers to the major peak (tubes 11–13) obtained by filtering through the Bio-Gel P-6 column (Fig. 6), the delipidized protein treated with alkaline borohydride.

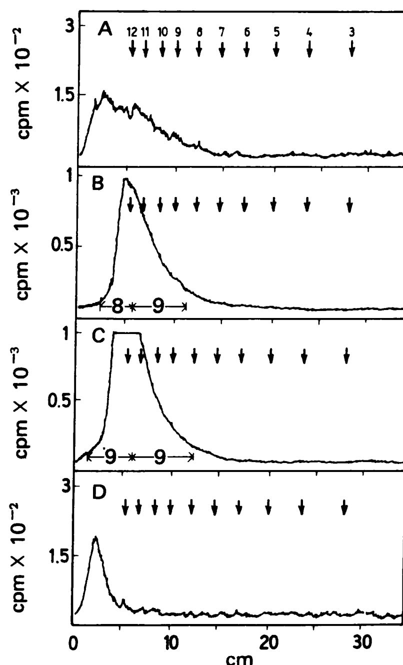

FIG. 8. Paper chromatography of the products of Fig. 7*A*. (*A*, *B*, and *C*) Peaks N, S, and F of Fig. 7*A*. (*D*) Peaks S plus F of Fig. 7*B* (G-oligosaccharide). The substances were eluted from the paper, *N*-acetylated, and chromatographed as in Fig. 5. The arrows show the position of malto-oligosaccharides containing 3–12 glucose units. The numbers under the peaks represent the ratio of radioactivities of mannose to glucose.

acid and heating 10 min at 96° followed by washing with cold trichloroacetic acid (6). These two samples and a G-oligosaccharide standard were then treated with alkali and borohydride, and the charged substances were acetylated. The three substances were chromatographed on paper with the butanol/pyridine/water, 4:3:4. The R_G values obtained were equal for the three substances: 0.063, 0.066, and 0.068, respectively. These results confirm the fact that oligosaccharides are not altered in size in the course of their transfer from dolichyl diphosphate to proteins.

DISCUSSION

The transfer of the saccharide moiety of dolichyl diphosphate G-oligosaccharide to endogenous protein was previously detected by using liver microsomes as enzyme source (9). In order to study the problem under conditions more similar to those prevailing *in vitro*, tissue slices were used. Thyroid tissue was selected because, according to Spiro *et al.* (17), its slices are one of the most efficient formers of dolichyl diphosphate oligosaccharide.

On incubation of the slices with glucose, a substance was formed with a solubility similar to that of dolichyl diphosphate oligosaccharide. The oligosaccharide moiety of this compound was found to be identical, as judged by paper chromatography, with that of the product that becomes labeled on incubation of liver microsomes with radioactive UDPG.

The next step was to detect glucose-containing oligosaccharides in the thyroid glycoproteins. For this purpose, the latter were broken down with Pronase and the glycopeptides were separated by gel filtration. The tubes containing mannose and glucose were then submitted to electrophoresis, treatment with alkali and sodium borohydride, electrophoresis of the positively charged compounds, acetylation, and paper chromatography. Comparison with G-oligosaccharide obtained from microsomes incubated with labeled UDPG was carried out at each step. The result was that the ex-glycopeptide that contained mannose and glucose migrated faster in electrophoresis and on paper chromatography. These results are most probably due to differences in molecular size. (Changes in mobility in paper chromatography may be attributed to differences in molecular size when compounds within homologous series are compared. This seems to be the case with the mannose- and glucose-containing oligosaccharides.) The difference in mobility between the G-oligosaccharide and the ex-glycopeptide corresponded to a change of three to five glucose units as judged by the behavior of the malto-oligosaccharides.

One explanation of the results is that the oligosaccharide of the glycoproteins does not arise from dolichyl diphosphate G-oligosaccharide. However, the fact that both compounds have mannose, glucose, and 2-hexosamine residues makes it reasonable to assume that they have a precursor-product relationship. If this is so, the oligosaccharide must be partially hydrolyzed after being transferred to protein. This partial hydrolysis does not seem to occur in the course of the transfer reaction because the protein glycosylated by the microsomal system contains a sugar moiety indistinguishable from the G-oligosaccharide. Although this point has been studied with rat liver enzymes, it is probably true for thyroid because the evidence at hand indicates that the reactions in which dolichyl phosphates are involved are similar in all mammalian tissues.

Another possibility to be considered is that contaminating hydrolases present in the Pronase preparation might degrade the glycopeptides. This possibility was excluded by incubating G-oligosaccharide with Pronase under the same conditions under which the glycopeptides were prepared and observing that practically no degradation occurred. Furthermore, experiments were performed in which the oligosaccharides were liberated directly from the protein with alkali and borohydride—that is, without using Pronase. The results were similar to those obtained with the glycopeptide.

The results indicate that, if dolichyl diphosphate G-oligosaccharide acts as saccharide donor *in vivo*, the transfer is followed by a process in which several hexose residues are removed. Similar alterations of the oligosaccharides have been suggested by Robbins *et al.* (18). This process of hydrolysis after synthesis reminds us of what occurs in the protein field where peptide chains are built up in the ribosomes and afterward various portions may be split off.

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