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Microsomal Glucosidases of Rat Liver. Partial Purification and Inhibition by Disaccharides

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Further work on microsomal glucosidases of rat liver has confirmed that at least two enzymes are involved in the removal of glucose from the glucose-containing oligosaccharide. One acts on the oligosaccharide containing three glucose residues and another on the oligosaccharide which has one or two glucoses.

The glucosidase which acts on $(Glc)_2(Man)_9(GlcNAc)_2$ could be purified with a Concanavalin-A–Sepharose column followed by electrofocusing. This purified preparation was active on the oligosaccharide containing one or two glucoses. Heat inactivation and inhibition by disaccharides was parallel for both activities. Inhibition of the glucosidase active on $(Glc)_3(Man)_9(GlcNAc)_2$ was obtained with kojibiose which has an α1-2 linkage, while the glucosidase acting on $(Glc)_{1-2}(Man)_9(GlcNAc)_2$ was inhibited by nigerose (α1-3 linkage), maltose (α1-4 linkage) and glucose at a higher concentration. None of the β anomers inhibited. These results are consistent with an α configuration of the three glucoses of the dolichyl-diphosphate-linked oligosaccharide. Kojibiose was found to inhibit glucosidase action not only on the free oligosaccharide but also on protein-bound one.

The glycosylation of asparagine residues in proteins occurs by transfer of an oligosaccharide from its Dol*PP* derivative [1, 2]. The oligosaccharide which is transferred contains three glucose, nine mannose and two *N*-acetylglucosamine residues [3]. In order to be transferred the oligosaccharide has to have three glucoses and presumably these serve as signals for the transfer [4, 5]. The step which immediately follows is the removal of the glucose residues after which other processing reactions may occur. These glucoses are linked as follows to the rest of the oligosaccharide: Glc(1–2)Glc(1–3)Glc(1–3)Man-R [3]. Glucosidases acting on $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ were first detected in the microsomal fraction of liver [6]. It was found later that at least two different membrane-bound glucosidases could be detected using as substrates oligosaccharides of different glucose content [7–9]. One of these enzymes acts on the Glc(1–2)Glc linkage. It can be extracted with phosphate buffer and a non-ionic detergent [8].

Another glucosidase acts on the Glc(1–3)Glc linkages. It has been partially purified and some of its properties have been described [7–9]. Further studies on these enzymes are described in this paper.

MATERIALS AND METHODS

Nigerose was prepared by partial hydrolysis of nigerian obtained from Koch Light Laboratories. Kojibiose and sophorose were supplied by Koch Light; maltose, isomaltose and cellobiose by Sigma.

Substrates

Dol*P*-[^{14}C]Glc was prepared by incubation of liver microsomes with UDP-[^{14}C]Glc and purified up to the DEAE-cellulose step [10, 11]. Dol*PP*-oligosaccharides labeled in glucose were similarly obtained with UDP-[^{14}C]Glc or Dol*P*-[^{14}C]Glc as substrates [5, 12]. Dol*PP*-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled both in glucose and mannose were prepared by incubating oviduct slices with [^{14}C]mannose [13]. Free $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ were obtained by mild acid hydrolysis [13] of the dolichyl diphosphate derivatives and separated by paper chromatography with 1-propanol/

Abbrevations. Glc, glucose; Man mannose; GlcNAc, *N*-acetylglucosamine; Dol*P*, dolichyl phosphate; Dol*PP*, dolichyl diphosphate; $(Glc)_1$, $(Glc)_2$, and $(Glc)_3$ glucosidases are the enzymes that act on the oligosaccharides containing one, two, and three glucose residues.

nitromethane/water (5:2:4) for 5–7 days. The three oligosaccharides were located with a scanner, eluted from the paper and used as substrates for the glucosi-

Estimation of the Glucosidases

Estimation of the glucosidases was carried out as described previously [8]. After incubation of the labeled oligosaccharides for 5 min at 37 °C with the enzyme in 40 mM phosphate buffer pH 7.0 and 0.1% Nonidet P-40 (total volume 50 µl) one volume of methanol was added. The supernatant fluid obtained by centrifugation was spotted on 2.5 × 14-cm papers and chromatograph for 3 h with isopropanol/acetic acid/water (29:4:9). The radioactive glucose ($R_f = 0.4$) and the oligosaccharide which remained at the origin were then measured in a scintillation counter. When Dol*PP*-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ was used as substrate the incubation was done as with the free oligosaccharides and the reaction was stopped by adding trichloroacetic acid (final concentration 5%). After heating to 90 °C for 10 min and centrifuging, the supernatant fluid was extracted with ethyl ether and chromatographed as described previously. One unit of $(Glc)_2$-glucosidase was defined as the amount of enzyme capable of releasing 10% of the glucose per min from $(Glc)_2(Man)_9(GlcNAc)_2$. Since $(Glc)_3$-glucosidase liberates only one of the three glucoses of $(Glc)_3(Man)_9(GlcNAc)_2$, one unit was taken as 3.3% of the glucose set free [8].

Solubilization of $(Glc)_3$-glucosidase

Liver microsomes were prepared [14] and extracted as described previously [8]. They were suspended in 80 mM triethylamine acetate buffer pH 7.0 containing 2% of Nonidet P-40 (final concentration 30 mg protein per ml). After 20 min the mixture was centrifuged and the supernatant fluid was removed. The extraction was repeated four times more. Under these conditions (without phosphate in the extraction buffer) the $(Glc)_2$-glucosidase activity was recovered in the supernatant and the $(Glc)_3$-glucosidase in the pellet. After the fifth extraction the pellet was suspended in 0.16 M phosphate buffer pH 7.0 containing 2% of Nonidet P-40 and centrifuged. The supernatant fluid contained $(Glc)_3$-glucosidase and a small amount of $(Glc)_2$- glucosidase.

RESULTS

Purification of $(Glc)_2$-Glucosidase

The extraction of microsomes was carried out as described above and the first supernatant fluids which contained hardly any $(Glc)_3$-glucosidase were passed through a column of Concanavalin-A – Sepharose 4B. As shown in Fig. 1 most of the protein appeared in

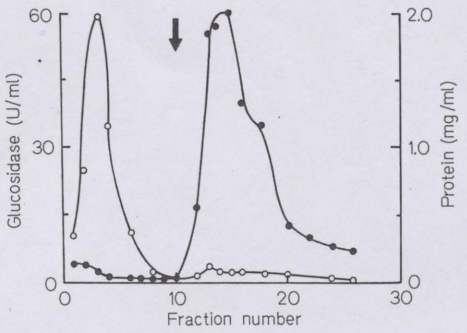

Fig. 1. *The binding of $(Glc)_2$-glucosidase to Concanavalin-A – Sepharose.* The $(Glc)_2$-glucosidase was solubilized as described in Materials and Methods. The extract (1.5 ml containing 15 mg of protein) was poured into a column of Concanavalin-A – Sepharose 4B (0.5 × 1 cm) equilibrated with 80 mM of triethylamine acetate pH 7, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 5 mM mercaptoethanol and 0.1% Nonidet P-40 (Buffer B). Fractions of 2.5 ml were collected. Glucosidase (●) was determined with a substrate of $(Glc)_2(Man)_9(GlcNAc)_2$ labeled in the glucose residue. The arrow shows when 2% of α-methyl mannoside in buffer B was added. After precipitation with 10% of trichloroacetic acid, protein (○) was determined by the method of Lowry et al. [15]

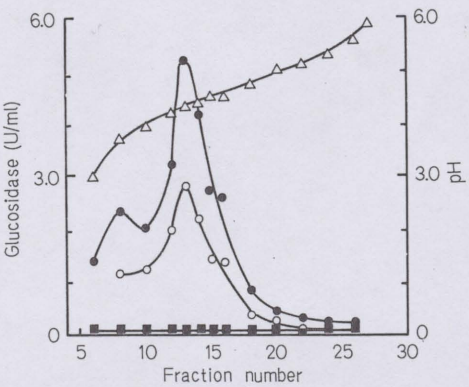

Fig. 2. *Electrofocussing of $(Glc)_2$-glucosidase.* $(Glc)_2$-glucosidase (Concanavalin A step) (10 ml) was dialyzed against buffer A and poured into an LKB 8101 Ampholine Electrofocussing apparatus (column of 110 ml). The isoelectric focussing was carried out as described in the LKB instruction manual with the addition of 0.1% Nonidet P-40 to the ampholite solution (pH 3 – 6). Electrofocussing was carried out at 4 °C for 12 h; maximum load 10 W, 1600 V. Fractions of 3 ml collected and the pH △ was determined. After dialysis against buffer A for 12 h at 4 °C the glucosidases were determined on $(Glc)_3(Man)_9(GlcNAc)_2$ (■), $(Glc)_2(Man)_9(GlcNAc)_2$ (●), and $(Glc)_1(Man)_9(GlcNac)_2$ (○) labeled in the glucose residue.

the percolate while $(Glc)_2$-glucosidase activity was retained in the column and could be eluted with 2% α-methylmannoside. A pool of fractions 13 to 15 were dialysed against 5 mM phosphate buffer pH 7.0, 5 mM EDTA, 5 mM 2-mercaptoethanol and 0.1% of Nonidet P-40 (buffer A). This preparation is referred to as $(Glc)_2$-glucosidase (Concanavalin A step). The protein content of these fractions was so low it could not be determined accurately. $(Glc)_2$-glucosidase purified as described was electrofocussed and the results are shown in Fig. 2. The activity on $(Glc)_{1-2}(Man)_9$

$(GlcNAc)_2$ appeared as sharp peaks and in the position corresponding to an isoelectric point of 4.25. A pool of fractions 12–16 was used as electrofocussed enzyme.

Stability of the Glucosidases

The Concanavalin-A – Sepharose purified glucosidases were found to be unstable. At 5 °C half the activity was lost in one day. Different compounds were tested as stabilizers of the enzyme with the results shown in Table 1. $(Glc)_2$-glucosidase activity was protected by the addition of 20% of glycerol in phosphate buffer of pH 7 and better protection was obtained by dialysis against buffer A. However, purified preparations were more labile than the crude extracts. The stability of the glucosidase acting on $(Glc)_{1-2}(Man)_9(GlcNAc)_2$ was studied by incubating the enzyme purified up to the Concanavalin A step for different times at 40 °C. The activity on the compound containing one and two glucoses decreased in the same manner following a first-order function.

The Action of $(Glc)_2$-Glucosidase

$(Glc)_2$-glucosidase purified by electrofocussing was incubated for various times with $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled in the glucose residue. As shown in Fig. 3A $(Glc)_2$-glucosidase released glucose from $(Glc)_{1-2}(Man)_9(GlcNAc)_2$ but not from $(Glc)_3(Man)_9(GlcNAc)_2$. In another experiment the substrate was $(Glc)_2(Man)_9(GlcNAc)_2$ labeled in the glucose and mannose residues. After the incubation for the different times the samples were chromatographed on paper with butanol/pyridine/water (6:4:3) and the radioactivity was located with a scanner. The pieces of paper corresponding to the oligosaccharides, glucose and mannose were cut out and the hexoses were counted by scintillation. No mannose could be detected showing that mannosidase was absent (Fig. 3B). The oligosaccharides were eluted from the papers and rechromatographed with 1-propanol/nitromethane/water (5:2:4). The results are shown in Fig. 4. The peak of $(Glc)_2(Man)_9(GlcNAc)_2$ disappeared progressively. A small peak of $Glc(Man)_9(GlcNAc)_2$ appeared at 5 min of incubation, and then increased in size and finally was converted to a substance with the mobility of $(Man)_9(GlcNAc)_2$. As shown in Fig. 4F the $(Glc)_3(Man)_9(GlcNAc)_2$ did not change when it was incubated 120 min with $(Glc)_2$-glucosidase.

Effect of Disaccharides on the Glucosidase

Several disaccharides were tested as inhibitors of the $(Glc)_2$ and $(Glc)_3$ glucosidases. As shown in Table 2 the most potent inhibitor for $(Glc)_3$-glucosidase was kojibiose which has the same linkage as the outermost residue of $(Glc)_3(Man)_9(GlcNAc)_2$. A slight inhibition was obtained with maltose and nigerose and none with sophorose, isomaltose, cellobiose and glucose. The results were quite different when $(Glc)_2$-glucosidase was tested on $(Glc)_2(Man)_9(GlcNAc)_2$. A marked inhibition was obtained with 1 mM nigerose, which has the same linkage, $\alpha 1-3$, as the glucoses of $(Glc)_2(Man)_9(GlcNAc)_2$, but maltose which is $\alpha 1-4$ had about the same inhibitory effect. Free glucose also inhibited but a higher concentration. Thus at 12 mM a 54% inhibition was obtained. In contrast $(Glc)_3$-glucosidase was not affected by glucose at the same concentration. When the substrate was $Glc(Man)_9(GlcNAc)_2$ oligosaccharide the spectrum of inhibition was the same as that obtained with $(Glc)_2(Man)_9(GlcNAc)_2$. The inhibiting effect at different concentrations of some saccharides is shown in Fig. 5. The apparent K_i value for kojibiose acting on $(Glc)_3$-glucosidase is 0.1 mM. No inhibition was produced by sophorose and maltose. A similar experiment carried

Table 1. *Effect of various compounds on the stability of the $(Glc)_2$-glucosidase*
Glucosidase activity was determined on glucose labeled $(Glc)_2(Man)_9(GlcNAc)_2$ as described in Materials and Methods after different times at 5 °C. For dialysis the enzyme was dialyzed against buffer A

Additions	Half-life
	days
None	1
20% glycerol	3
20% glycerol + 5 mM phosphate pH 7	8
5 mM EDTA + 5 mM phosphate pH 7	6
Dialysis	Stable up to 28 days

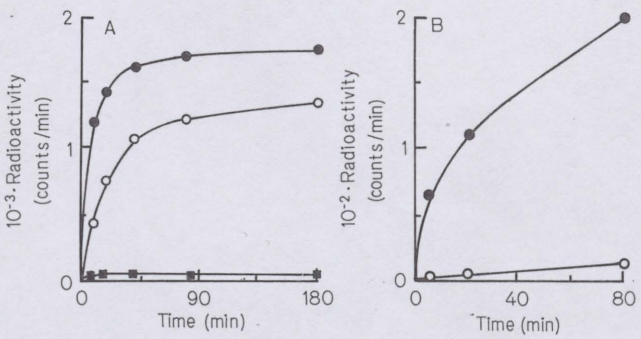

Fig. 3. *The action of $(Glc)_2$-glucosidase on $(Glc)_{1-3}(Man)_9(GlcNAc)_2$.* $(Glc)_2$-glucosidase purified by electrofocussing and dialyzed against buffer A was incubated as described in Materials and Methods with different substrates. (A) with $(Glc)_3(Man)_9(GlcNAc)_2$ (■); $(Glc)_2(Man)_9(GlcNAc)_2$ (●); and $Glc(Man)_9(GlcNAc)_2$ (○) (all labeled only in the glucose residues) for different times at 37 °C. (B) With $(Glc)_2(Man)_9(GlcNAc)_2$ labeled in both glucose and mannose residues. Mannose (○) and glucose (●) were separated by paper chromatography with butanol/pyridine/water (6:4:3) and counted in a scintillation counter. The oligosaccharides remaining at the origin were used in the experiment of Fig. 4

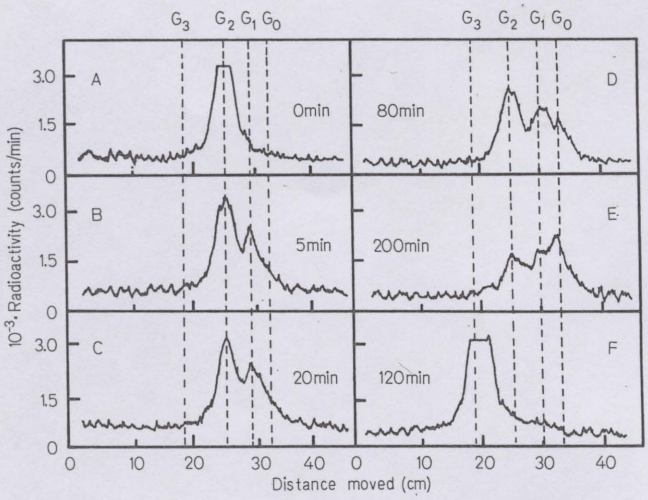

Fig. 4. *Action of $(Glc)_2$-glucosidase on the $(Glc)_3(Man)_9(GlcNAc)_2$ and $(Glc)_2(Man)_9(GlcNAc)_2$.* $(Glc)_2$-glucosidase was incubated wit $(Glc)_2(Man)_9(GlcNAc)_2$ (A, B, C, D and E) or $(Glc)_3(Man)_9(GlcNAc)_2$ (F) for the indicated times. The oligosaccharides labeled in the glucos and mannose residues were separated as described in Fig. 3B and chromatographed with 1-propanol/nitromethane/water (5:2:4). G_3 $(Glc)_3(Man)_9(GlcNAc)_2$; G_2, $(Glc)_2(Man)_9(GlcNAc)_2$; G_1, $Glc(Man)_9(GlcNAc)_2$; G_0, $(Man)_9(GlcNAc)_2$

Table 2. *The action of disaccharides on $(Glc)_1$, $(Glc)_2$ and $(Glc)_3$ glucosidases*
$(Glc)_3$-glucosidase, solubilized with detergent and phosphate buffer, was tested with $(Glc)_3(Man)_9(GlcNAc)_2$ labeled in the glucose residue $(Glc)_2$-glucosidase (Con canavalin A step) was used for $Glc(Man)_9(GlcNAc)_2$ and $(Glc)_2(Man)_9(GlcNAc)_2$

Additions	Linkage	Concentration	Inhibition of glucose release		
			$Glc(Man)_9(GlcNAc)_2$	$(Glc)_2(Man)_9(GlcNAc)_2$	$(Glc)_3(Man)_9(GlcNAc)$
		mM	%		
Nigerose	Glc(α1-3)Glc	1 (2)	65 (73)	56 (73)	7 (10)
Kojibiose	Glc(α1-2)Glc	1 (2)	9 (16)	12 (15)	91 (94)
Maltose	Glc(α1-4)Glc	1 (2)	36 (55)	40 (55)	5 (7)
Isomaltose	Glc(α1-6)Glc	4	–	12	–
Sophorose	Glc(β1-2)Glc	1	–	–	0
Cellobiose	Glc(β1-4)Glc	4	–	9	0
Trehalose	Glc(α1-1)Glc	4	–	2	–
Glucose		4 (8)	(40)	27 (32)	0
Glucose		12	–	54	4
Galactose		16	–	7	–
Mannose		16	–	28	–
Xylose		16	–	0	–

out with $(Glc)_2$-glucosidase gave the following apparent K_i values: maltose, 1.8 mM; glucose, 11 mM.

The Action of Kojibiose on the Disappearance of DolPP-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$

When Dol*P*-[^{14}C]Glc was incubated with liver microsomes and 0.5% of sodium deoxycholate without Mn^{2+} ions, Dol*PP*-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ (measured by the radioactivity in the 1:1:0.3 fraction, Fig. 6A) was formed rapidly but disappeared immediately. If 80 mM kojibiose was also added Dol*PP*-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ formation was not affected but the disappearance was much slower and the radioactivity in the aqueous phase did not increase as fast (Fig. 6B). This shows that kojibiose does not inhib the transfer of glucose from DolP-Glc to the endoge neous acceptor. If the action of kojibiose were o $(Glc)_3$-glucosidase, the DolPP-$(Glc)_{1-3}(Man)_9(Glc$ $NAc)_2$ which remained at the end of the incubatio should have had mostly three glucoses. This wa checked by hydrolyzing the DolPP-$(Glc)_{1-3}(Man)_9$ $(GlcNAc)_2$ formed in the experiment of Fig. 6B an separating the oligosaccharides by paper chromato graphy. The results showd that the oligosaccharide increased in the first 5–10 min and decreased after wards. However, $(Glc)_{1-2}(Man)_9(GlcNAc)_2$ decrease much faster than the $(Glc)_3(Man)_9(GlcNAc)_2$. It ma be concluded that the kojibiose inhibited the hydro lysis of the external glucose of Dol*PP*-$(Glc)_3(Man)_9$

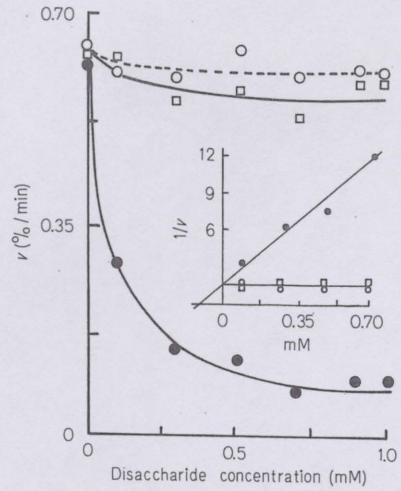

Fig. 5. *Effect of disaccharides on $(Glc)_3$-glucosidase activity.* Liver microsomal $(Glc)_3$-glucosidase solubilized as described in Materials and Methods was incubated with $(Glc)_3(Man)_9(GlcNAc)_2$ (1500 counts/min) and 0.2% Nonidet P-40 under standard conditions with different concentrations of disaccharides: kojibiose (●), maltose (□) and sophorose (○). The rate v is expressed as percentage glucose released $\times$ min^{-1}

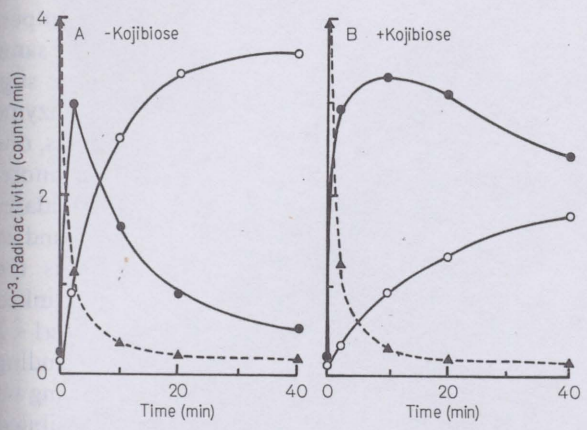

Fig. 6. *Formation of dolichyl diphosphate $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ from dolichyl phosphate glucose in the presence or absence of kojibiose.* Dol*P*-[^{14}C]Glc (5000 counts/min) was dried in a test tube and the following components were added: 50 mM Tris maleate pH 7.7, 100 mM 2-mercaptoethanol, 40 mM EDTA, 0.5% of sodium deoxycholate, 2 mg of microsomal protein in the absence (A) or presence (B) of 80 mM of kojibiose. After incubation at 30 °C for different times, 0.4 ml methanol, 0.15 ml of 4 mM $MgCl_2$ and 0.6 ml chloroform were added. After centrifugation the lower and upper phase were separated. The protein interphase was washed twice with chloroform/methanol/water (3/48/47) and extracted with cloroform/methanol/water (1/1/0.3). The lower phase (▲), upper phase (○) and 1/1/0.3-extract (●) were counted in a flow counter

$(GlcNAc)_2$. As mentioned before maltose is a good inhibitor of $(Glc)_2$-glucosidase. An experiment similar to that reported in Fig. 6 was carried out with maltose instead of kojibiose. In this case Dol*PP*-$(Glc)_{1-3}$-$(Man)_9(GlcNAc)_2$ disappeared only a little slower than in the control. Considerable maltase activity was detected in the enzyme preparation and this may have influenced the results.

The Action of Kojibiose on the Transfer to Protein

When glucosidase activity is measured in a microsomal preparation an activation is obtained on adding Nonidet P-40 up to 0.2% of concentration. This activation occurs using either free $(Glc)_3(Man)_9(GlcNAc)_2$ or Dol*PP*-$(Glc)_3(Man)_9(GlcNAc)_2$ as substrates. However the action on the two substrates is different when the detergent concentration is higher (1–4%). While activity on the free $(Glc)_3(Man)_9(GlcNAc)_2$ is not affected by detergent, that on the Dol*PP* derivative is strongly inhibited.

These experiments are not shown here but the results may help to understand better what follows. Some experiments were carried out in order to detect the action of kojibiose on $(Glc)_3$-glucosidase with protein-bound $(Glc)_3(Man)_9(GlcNAc)_2$ as substrate. The latter was prepared by transfer of $(Glc)_3(Man)_9$-$(GlcNAc)_2$ to endogeneous acceptor, in the presence of Mn^{2+} and a relatively high concentration of Nonidet P-40 so as to inhibit the action of glucosidase on Dol*PP*-$(Glc)_3(Man)_9(GlcNAc)_2$. A comparison of the transfer of oligosaccharide from Dol*PP*$(Glc)_3$-$(Man)_9(GlcNAc)_2$ to protein with and without kojibiose is shown in Fig. 7. Dol*PP*-$(Glc)_3(Man)_9$-$(GlcNAc)_2$ disappeared rapidly in the presence of Mn^{2+} which is necessary for transfer to protein. Kojibiose did not affect the disappearance of the Dol*PP* derivative. However kojibiose greatly retarded the rate of disappearance of radioactivity from the protein fraction as would be expected if the action of $(Glc)_3$-glucosidase on the protein-bound oligosaccharide were inhibited. In order to ascertain the composition of the protein-bound oligosaccharide remaining at the end of the incubation period a similar experiment was carried out but the Dol*PP*-$(Glc)_{1-3}$-$(Man)_9(GlcNAc)_2$ was labeled in the glucose and mannose residues. After an incubation period of 30 min the protein was digested with pronase passed through Bio-Gel P6 and submitted to paper electrophoresis. The charged substances were treated with endo-β-*N*-acetylglucosaminidase H and chromatographed on paper.

The results are shown in Fig. 8B. The sample which had kojibiose during the incubation showed a large peak of $(Glc)_3(Man)_9(GlcNAc)_2$ and hardly any $(Glc)_{1-2}(Man)_9(GlcNAc)_2$. This is in agreement with the finding that only the oligosaccharide with three glucose residues is transferred to protein [4, 5]. The sample incubated without kojibiose gave a small peak of $(Glc)_3(Man)_9(GlcNAc)_2$ and a similar amount of $Glc(Man)_9(GlcNAc)_2$ with a shoulder presumably corresponding to $(Man)_9(GlcNAc)_2$.

DISCUSSION

The presence of two different microsomal glucosidases, has been reported by several workers [7–9].

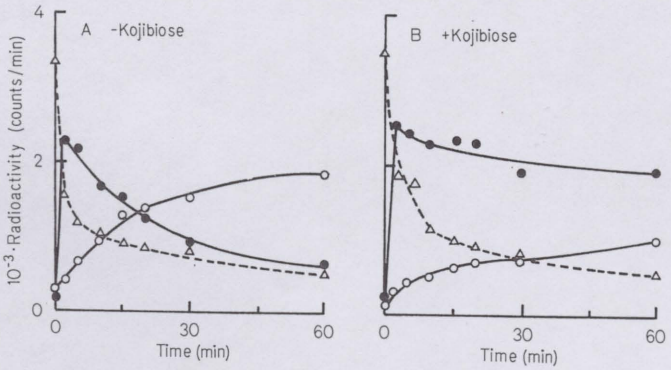

Fig. 7. *Transfer of $(Glc)_3(Man)_9(GlcNAc)_2$ to protein.* Dol*PP*-$(Glc)_3(Man)_9(GlcNAc)_2$, labeled with ^{14}C in the glucose residue (340 counts/min) were dried and the following components were added: 50 mM Tris maleate buffer pH 7.7, 10 mM 2-mercaptoethanol, 10 mM $MnCl_2$, 2% Nonidet P-40 and 2 mg of microsomal protein in a total volume of 50 µl. After incubations for different times at 30 °C the mixture was processed as described in the legend to Fig. 6. The protein precipitate was washed with methanol, dissolved in 0.2 ml of Protosol and counted in a scintillation counter. (△) 1/1/0.3-extract; upper phase (○); protein (●). (A) No addition; (B) plus 100 mM of kojibios

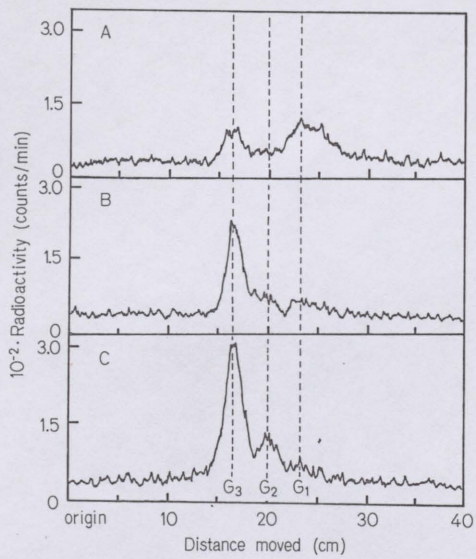

Fig. 8. *Effect of kojibiose on the oligosaccharides bound to protein.* Dol*PP*-$(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled in the glucose and mannose residues were incubated with liver microsomes as described in the legend to Fig. 7. After 30 min at 30 °C, 0.5 ml of 5% trichloroacetic acid was added and the samples were heated for 5 min at 96 °C. The protein precipitate was washed twice with 5% of trichloroacetic acid, four times with ethyl ether, and once with methanol. The protein residue was digested with pronase and the glycopeptides separated by passage through a gel filtration column as described previously [13]. The glycopeptides were submitted to electrophoresis in 5% formic acid in order to remove any neutral substances. The charged compounds were treated with endo-*β*-*N*-acetylglucosaminidase H (0.005 units) in 50 µl of 50 mM triethylamine acetate buffer pH 5.5 for 12 h at 37 °C in a toluene atmosphere. The neutral substances freed from charged compounds by electrophoresis were chromatographed on paper with 1-propanol/nitromethane/water (5/2/4). (A) Incubated with no addition; (B) incubated with 0.1 M kojibiose; (C) $(Glc)_{1-3}(Man)_9(GlcNAc)_2$ labeled in the glucose residue treated with endo-*β*-*N*-acetylglucosaminidase. G_1, $Glc(Man)_9(GlcNAc)_2$; G_2, $(Glc)_2(Man)_9(GlcNAc)_2$; G_3, $(Glc)_3(Man)_9(GlcNAc)_2$

One of these glucosidases acts on $(Glc)_3(Man)_9(GlcNAc)_2$ and another on $Glc(Man)_9(GlcNAc)_2$ or $(Glc)_2(Man)_9(GlcNAc)_2$ or on mixtures of the two oligosaccharides. The latter enzyme is presumably a glycoprotein since it binds to Concanavalin A. Making use o this fact, the enzyme has now been purified consider ably and found to continue acting on the two sub strates, $Glc(Man)_9(GlcNAc)_2$ and $(Glc)_2(Man)_9(Glc NAc)_2$. Furthermore when the enzyme was submitte electrofocussing both activities were recovered in th same position. The rate of inactivation with temper ature and the inhibition by disaccharides was the sam for the two activities. These results confirm the sug gestion of other workers [7, 9] that only one enzym catalyzes the removal of the two internal glucoses, on of which is linked to glucose and another to mannose

The disaccharides which inhibit the glucosidase have the same linkages as those which are found i the glucoses of $(Glc)_3(Man)_9(GlcNAc)_2$. Thus th removal of the most external glucose which is linke $1-2$ is inhibited by kojibiose which has the $\alpha 1-2$ linkage, but not by other disaccharides including sophorose which is $\beta 1-2$. The glucosidases acting on the innermost glucose has been found to be inhibited more strongly by *p*-nitrophenyl-α-D-glucoside than by the *β* anomer [9]. We have found that none of the disaccharides with *β* linkage inhibited $(Glc)_2$-glucosidase but that nigerose which is $\alpha 1-3$ is a good inhibitor. This is as expected since the inner glucoses of $(Glc)_3$-$(Man)_9(GlcNAc)_2$ are $1-3$. However maltose, which is $\alpha 1-4$, also inhibited. The conclusion that three glucoses of $(Glc)_3(Man)_9(GlcNAc)_2$ have the α configuration agrees with the published evidence which is not all very convincing [6, 16, 17].

The substrates used in our studies, and those of other workers on the microsomal glucosidases, have been free oligosaccharides because this is the easily available substrate. However, it was assumed that the same enzymes acted on the protein-bound oligosaccharides which should the natural substrates. The finding that kojibiose inhibits the deglucosylation of the free and protein-bound substrate lends support to the belief that the same enzyme is involved in both processes.

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