Different effects of the glucosidase inhibitors 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin and castanospermine on the glycosylation of rat a_1-proteinase inhibitor and a_1-acid glycoprotein*

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The glucosidase inhibitors 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin and castanospermine were used to inhibit oligosaccharide processing in primary cultures of rat hepatocytes. Their effect on the glycosylation of a_1-proteinase inhibitor (a_1PI) and a_1-acid glycoprotein (a_1AGP) was studied. Of the three glucosidase inhibitors examined, 1-deoxynojirimycin inhibited not only oligosaccharide trimming but also glycosylation de novo of newly synthesized proteins, resulting in the formation of a_1PI with two and three (normally carrying three) and a_1AGP with two to five (normally carrying six) oligosaccharide side chains. In the presence of the glucosidase inhibitors, glucosylated high-mannose-type oligosaccharides accumulated. Whereas most of the endoglucosaminidase-H-sensitive oligosaccharides formed in the presence of 1-deoxynojirimycin contained only one glucose residue, N-methyl-1-deoxynojirimycin and castanospermine led mainly to the formation of oligosaccharides with three glucose residues. None of the three glucosidase inhibitors completely prevented the formation of complex-type oligosaccharides. Thus, in their presence, a_1PI and a_1AGP with a mixture of both high-mannose and complex-type oligosaccharides were secreted.

INTRODUCTION

The major pathway for the biosynthesis of complex-type oligosaccharides begins with the co-translational transfer of oligosaccharides of the composition $Glc_3Man_9GlcNAc_2$ from their dolichol derivatives to certain asparagine residues of nascent proteins (Hubbard & Ivatt, 1981; Snider & Robbins, 1981; Hanover & Lennarz, 1981; Kornfeld, 1982). The processing of these oligosaccharides starts in the endoplasmic reticulum with the removal of three glucose residues. Thus, glucosidase I removes the peripheral $a1,2$-linked glucose, whereas glucosidase II removes the two inner $a1,3$-linked glucose residues (Kornfeld et al., 1978; Ugalde et al., 1978; Hubbard & Robbins, 1979; Grinna & Robbins, 1979; Burns & Touster, 1982). Subsequently, six mannose residues are removed by mannosidases I and II in the Golgi complex (Tabas & Kornfeld, 1978, 1979; Tulsiani et al., 1982a). More recently, mannosidase activity has been found in the endoplasmic reticulum, which might also be involved in the oligosaccharide trimming (Bischoff & Kornfeld, 1983). The formation of the complex-type oligosaccharides is completed in the Golgi complex by the addition of GlcNAc, galactose, fucose and sialic acid (Hubbard & Ivatt, 1981).

Specific inhibitors of the individual steps of the trimming pathway have now been described (for a review, see Schwarz & Datema, 1984). Swainsonine has been found to be a potent inhibitor of mannosidase II (Elbein

et al., 1981; Tulsiani et al., 1982b), and 1-deoxymanno-jirimycin was found to inhibit Golgi mannosidase I (Fuhrmann et al., 1984; Bischoff & Kornfeld, 1984). Various compounds have been described as inhibitors of the glucosidase reactions: nojirimycin (Peyrieras et al., 1983), bromoconduritol (Datema et al., 1982), 1-deoxynojirimycin (Saunier et al., 1982), castanospermine (Saul et al., 1983), N-methyl-1-deoxynojirimycin (Romero et al., 1983), 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (Elbein et al., 1984).

In previous experiments we have studied the effect of several inhibitors of the trimming pathway on the glycosylation of a_1PI in primary cultures of rat hepatocytes. We have found that, in the presence of swainsonine, hepatocytes secreted a_1PI carrying oligosaccharides of the hybrid type (Gross et al., 1983a). Hepatocytes treated with 1-deoxymannojirimycin secreted a_1PI of the high-mannose type (Gross et al., 1985). Addition of 1-deoxynojirimycin to hepatocyte primary cultures led to the formation of a_1PI carrying both high-mannose and complex-type oligosaccharides (Gross et al., 1983b). From the work of Hettkamp et al. (1984) it is known that N-methyl-1-deoxynojirimycin has a lower K_i for glucosidase I than does 1-deoxynojirimycin, whereas 1-deoxynojirimycin has a lower K_i for glucosidase II than does N-methyl-1-deoxynojirimycin. A similar K_i to that of N-methyl-1-deoxynojirimycin for glucosidase I can be estimated for castanospermine (Pan et al., 1983).

Since 1-deoxynojirimycin did not lead to a complete

inhibition of glucose trimming in our hepatocyte system (Gross et al., 1983b), we have studied in addition the two glucosidase inhibitors N-methyl-1-deoxynojirimycin and castanospermine in respect to their effect on the glycosylation and trimming of α_1PI and α_1AGP in rat hepatocyte primary cultures. We found that, in spite of their low K_i values reported, none of the glucosidase inhibitors totally inhibited the processing of high-mannose-type to complex-type oligosaccharides. 1-Deoxynojirimycin led mainly to the formation of oligosaccharides containing one glucose residue, and N-methyl-1-deoxynojirimycin and castanospermine to the formation of oligosaccharides containing three glucose residues.

MATERIALS AND METHODS

Chemicals

L-[^{35}S]Methionine (> 600 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. D-[2-^{3}H]Mannose (24 Ci/mmol), D-[4,5-^{3}H]galactose (52 Ci/mmol) and Protosol were from New England Nuclear; endoglucosaminidase H from Streptomyces plicatus was obtained from Miles, Frankfurt, Germany; tunicamycin was from Calbiochem–Behring, Giessen, Germany; protein A–Sepharose CL-4B and concanavalin A–Sepharose were from Pharmacia, Freiburg, Germany. Proteinase K was purchased from Merck, Darmstadt, Germany. 1-Deoxynojirimycin (Bay h 5595) was generously given by Bayer A. G., Wuppertal-Elberfeld, Germany, and N-methyl-1-deoxynojirimycin was prepared by N-methylation of 1-deoxynojirimycin as described by Murai et al. (1979). Swainsonine was a present from Dr. K. Vosbeck, Ciba–Geigy, Basel, Switzerland.

Preparation of rat hepatocyte monolayers

This was done as previously described (Bischoff et al., 1976). After the cells were washed with Krebs–Henseleit buffer, they were suspended in a modified Waymouth medium (Gebhardt & Mecke, 1979) containing 10% (v/v) fetal-calf serum, 50 units of penicillin/ml, 50 μg of streptomycin/ml, 1 μM-dexamethasone and 1 μM-insulin. Samples of cell suspension (3 ml; 4×10^6 cells) were added to 55 mm-diam. contour-bottom Falcon plastic tissue-culture dishes. The dishes were incubated at 37 °C in a humid atmosphere of 5% CO_2 in air for 3 h. The plates were then washed with Krebs–Henseleit buffer, and 3 ml of culture medium (Waymouth medium containing 5% fetal-calf serum, penicillin, streptomycin, dexamethasone and insulin in the same concentrations as above) was added. Confluent monolayers were formed after overnight incubation at 37 °C in a humid atmosphere of 5% CO_2 in air.

Labelling of hepatocytes

Modified Waymouth medium without fetal-calf serum, bovine serum albumin and oleic acid was used for the radioactive labelling of the hepatocyte monolayers obtained after overnight incubation. Tunicamycin, the glucosidase inhibitors 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin and castanospermine, and the mannosidase II inhibitor swainsonine were added to the culture medium 1 h before the addition of [^{35}S]methionine or a ^{3}H-labelled monosaccharide. For the labelling of proteins, 25 μCi of [^{35}S]methionine was added to 3 ml of

methionine-free culture medium. To label carbohydrates, 80 μCi of D-[2-^{3}H]mannose or 60 μCi of D-[4,5-^{3}H]galactose was added to each dish. For the labelling with D-[2-^{3}H]mannose, medium containing only one-tenth of the normal glucose concentration was used. The incubation times were 2.5 h for [^{35}S]methionine and 4 h for the ^{3}H-labelled sugars. After incubation at 37 °C, the media were separated from the cells. The cells of each dish were carefully washed with 0.15 M-NaCl/10 mM-Tris/ HCl (pH 7.6), homogenized in 1 ml of 25 mM-Tris/HCl buffer (pH 7.5)/20 mM-NaCl/1% sodium deoxycholate/ 1% Triton X-100, with a Potter/Elvehjem homogenizer at 800 rev./min, and centrifuged for 10 min at 12000 g. The supernatant from this centrifugation and the culture medium were used for the immunoprecipitations.

Immunoprecipitation

For this, 1.5–2.5 ml of medium or 0.4–0.8 ml of the supernatant obtained from the cell homogenate were added to 5 ml of 20 mM-Tris/HCl (pH 7.6)/0.14 M-NaCl/5 mM-EDTA/1% Triton X-100 containing 1 mM-phenylmethanesulphonyl fluoride and 0.1 mg of kallikrein trypsin inhibitor (kindly provided by Bayer A. G., Wuppertal-Elberfeld). After addition of 7.5 μl of a specific antiserum against rat α_1PI (Gross et al., 1983c) or rat α_1AGP (Gross et al., 1984) and incubation at 0 °C overnight, the antigen–antibody complexes were bound to 7 mg (dry wt.) of protein A–Sepharose and washed four times with the above-mentioned buffer and twice with 50 mM-sodium phosphate buffer, pH 7.5. Elution was performed by incubation with 0.1 M-Tris/HCl (pH 6.8)/5% β-mercaptoethanol/5% SDS/10% glycerol at 95 °C for 5 min. The eluted proteins were analysed by electrophoresis in SDS/polyacrylamide slab gels (King & Laemmli, 1971) and fluorography (Bonner & Laskey, 1974).

Treatment of a_1-proteinase inhibitor and a_1-acid glycoprotein with endoglucosaminidase H

The glycoprotein–IgG complexes eluted from the protein A–Sepharose were dialysed exhaustively against 50 mM-phosphate buffer (pH 6.0)/0.01% SDS and incubated in a total volume of 0.1 ml with 5 munits of endoglucosaminidase H at 37 °C for 16 h.

Bio-Gel P-4 chromatography of oligosaccharides

[^{3}H]Mannose-labelled oligosaccharides of α_1PI obtained after digestion with endoglucosaminidase H were subjected to Bio-Gel P-4 chromatography. Bio-Gel P-4 (-400 mesh) columns (1 cm × 150 cm), eluted with water containing 0.02% NaN_3, were used. The void-volume marker was bovine serum albumin (Datema & Schwarz, 1981). The volume of the fractions was 0.35 ml. Radioactivity was determined with a Packard Tri-Carb liquid-scintillation spectrophotometer model 460 C. Treatment of oligosaccharides with α-glucosidase was done as described by Grinna & Robbins (1979), modified by addition of 10 mM-EDTA to inhibit residual mannosidase as described by Datema et al. (1982).

Concanavalin A–Sepharose chromatography of glycopeptides from a_1-proteinase inhibitor and a_1-acid glycoprotein

After immunoprecipitation, the glycoprotein–IgG complexes were eluted from the protein A–Sepharose by incubation with 20 mM-Tris/HCl (pH 7.5)/2% SDS at 95 °C for 5 min. Glycopeptides were prepared by

digestion with 1 mg of proteinase K/ml in 20 mM-
Tris/HCl (pH 7.5)/0.15 M-NaCl/0.2% SDS at 37 °C for
18 h. The reaction was stopped by the addition of
1 mM-phenylmethanesulphonyl fluoride and subsequent
treatment at 95 °C for 10 min. The incubation mixture,
diluted with concanavalin A buffer (10 mM-Tris/HCl,
pH 7.5, 0.3 M-NaCl, 1 mM-CaCl$_2$, 1 mM-MgCl$_2$, 1 mM-
MnCl$_2$), to a final SDS concentration of 0.02%, was then
loaded on to a concanavalin A–Sepharose column (3 ml
bed volume) equilibrated with the same buffer. The
column was washed with equilibration buffer, followed
by elution with 9.75 ml of 10 mM-α-methyl glucoside and
then 9.75 ml of 500 mM-α-methyl mannoside. Fractions
of volume 0.65 ml were collected and their radioactivity
was determined.

RESULTS

Fig. 1 shows the effect of the glucosidase inhibitors
1-deoxynojirimycin, N-methyl-1-deoxynojirimycin and
castanospermine on the intra- and extra-cellular forms of
α$_1$PI and α$_1$AGP. After a 3 h labelling period with
[^{35}S]methionine, two intracellular forms of α$_1$PI with
different M_r values were found: a 49000-M_r form and a
54000-M_r form, the latter being secreted into the
medium. Similarly, two forms of α$_1$AGP exist: a 39000-M_r
form found only in cells and a 43000–60000-M_r form
found in cells and medium. In the presence of the
glucosidase inhibitors, the intracellular forms of α$_1$PI and
α$_1$AGP were different from those of control cells.
Apparent M_r values of 51000 and 49000 were determined
for the largest forms of α$_1$PI and α$_1$AGP respectively. α$_1$PI
and α$_1$AGP secreted by hepatocytes treated with
glucosidase inhibitors were of smaller apparent M_r than
the same glycoproteins secreted by control cultures
(51000 instead of 54000 for α$_1$PI; 43000 instead of
43000–60000 for α$_1$AGP). In the presence of N-methyl-
1-deoxynojirimycin and castanospermine, intracellular
α$_1$AGP has a lower electrophoretic mobility than the
secreted α$_1$AGP. The latter, however, is also detected
intracellularly (Fig. 1b, lanes marked 'mdN' and 'CS').
This effect can hardly be seen with α$_1$PI, because of the
much smaller carbohydrate content (13.2%; Ikehara
et al., 1981), compared with α$_1$AGP (34–37%; Nagashima
et al., 1980). In contrast with N-methyl-1-deoxynojiri-
mycin and castanospermine, 1-deoxynojirimycin led to
the formation of α$_1$PI and α$_1$AGP with less than the
number of oligosaccharide chains normally found,
namely three for α$_1$PI (Gross et al., 1982; Carlson &
Stenflo, 1982) and six for α$_1$AGP (Yoshima et al., 1981;
Baumann & Jahreis, 1983). In the presence of 5 mM-
1-deoxynojirimycin, α$_1$PI with two and three, and α$_1$AGP
with two to five, carbohydrate side chains were found.
This is evident from Fig. 1, where the forms of α$_1$PI and
α$_1$AGP with a lower number of oligosaccharide side
chains represent distinct bands of lower M_r (see lanes
marked 'dN'). Measurements of the radioactivity in
individual bands of Fig. 1 showed that, of the three
glucosidase inhibitors, only 1-deoxynojirimycin led to a
marked inhibition of the secretion of α$_1$PI and α$_1$AGP,
by about 50%, whereas the secretion of the two glyco-
proteins was not affected by N-methyl-1-deoxynojiri-
mycin or castanospermine. The inhibition of glycoprotein
secretion by 1-deoxynojirimycin was not due to its
inhibitory effect on glycosylation de novo, since the
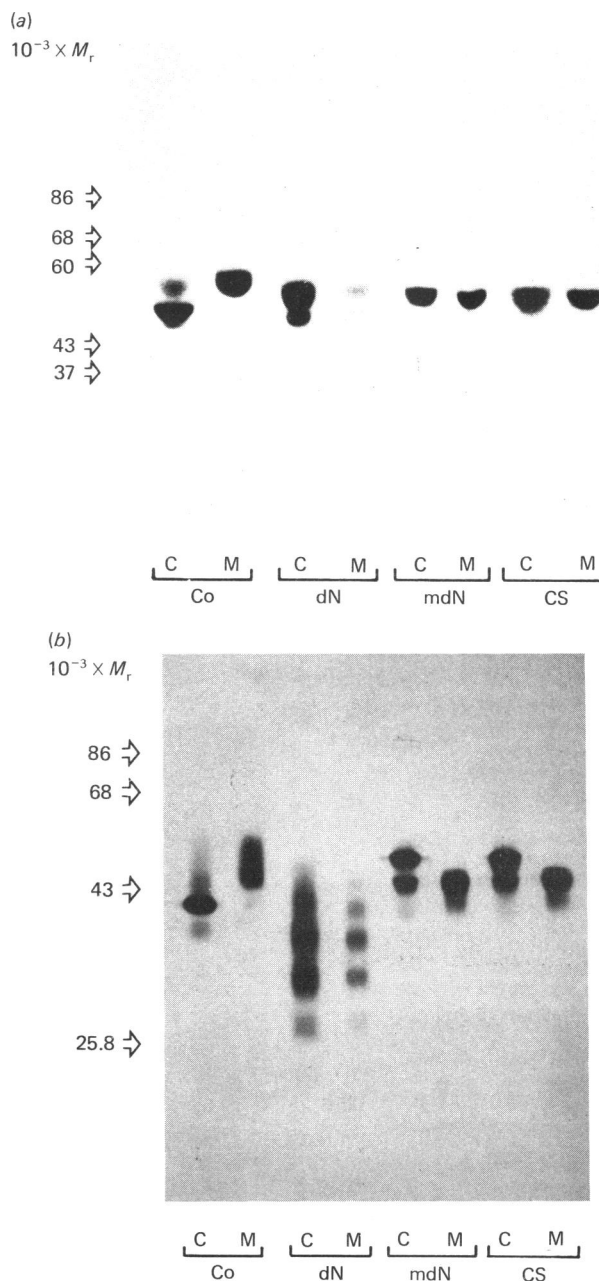underglycosylated forms of α$_1$PI with two carbohydrate

Fig. 1. **Effect of the glucosidase inhibitors 1-deoxynojirimycin,
N-methyl-1-deoxynojirimycin and castanospermine on the
intra- and extra-cellular forms of a$_1$-proteinase inhibitor
and a$_1$-acid glycoprotein**

Rat hepatocyte primary cultures were incubated without
(Co) or with 5 mM-1-deoxynojirimycin (dN), 5 mM-N-
methyl-1-deoxynojirimycin (mdN) or 100 µg of castano-
spermine/ml (CS). After 1 h the cells were labelled with
[^{35}S]methionine (25 µCi/dish, containing about 4 × 10^6
cells). After incubation at 37 °C for 3 h in the presence of
the glucosidase inhibitors, α$_1$PI (a) or α$_1$AGP (b) was
immunoprecipitated from the cells (C) and the media (M)
and analysed by SDS/polyacrylamide-gel electrophoresis
and fluorography as described in the Materials and
methods section. M_r markers were conalbumin (86000),
bovine serum albumin (68000), catalase (60000), ovalbumin
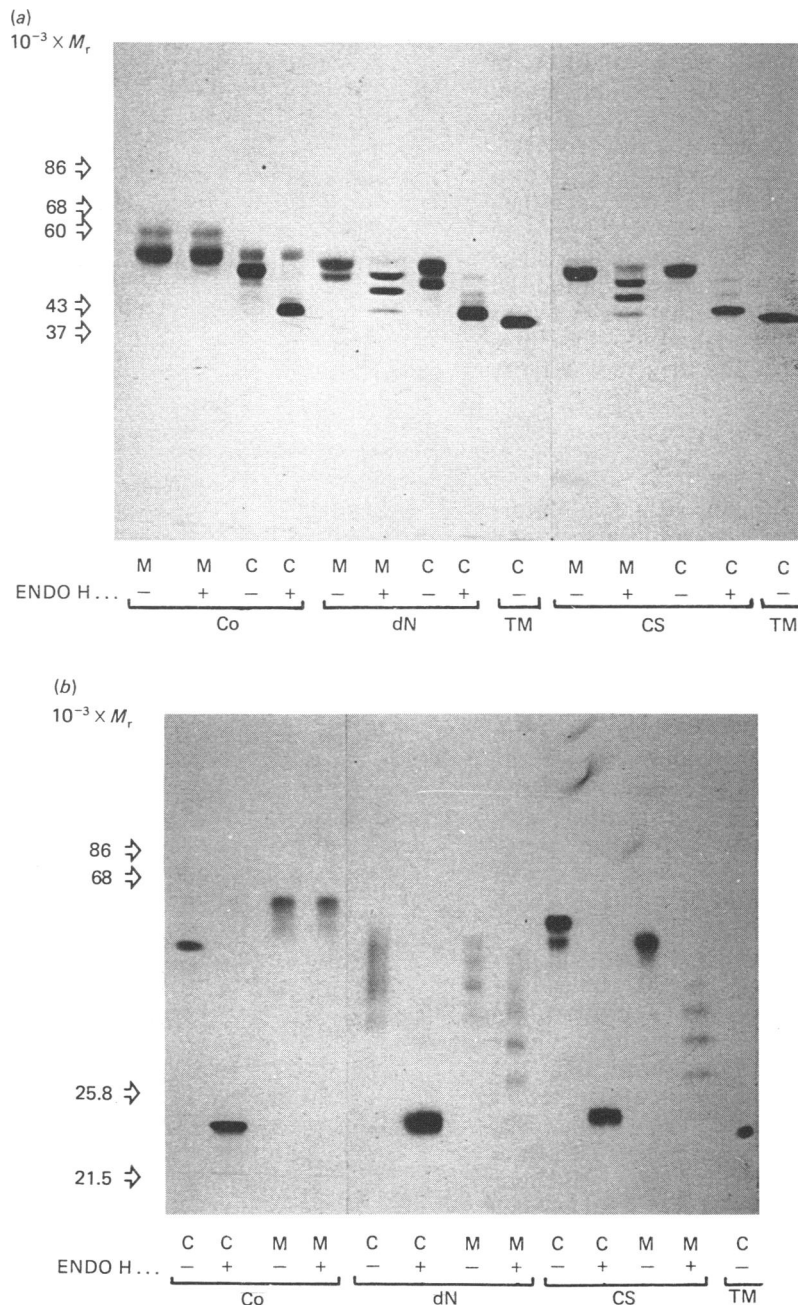(43000), alcohol dehydrogenase (37000) and elastase
(25800).

Fig. 2. Endoglucosaminidase H digestions of a_1-proteinase inhibitor and a_1-acid glycoprotein synthesized in the presence of glucosidase inhibitors

α_1PI (*a*) and α_1AGP (*b*) immunoprecipitated from the cells (C) and media (M) of control hepatocyte cultures (Co) or cultures incubated with 5 mM-1-deoxynojirimycin (dN) or 100 μg of castanospermine/ml (CS) were incubated without (−) or with (+) endoglucosaminidase H (ENDO H) as described in the Materials and methods section. α_1PI or α_1AGP immunoprecipitated from hepatocytes treated with 3 μg of tunicamycin/ml of medium (TM) were used as reference. M_r markers were the same as in Fig. 1; in addition soya-bean trypsin inhibitor (21 500) was used.

chains and of α_1AGP with two to five carbohydrate chains were present in the same ratios intra- and extra-cellularly.

α_1PI (M_r 54000) and α_1AGP (M_r 43000–60000) secreted by control hepatocytes carry exclusively oligo-saccharide chains of the complex type, resistant to endoglucosaminidase H. Intracellularly both the high-mannose-type precursors (M_r 49000 for α_1PI, M_r 39000

for α_1AGP) sensitive to endoglucosaminidase H and the processed complex-type glycoproteins could be found (Fig. 2). The results of endoglucosaminidase H digestions were different when the cells were incubated with a glucosidase inhibitor. The intracellular forms of α_1PI and α_1AGP synthesized by hepatocytes treated with 5 mM-1-deoxynojirimycin or 100 μg of castanospermine/ml were sensitive to endoglucosaminidase H, indicating the
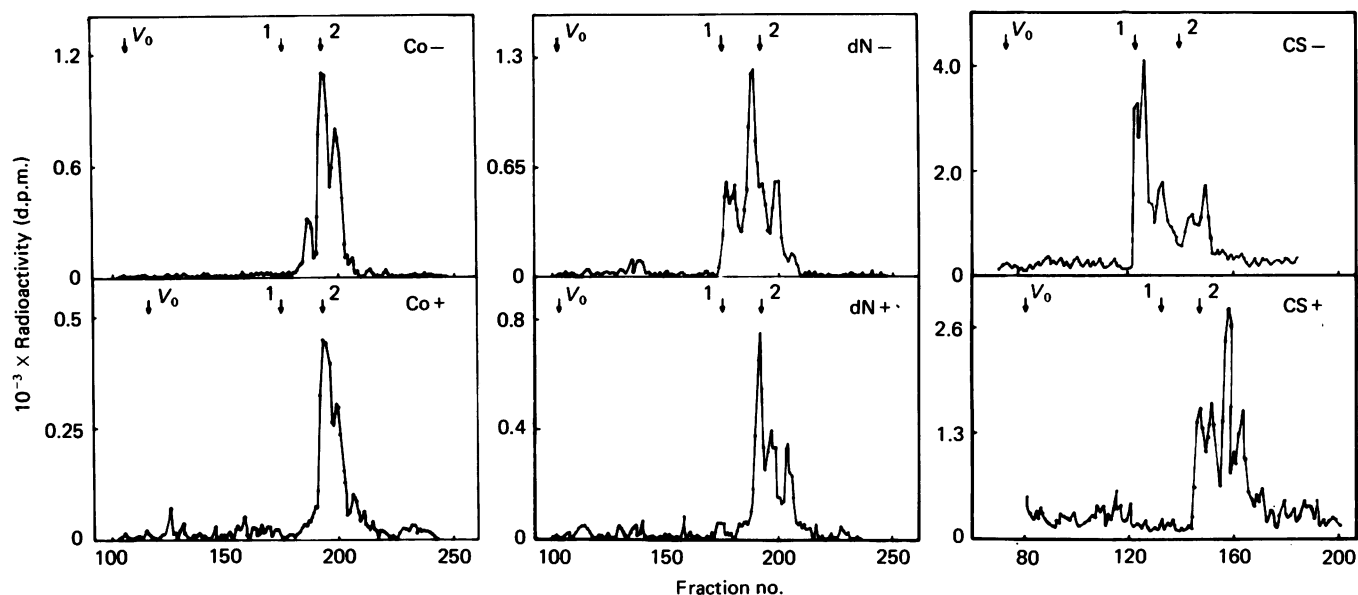
Fig. 3. Size of oligosaccharides of intracellular a_1-proteinase inhibitor

After labelling with [^{3}H]mannose, a_1PI was immunoprecipitated from the cells of control hepatocyte cultures (Co) or cultures treated with 5 mM-1-deoxynojirimycin (dN) or 100 μg of castanospermine/ml (CS). The oligosaccharides were cleaved off by endoglucosaminidase H and analysed on Bio-Gel P4 columns either directly (−) or after incubation with rat liver α-glucosidase for 120 min (+). The reaction was stopped by boiling the mixture for 3 min, and ^{14}C-labelled standards (1, Glc$_3$Man$_9$GlcNAc; 2, Man$_9$GlcNAc) were added. V_0, elution volume of bovine serum albumin. Fraction volume was 350 μl.

presence of high-mannose-type oligosaccharides, whereas the secreted forms could only partially be deglycosylated by endoglucosaminidase H, suggesting the presence of both high-mannose and complex-type oligosaccharides. With N-methyl-1-deoxynojirimycin (results not shown), similar results to those with castanospermine were obtained.

The size of the endoglucosaminidase-H-sensitive oligosaccharides of intracellular a_1PI synthesized by control hepatocytes or by cells incubated with 5 mM-1-deoxynojirimycin or 100 μg of castanospermine/ml was determined by Bio-Gel P-4 chromatography after labelling of the cells with [^{3}H]mannose (Fig. 3). Glc$_3$Man$_9$GlcNAc and Man$_9$GlcNAc were used as standards (arrows). The oligosaccharides of a_1PI from control cells contained mainly eight and nine mannose residues and, except for the small peak ahead of Man$_9$GlcNAc, were resistant to α-glucosidase. When cells were treated with 5 mM-1-deoxynojirimycin, most of the oligosaccharides of a_1PI were eluted under three peaks ahead of Man$_9$GlcNAc; the one corresponding to the highest M_r co-eluted with Glc$_3$Man$_9$GlcNAc. After digestion with α-glucosidase the major species was Man$_9$GlcNAc. Therefore, we conclude that in the presence of 1-deoxynojirimycin Glc$_3$Man$_9$GlcNAc, Glc$_2$Man$_9$GlcNAc and Glc$_1$Man$_9$GlcNAc (the last being the predominant species) are the major oligosaccharides of intracellular accumulated a_1PI.

Incubation of hepatocytes with castanospermine led to a different pattern of oligosaccharides as compared with 1-deoxynojirimycin. In the presence of castanospermine mainly three glucosylated species, the largest co-eluted with Glc$_3$Man$_9$GlcNAc, could be found. After digestion with α-glucosidase four peaks (Man$_9$GlcNAc, Man$_8$GlcNAc, Man$_7$GlcNAc and prob-

ably Man$_6$GlcNAc) were found. From the two profiles we conclude that the glucosylated peaks were Glc$_3$Man$_9$GlcNAc, Glc$_3$Man$_8$GlcNAc and Glc$_3$Man$_7$GlcNAc, since Man$_9$GlcNAc, Man$_8$GlcNAc and Man$_7$GlcNAc appeared after α-glucosidase treatment. Similar results to those with castanospermine were obtained with N-methyl-1-deoxynojirimycin (not shown).

The oligosaccharides of a_1PI and a_1AGP secreted by hepatocytes treated with a glucosidase inhibitor were further characterized by affinity chromatography on concanavalin A–Sepharose (Fig. 4). a_1PI and a_1AGP labelled in their oligosaccharide part with [^{3}H]mannose were immunoprecipitated from the media of control hepatocyte cultures or from the media of cultures incubated with the glucosidase inhibitors 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin or castanospermine, and/or the mannosidase II inhibitor swainsonine. After digestion of the denatured proteins by proteinase K, the glycopeptides were subjected to concanavalin A–Sepharose chromatography and divided into three fractions (Reitman et al., 1982): A, glycopeptides that did not bind to concanavalin A (mainly three- and higher-antennary complex-type oligosaccharides); B, glycopeptides that could be eluted by 10 mM-α-methyl glucoside (mainly biantennary complex-type oligosaccharides); C, glycopeptides that could be eluted by 500 mM-α-methyl mannoside (high-mannose type and hybrid-type oligosaccharides). Fig. 4 shows that in control media a_1PI and a_1AGP carried complex-type oligosaccharides (fractions A and B). After incubation with the glucosidase inhibitors 1-deoxynojirimycin, N-methyl-1-deoxynojirimycin or castanospermine, a_1PI and a_1AGP with both complex- and high-mannose-type oligosaccharides were found (fractions A–C). After incubation with swainsonine, which leads to the
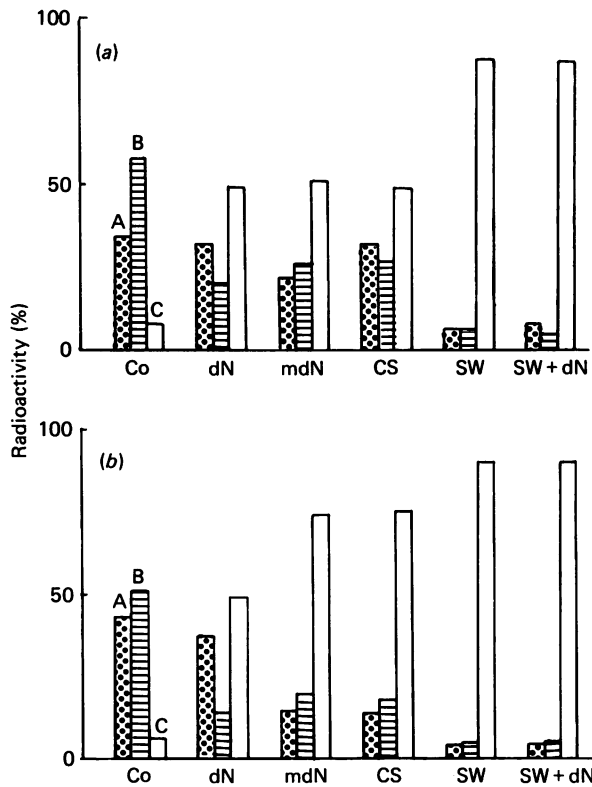
Fig. 4. Concanavalin A–Sepharose chromatography of [³H]-mannose-labelled glycopeptides of a_1-proteinase inhibitor and a_1-acid glycoprotein

Hepatocyte primary cultures were labelled with [³H]mannose and incubated without (Co) or with 5 mM-1-deoxynojirimycin (dN), 5 mM-N-methyl-1-deoxy-nojirimycin (mdN), 100 μg of castanospermine/ml (CS), 2 μg of swainsonine/ml (SW) or 2 μg of swainsonine/ml + 5 mM-1-deoxynojirimycin (SW+dN). a_1PI (a) and a_1AGP (b) were immunoprecipitated from the hepatocyte media and digested with proteinase K as described in the Materials and methods section. The glycopeptides were subjected to concanavalin A–Sepharose chromatography. Three fractions, A, B and C, were collected: ▓, glycopeptides not binding to concanavalin A; ▤, glycopeptides that could be eluted by 10 mM-α-methyl glucoside; □, glycopeptides that could be eluted by 500 mM-α-methyl mannoside.

formation of hybrid-type oligosaccharides, radioactivity was found in fraction C. An identical distribution of radioactivity was obtained after simultaneous incubation with the mannosidase inhibitor swainsonine and the glucosidase inhibitor 1-deoxynojirimycin, indicating that no complex-type oligosaccharides had been synthesized in the simultaneous presence of the two inhibitors.

We have shown that hepatocytes incubated with a glucosidase inhibitor secreted a_1PI and a_1AGP which both carried oligosaccharides that were sensitive to endoglucosaminidase H and had a high affinity for concanavalin A, as well as oligosaccharides that were resistant to endoglucosaminidase H and had no or low affinity for concanavalin A. The question arose whether the oligosaccharides that were resistant to endoglucos-aminidase H and had no or low affinity to concanavalin A were due either to an incomplete inhibition of the

processing of the glucosylated $\alpha1,3$-linked antenna of newly synthesized oligosaccharides or to a further processing of the $\alpha1,6$-linked antenna, while trimming of the glucosylated antenna would be blocked completely. To answer this question we incubated hepatocytes simultaneously with a glucosidase inhibitor and the mannosidase II inhibitor swainsonine, and labelled them with [³H]galactose. In a previous study (Gross *et al.*, 1983a) we have shown that, after labelling of hepatocytes with [³H]galactose for 3 h, [³H]galactose is incorporated into a_1PI. After hydrolysis in 2 M-HCl at 100 °C for 2 h, no other radioactively labelled sugars could be detected
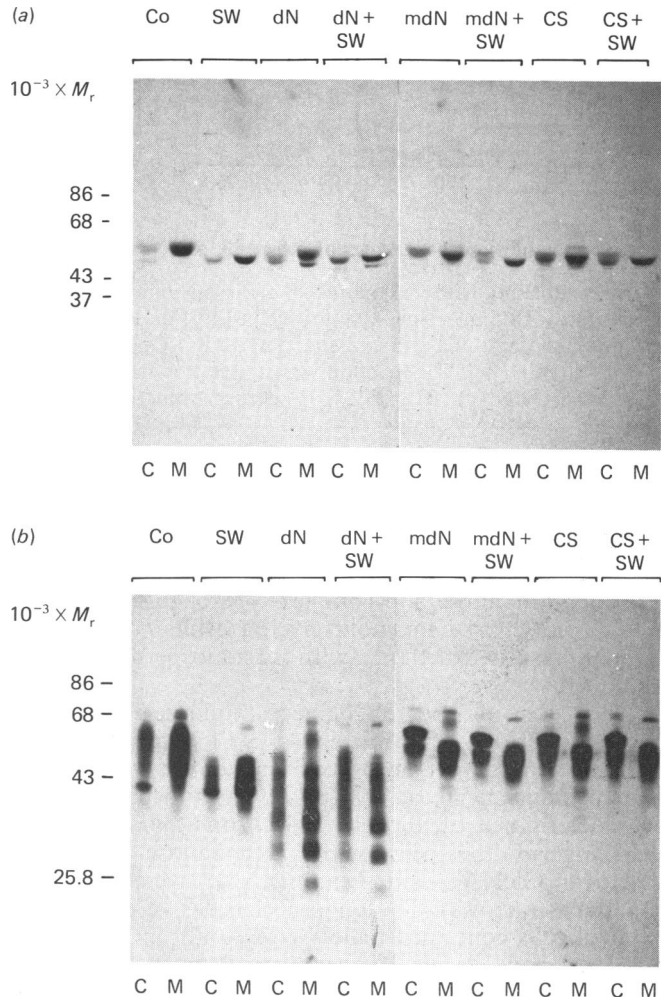

Fig. 5. Simultaneous inhibition of processing glucosidases and mannosidase II

Rat hepatocyte primary cultures were incubated without (Co) or with 2 μg of swainsonine/ml (SW), 5 mM-1-deoxynojirimycin (dN), 5 mM-1-deoxynojirimycin + 2 μg of swainsonine/ml (dN+SW), 5 mM-N-methyl-1-deoxy-nojirimycin (mdN), 5 mM-N-methyl-1-deoxynojirimycin + 2 μg of swainsonine/ml (mdN+SW), 100 μg of castanospermine/ml (CS), or 100 μg of castano-spermine/ml + 2 μg of swainsonine/ml (CS+SW). Cells were labelled with [³H]galactose for 4 h (60 μCi/dish containing about 4 × 10⁶ cells). a_1PI (a) and a_1AGP (b) were immunoprecipitated from the cells (C) and their media (M) and analysed by SDS/polyacrylamide-gel electrophoresis and fluorography as described in the Materials and methods section. The M_r standards were the same as those described in the legend to Fig. 1.

by paper chromatography. As shown in Fig. 5, [^{3}H]galactose-labelled α_1PI and α_1AGP were detected under these conditions. Since inhibition of mannosidase II by swainsonine is complete (Gross *et al.*, 1983*a*), it must be concluded that the glucosidase inhibitors do not completely block processing of the glucosylated α1,3-linked antenna of newly synthesized oligosaccharides.

DISCUSSION

Nojirimycin (Peyrieras *et al.*, 1983), bromoconduritol (Datema *et al.*, 1982), 1-deoxynojirimycin (Saunier *et al.*, 1982), castanospermine (Saul *et al.*, 1983) and *N*-methyl-1-deoxynojirimycin (Romero *et al.*, 1983) have been described as inhibitors of the processing glucosidases involved in the biosynthesis of *N*-linked complex type oligosaccharides. Several authors have studied the effect of these inhibitors in different systems (Datema *et al.*, 1982, 1984; Gross *et al.*, 1983*b*; Hori *et al.*, 1984; Lemansky *et al.*, 1984; Pan *et al.*, 1983; Peyrieras *et al.*, 1983; Romero *et al.*, 1983, 1985*a,b*; Saul *et al.*, 1983; Saunier *et al.*, 1982; Schlesinger *et al.*, 1984; Burke *et al.*, 1984). In the present study we have compared the effects of 1-deoxynojirimycin, *N*-methyl-1-deoxynojirimycin and castanospermine on the glycosylation of α_1PI and α_1AGP by rat hepatocytes in primary culture. We obtained different results for 1-deoxynojirimycin on the one hand and for *N*-methyl-1-deoxynojirimycin and castanospermine on the other. Bromoconduritol was not included in this study, since it turned out to be toxic for the hepatocytes when used in the concentrations needed for glucosidase inhibition (Datema *et al.*, 1982). Nojirimycin was excluded also, since it leads to the formation of drastically shortened oligosaccharides (Peyrieras *et al.*, 1983).

Whereas *N*-methyl-1-deoxynojirimycin and castanospermine did not affect glycosylation *de novo* of α_1PI and α_1AGP, 1-deoxynojirimycin led to the formation of underglycosylated α_1PI carrying two and three instead of three, and α_1AGP with two to five instead of six, oligosaccharide chains. A similar observation has been published by Lemansky *et al.* (1984), who found increased amounts of a β-chain precursor of β-hexosaminidase lacking one of its carbohydrate side chains in the presence of 1-deoxynojirimycin. The decrease in the number of oligosaccharide side chains observed after 1-deoxynojirimycin might be explained by the inhibition of the formation of $Glc_3Man_9GlcNAc_2$-*PP*-dolichol, which has been observed in intestinal epithelial cells by Romero *et al.* (1985*b*). Since $Glc_3Man_9GlcNAc$ is the preferred substrate for the transfer to protein (Hubbard & Ivatt, 1981), the decrease in $Glc_3Man_9GlcNAc_2$-*PP*-dolichol donor in 1-deoxynojirimycin-treated cells may account for the incomplete glycosylation of α_1PI and α_1AGP.

Oligosaccharides of different compositions were found to accumulate in the presence of 1-deoxynojirimycin and the other two glucosidase inhibitors. In the presence of 1-deoxynojirimycin, mainly oligosaccharides partially processed in their glucose but not in their mannose moieties were found, the predominant species having the composition $Glc_1Man_9GlcNAc$. Castanospermine and *N*-methyl-1-deoxynojirimycin, however, led mainly to the formation of oligosaccharides processed not in their glucose but in their mannose

moieties, i.e. $Glc_3Man_9GlcNAc$, $Glc_3Man_8GlcNAc$, $Glc_3Man_7GlcNAc$ and possibly $Glc_3Man_6GlcNAc$. Obviously, *N*-methyl-1-deoxynojirimycin and castanospermine are more effective inhibitors of glucosidase I in our hepatocyte system than is 1-deoxynojirimycin. Our results are in agreement with those of Romero *et al.* (1985*a*), who found in rat intestinal epithelial cells that about 70% of the endoglucosaminidase-H-sensitive oligosaccharides formed in the presence of *N*-methyl-1-deoxynojirimycin contain three glucose residues, compared with only about 20% of the corresponding oligosaccharides of the 1-deoxynojirimycin-treated cells.

Of the three glucosidase inhibitors, only 1-deoxynojirimycin inhibited the secretion of α_1AGP and α_1PI by about 50%, whereas castanospermine and *N*-methyl-1-deoxynojirimycin had no comparable effect. This finding suggests that α_1PI and α_1AGP with oligosaccharides containing only one glucose residue are less effectively secreted than the same glycoproteins carrying carbohydrate side chains containing three glucose residues. The fact that there is hardly any mannose trimming in the endoglucosaminidase-H-sensitive oligosaccharide chains of intracellular α_1PI in the presence of 1-deoxynojirimycin reflects the inhibition of the intracellular transport of this protein in the rough endoplasmic reticulum. This has been demonstrated directly by Lodish & Kong (1984) with HepG$_2$ cells. A general inhibitory effect of 1-deoxynojirimycin on protein secretion is not probable. It was shown that 1-deoxynojirimycin has no effect on the secretion of albumin in primary cultures of rat hepatocytes (Gross *et al.*, 1983*b*). Whereas 1-deoxynojirimycin greatly decreased the rate of secretion of α_1PI and α_1-antichymotrypsin by human hepatoma HepG$_2$ cells, it had only marginal effects on the secretion of the glycoproteins C3 and transferrin or of albumin (Lodish & Kong, 1984). In IgD- and IgM-producing hybridoma cells, Peyrieras *et al.* (1983) found that the secretion of IgD, but not that of IgM, was blocked by 1-deoxynojirimycin.

In our system none of the three glucosidase inhibitors totally blocked the formation of complex-type oligosaccharides in the secreted form of α_1PI and α_1AGP. This was demonstrated by the existence of endoglucosaminidase-H-resistant oligosaccharides (Fig. 2) and by experiments where, in addition to the processing glucosidases, mannosidase II was inhibited by swainsonine (Figs. 4 and 5). Similar findings have been reported by Reitman *et al.* (1982), using a mouse lymphoma cell line deficient in glucosidase II. In spite of the nearly complete lack of glucosidase II in these cells, the synthesis of complex-type oligosaccharides was only decreased by 75%.

The existence of endoglucosaminidase-H-resistant oligosaccharides has been observed in the presence of castanospermine by Pan *et al.* (1983) and in the presence of 1-deoxynojirimycin by Lemansky *et al.* (1984). The incomplete inhibition of oligosaccharide processing by 1-deoxynojirimycin, *N*-methyl-1-deoxynojirimycin or castanospermine, resulting in the secretion of α_1PI and α_1AGP molecules carrying together high-mannose and complex-type oligosaccharide side chains, might be explained either by incomplete inhibition of the trimming glucosidases by these inhibitors *in vivo*, which would be in contrast with their lower K_i values measured *in vitro*, or by the fact that only a part of the oligosaccharides is transferred as glucosylated species from their dolichol derivatives to the nascent polypeptide chains.

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