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EFFECT OF THE ALPHA-GLUCOSIDASE INHIBITOR *N*-HYDROXYETHYL-1-DEOXYNOJIRIMYCIN (BAY M 1099) ON THE BIOSYNTHESIS OF LIVER SECRETORY GLYCOPROTEINS

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Abstract—The effect of the alpha-glucosidase inhibitor *N*-hydroxyethyl-1-deoxynojirimycin (Bay m 1099) on the glycosylation and secretion of α_1-antitrypsin (three complex type oligosaccharide chains) and of α_1-acid glycoprotein (six complex type oligosaccharide chains) was studied in rat hepatocyte primary cultures. In the presence of 4 mM Bay m 1099 the processing of high-mannose to complex type oligosaccharides was partially inhibited leading to the secretion of α_1-antitrypsin and α_1-acid glycoprotein carrying a mixture of both high-mannose and complex type oligosaccharides. The major part of α_1-antitrypsin secreted by Bay m 1099 treated cells still carried two complex type oligosaccharide chains, the majority of α_1-acid glycoprotein carried three to five. Despite its effects on protein glycosylation Bay m 1099 did not lead to pronounced changes in the synthesis or secretion of α_1-antitrypsin, α_1-acid glycoprotein or albumin. At concentrations of Bay m 1099 lower than 0.5 mM no inhibitory effect on oligosaccharide trimming could be observed. After removal of Bay m 1099 from hepatocytes its inhibitory effect on protein glycosylation was immediately reversible.

Alpha-glucosidase inhibitors are drugs of potential use in the treatment of diabetes mellitus. They diminish postprandial plasma glucose levels by inhibition of intestinal α-glucosidases. In recent years derivatives of the glucose analogue 1-deoxynojirimycin have been developed as glucosidase inhibitors [1–4]. Since these substances can be absorbed from the intestines [5] they might interfere with glucosidases in other organs than the intestinal mucosa.

Microsomal glucosidases are involved in the biosynthesis of glycoproteins. After the co-translational transfer of oligosaccharides of the composition of $Glc_3Man_9GlcNAc_2$ from their dolichol derivatives to certain asparagine residues of nascent glycoproteins [6, 7] these oligosaccharides are further processed in the endoplasmatic reticulum. The peripheral α 1,2-linked glucose is cleaved off by endoplasmatic reticulum glucosidase I, the two inner α 1,3-linked glucose residues by endoplasmatic reticulum glucosidase II [8–12]. Removal of the glucose residues is the prerequisite for the further processing of the oligosaccharide chains in the Golgi-complex [6, 7] which finally leads to the synthesis of oligosaccharides of the complex type. In previous experiments we have studied the effect of 1-deoxynojirimycin and its derivative *N*-methyl-1-deoxynojirimycin on the glycosylation of rat α_1-antitrypsin and α_1-acid glycoprotein [13, 14]. While 1-deoxynojirimycin was found to impair *de novo* glycosylation, glucose trimming and secretion of α_1-antitrypsin [13], *N*-methyl-1-deoxynojirimycin inhibited only oligosaccharide processing without affecting *de novo* glycosylation or secretion of α_1-antitrypsin or α_1-acid glycoprotein [14].

In the present paper we studied whether the α-glucosidase inhibitor Bay m 1099 affects the biosynthesis and/or secretion of the two glycoproteins α_1-antitrypsin (three complex type oligosaccharide chains) [15, 16] and α_1-acid glycoprotein (six complex type oligosaccharide chains) [17, 18] in rat hepatocyte primary cultures.

MATERIALS AND METHODS

Chemicals. L-[^{35}S]Methionine (>3,7 × 10^{13} Bq/mmol) was purchased from Amersham-Buchler (Braunschweig). Protosol was from New England Nuclear (Dreieich). Medium 199 and endo β-*N*-acetylglucosaminidase H from *Streptomyces plicatus* were from Boehringer (Mannheim). Protein A-Sepharose was obtained from Pharmacia (Freiburg). Bay m 1099 was a generous gift of the Bayer AG, Wuppertal.

Preparation of rat hepatocyte primary cultures. Hepatocytes were prepared as previously described [19, 20]. They were isolated from fed adult female Wistar rats (180 g) by recirculating perfusion with collagenase *in situ*. Non-paranchymal cells and debris were removed by several washings and sedimentation of the hepatocytes at 30 *g* for 2 min. The parenchymal cells (3 × 10^6/60-mm dish) were cultured in basic medium 199 containing glucose

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(5.5 mM), insulin (0.5 nM), dexamethasone (5 nM), bovine serum albumin (2 g/l.), penicillin (60 mg/l.) streptomycin sulfate (120 mg/l.) and $NaHCO_3$ (18 mM). 3% neonatal calf serum was present only during the first 4 hr. Incubation was performed at 36.5° and 5% CO_2 in humidified air.

For the experiments 48-hr-old hepatocyte primary cultures were used. Cells were radiolabeled with [^{35}S]methionine (925 kBq/3 × 10^6 cells) in basic medium 199 without methionine containing glucose, insulin, dexamethasone, bovine serum albumin, penicillin, streptomycin sulfate, and $NaHCO_3$ in the same concentrations as mentioned above. If not otherwise stated in the text, the labeling time was 2 hr. In pulse-chase experiments hepatocytes were labeled with [^{35}S]methionine for 10 min, then the medium was discarded and fresh medium containing 3 mM methionine was added to the cells. Bay m 1099 was added to the culture media in different concentrations up to 4 mM. If not stated otherwise the hepatocytes were preincubated with Bay m 1099 for 1 hr before adding the radio-labeled metabolite. The same concentration of Bay m 1099 was kept during the labeling period.

At the end of the incubation the media were separated from the cells. The cells of each dish were washed twice with 0.15 M NaCl, 10 mM Tris–HCl buffer, pH 7.5, homogenized by stirring for 20 min in 1 ml of 20 mM NaCl, 25 mM Tris–HCl buffer, pH 7.5 containing 1% sodium deoxycholate, 1% Triton X-100 and centrifuged for 15 min at 12000 *g*. The supernatant from this centrifugation and the culture medium were used for the immunoprecipitations.

Immunoprecipitation and electrophoresis. Immunoprecipitations were essentially carried out according to Macchecchini *et al.* [21]. 0.8 ml of medium or 0.3 ml of the supernatant obtained from the cell homogenate were added to 5 ml of 0.14 M NaCl, 20 mM Tris–HCl buffer, pH 7.5, containing 5 mM EDTA, 1% Triton X-100. After addition of 10 μl of a specific antiserum against rat α_1-antitrypsin [22] or rat α_1-acid glycoprotein [23] and incubation at 0° overnight the antigen–antibody complexes were bound to 10 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 buffer, pH 6.8, containing 5% β-mercaptoethanol, 5% sodium dodecyl sulfate, 10% glycerol at 95° for 5 min. The eluted proteins were analysed by electrophoresis in sodium dodecyl sulfate/polyacrylamide slab gels [24] and fluorography [25].

Protein and radioactivity determinations. Protein concentrations were determined according to Lowry *et al.* [26]. TCA-precipitable radioactivity was determined according to Mans and Novelli [27]. To determine the radioactivity of individual bands of sodium dodecyl sulfate/polyacrylamide slab gels the corresponding regions were cut from the dried gels and incubated with 1 ml of Protosol/water (9/1, v/v) at 45° overnight. After addition of 0.1 ml acetic acid radioactivity was determined in a liquid scintillation spectrophotometer.

Treatment of α_1 antitrypsin and α_1 acid glycoprotein with endo β-N-acetyglucosaminidase H. The glycoprotein–IgG complexes eluted from the protein A-Sepharose were dialysed exhaustively against 50 mM phosphate buffer, pH 6.0 containing 0.1 mg/ml sodium dodecyl sulfate and incubated in a total volume of 0.1 ml with 5 mU of endo β-*N*-acetylglucosaminidase H at 37° for 16 hr.

RESULTS

To find out whether Bay m 1099 affects the biosynthesis of complex type oligosaccharide chains glycosylation of the two secretory liver glycoproteins α_1-antitrypsin and α_1-acid glycoprotein was studied in rat hepatocyte primary cultures. The type of their carbohydrate side chains was characterized by their susceptibility to endo β-*N*-acetylglucosaminidase H. Endo β-*N*-acetylglucosaminidase H cleaves high-mannose type but not complex type oligosaccharides between the two proximal GlcNAc residues [28]. Figure 1A shows that in control cultures the main intracellular form of α_1-antitrypsin has an apparent molecular weight of 49 kD (lane 1). This form of α_1-antitrypsin can be cleaved by endo β-*N*-acetylglucosaminidase H, indicating that it represents the high-mannose type precursor form of this protein (lane 2). α_1-Antitrypsin secreted by control hepatocytes has an apparent molecular weight of 54 kD. It carries oligosaccharide chains which cannot be cleaved off by endo β-*N*-acetylglucosaminidase H (lanes 3–4). A small amount of this 54 kD form of α_1-antitrypsin can also be found in the cells (lanes 1, 2). It is known from previous studies that secreted rat α_1-antitrypsin carries three complex type carbohydrate side chains [15, 16]. In contrast to α_1-antitrypsin secreted by control cells, α_1-antitrypsin secreted by hepatocytes incubated with 4 mM Bay m 1099 was partly deglycosylated by endo β-*N*-acetylglucosaminidase H (Fig. 1, lanes 8, 9) leading to three lower molecular weight forms (49 kD, 46 kD, 43 kD). These lower molecular weight forms represent α_1-antitrypsin with 2, 1 or 0 carbohydrate side chains. The majority of α_1-antitrypsin secreted by 4 mM Bay m 1099 treated cells carries one endo β-*N*-acetylglucosaminidase H sensitive and two insensitive oligosaccharide chains. For comparison, Fig. 1A, lanes 5 and 10 show unglycosylated α_1-antitrypsin from tunicamycin treated hepatocytes (41 kD).

For α_1-acid glycoprotein similar results as for α_1-antitrypsin were obtained (Fig. 1B). An endo β-*N*-acetylglucosaminidase H susceptible form of α_1-acid glycoprotein with an apparent molecular weight of 39 kD could be found in the cells, where it represented the major form of this protein (Fig. 1B lanes 1, 2). The cells contained only small amounts of the 43–50 kD form of α_1-acid glycoprotein, which is resistant to the action of endo β-*N*-acetylglucosaminidase H and represents the secreted form of the protein. Whereas α_1-acid glycoprotein secreted by control hepatocytes could not be deglycosylated by endo β-*N*-acetylglucosaminidase H (Fig. 1B lanes 3, 4) α_1-acid glycoprotein secreted by cells incubated with Bay m 1099 could partly be deglycosylated by endo β-*N*-acetylglucosaminidase H leading to at least four distinct forms of lower molecular weights (Fig. 1B, lanes 8, 9). Unglycosylated α_1-acid glycoprotein

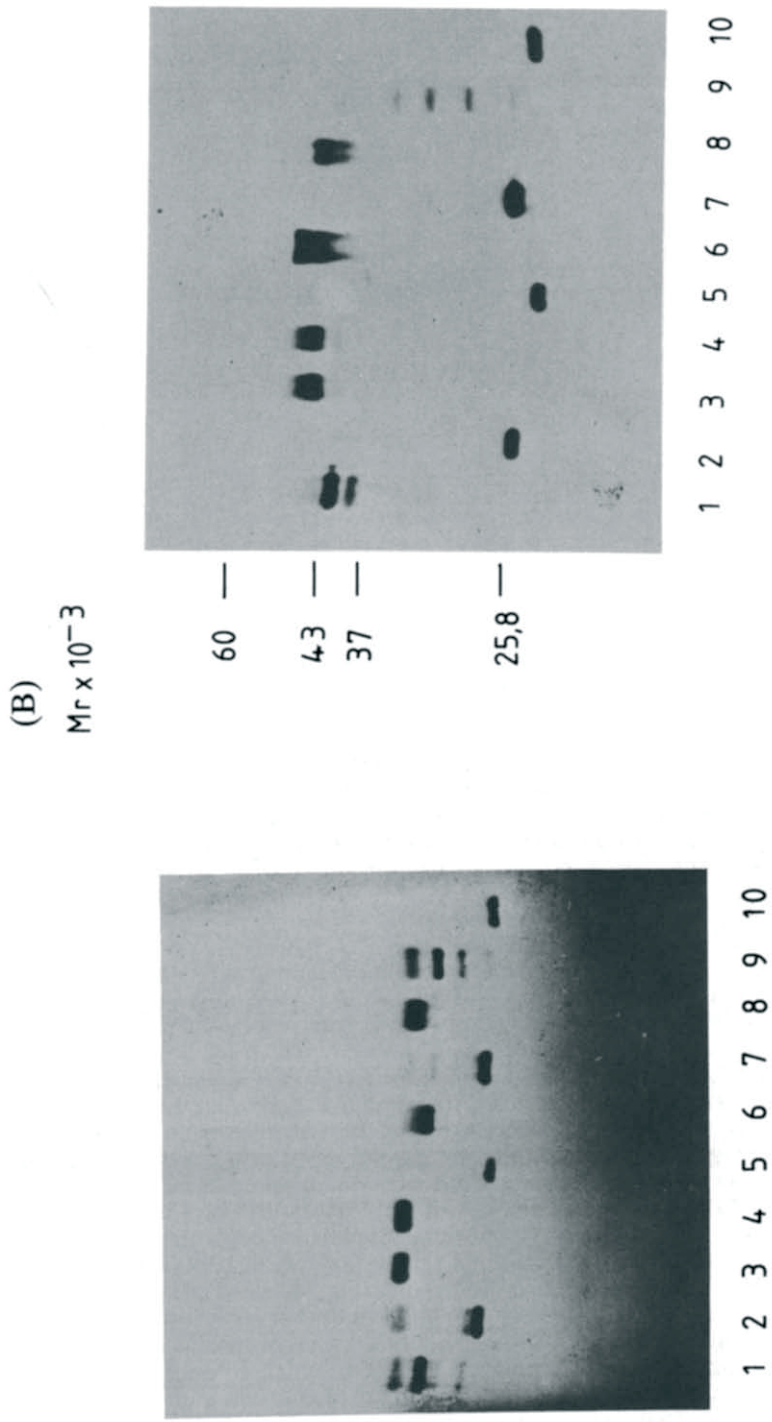

Fig. 1. Endo *β-N*-acetylglucosaminidase H treatment of α_1-antitrypsin and α_1-acid glycoprotein. Rat hepatocyte primary cultures were incubated without (lanes 1–4) or with 4 mM Bay m 1099 (lanes 6–9) or 3 μg/ml tunicamycin (lanes 5 and 10) and labeled with [^{35}S]methionine (925 kBq/dish) for 2 hr. α_1-Antitrypsin (A) and α_1-acid glycoprotein (B) were immunoprecipitated from the cells (lanes 1, 2, 6, 7) and media (lanes 3–5, 8–10) and incubated without (lanes 1, 3, 5, 6, 8, 10) or with (lanes 2, 4, 7, 9) 5 mU of endo *β-N*-acetylglucosaminidase H as described in Materials and Methods.

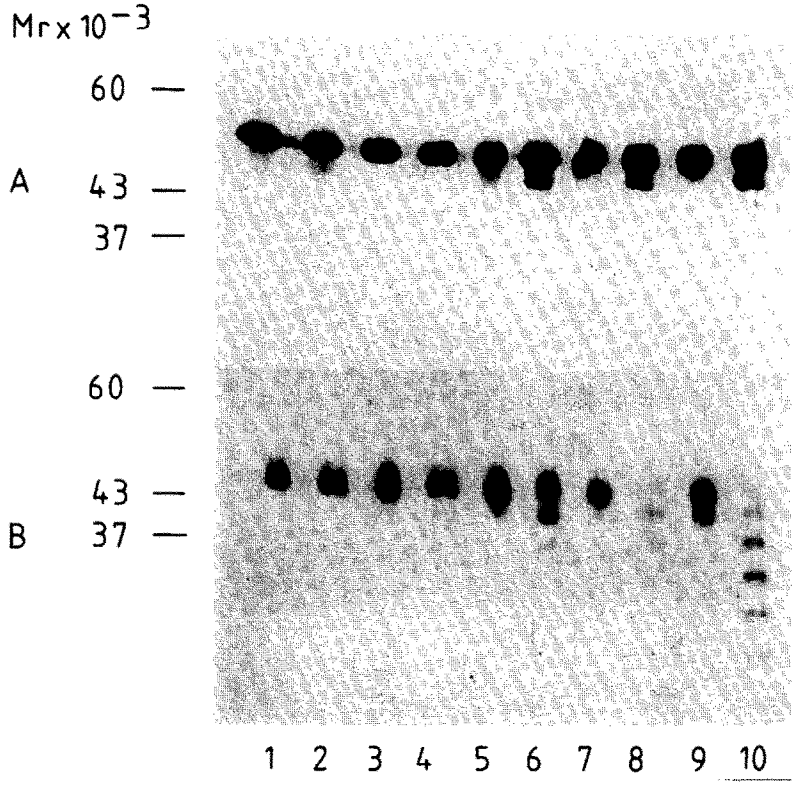

Fig. 2. Determination of the minimal concentration of Bay m 1099 required for the inhibition of complex type glycosylation. Rat hepatocyte primary cultures were incubated without (lanes 1 and 2) or with Bay m 1099 in a concentration of 0.25 mM (lanes 3 and 4), 0.5 mM (lanes 5 and 6), 1 mM (lanes 7 and 8) or 2 mM (lanes 9 and 10). After 2 hr of labelling with [^{35}S]methionine (925 kBq/dish) α_1-antitrypsin (A) and α_1 acid glycoprotein (B) were immunoprecipitated from the hepatocyte media and incubated without (lanes 1, 3, 5, 7, 9) or with (lanes 2, 4, 6, 8, 10) 5 mU of endo β-*N*-acetylglucosaminidase H.

(23 kD) immunoprecipitated from tunicamycin treated hepatocyte primary cultures is shown in Fig. 1B, lanes 5 and 10.

Despite its effect on oligosaccharide trimming 4 mM Bay m 1099 did not inhibit *de novo* *N*-glycosylation (Fig. 1) and protein synthesis in rat hepatocyte primary cultures as measured by the incorporation of [^{35}S]methionine in TCA-precipitable radioactive material. During a 2 hr labeling period with [^{35}S]methionine (925 kBq/3 × 10^6 cells) control cultures incorporated $26.2 \times 10^6 \pm 9.1 \times 10^6$ cpm/mg protein, Bay m 1099 treated cultures $31.2 \times 10^6 \pm 4.9 \times 10^6$ cpm/mg protein (mean ± SD). Furthermore, when hepatocyte primary cultures were incubated with Bay m 1099 for 48 hr essentially no LDH-leakage higher than in control cultures could be observed (not shown).

To determine the minimal concentration of Bay m 1099 required for the inhibition of oligosaccharide trimming, rat hepatocyte primary cultures were incubated with increasing concentrations of Bay m 1099 ranging from 0.25 to 2 mM. α_1-Antitrypsin and α_1-acid glycoprotein were immunoprecipitated from these hepatocyte media and the susceptibility of their oligosaccharide side chains to be released by endo β-*N*-acetylglucosaminidase H digestion was checked. As shown in Fig. 2, Bay m 1099 concentrations lower than 0.5 mM did not impair oligosaccharide processing. At a Bay m 1099 concentration of 2 mM (Fig. 2, A, B, lanes 9, 10) oligosaccharide processing of α_1-antitrypsin and α_1-acid glycoprotein is inhibited to a lower extent than at a Bay m 1099 concentration of 4 mM (see Fig. 1, A and B, lanes 9 and 10).

In further experiments we studied whether the effect of Bay m 1099 on the glycosylation of α_1-antitrypsin persists after removal of the drug. For this purpose, hepatocyte cultures were incubated with 4 mM Bay m 1099 for 1 hr. Radioactive labeling with [^{35}S]methionine was started either at the time of removal of Bay m 1099 (Fig. 3, lanes 3 and 4), after 1 hr (Fig. 3, lanes 5 and 6) or after 2 hr (Fig. 3, lanes 7 and 8). For comparison some cultures were labeled with [^{35}S]methionine in the presence of Bay m 1099 (Fig. 3, lanes 1 and 2). It is evident from Fig. 3 that the inhibitory effect of Bay m 1099 on oligosaccharide trimming is rapidly reversible when the drug is removed from the hepatocyte culture medium.

To study whether Bay m 1099 impairs the secretion of the altered glycoproteins pulse-chase experiments were carried out in the presence of 4 mM Bay m 1099. Hepatocyte cultures were pulse-labeled with [^{35}S]methionine for 15 min followed by a chase with unlabeled methionine for up to 120 min. α_1-Antitrypsin and α_1-acid glycoprotein were immunoprecipitated from control cells and from their

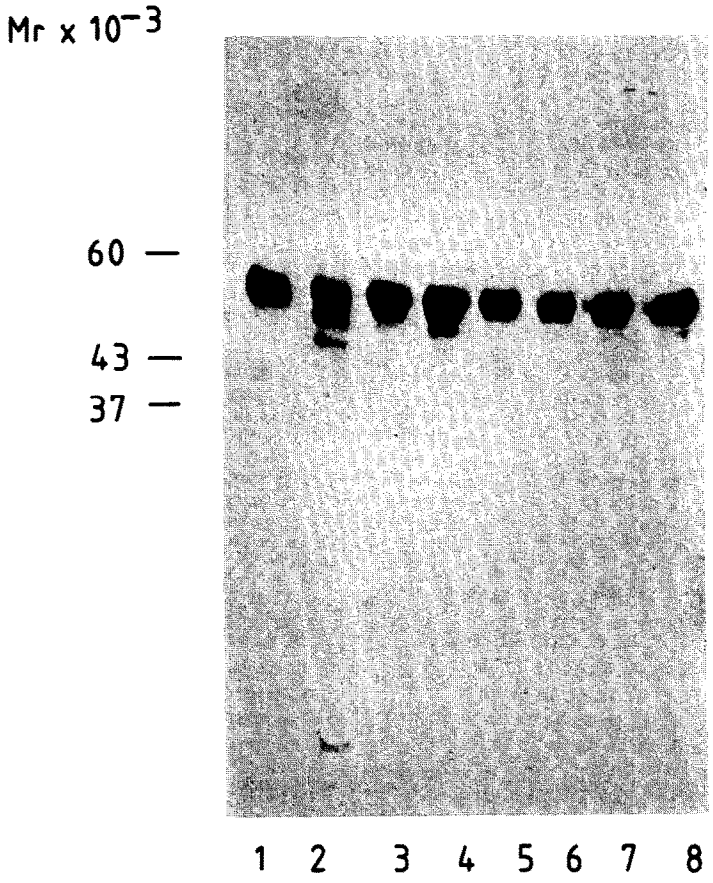

Fig. 3. Determination of the duration of the effect of Bay m 1099 on *N*-glycosylation after removal of the drug from hepatocyte cultures. Hepatocyte primary cultures were incubated with 4 mM Bay m 1099 for 1 hr. Then the medium was removed. The cells were further incubated with medium without Bay m 1099 for 0 hr (lanes 1–4), 1 hr (lanes 5 and 6) or 2 hr (lanes 7 and 8) and then labeled with [^{35}S]methionine (925 kBq/dish) for 2 hr in the presence (lanes 1 and 2) or absence (lanes 3–8) of 4 mM Bay m 1099. α_1-Antitrypsin was immunoprecipitated from all these media and incubated without (lanes 1, 3, 5, 7) or with (lanes 2, 4, 6, 8) 5 mU of endo β-*N*-acetylglucosaminidase H.

supernatants (Fig. 4, A and C) and from Bay m 1099-treated cells and from their supernatants (Fig. 4, B and D). A shift of the radioactive labeled proteins from the cells to the media can be seen in the course of the chase period. After 120 min the vast majority of the proteins has been secreted. The low molecular weight band seen in Fig. 4A and B, lane 9 (arrow) represents a contamination which is sometimes seen when α_1-antitrypsin is immunoprecipitated from cells. The results of pulse-chase experiments are quantitated in Fig. 5. For comparison, the secretion of the unglycosylated albumin is shown (Fig. 5C) in addition to that of α_1-antitrypsin (Fig. 5A) and of α_1-acid glycoprotein (Fig. 5B). It is obvious from Fig. 5 that Bay m 1099 does not lead to pronounced changes in protein secretion.

DISCUSSION

Nojirimycin is a glucose analogue in which the oxygen of the pyranose ring is substituted by nitrogen. Nojirimycin and its derivatives are α-glucosidase inhibitors. The nojirimycin derivatives *N*-hydroxyethyl-1-deoxynojirimycin (Bay m 1099) and *N* -|β - (4- ethoxycarbonylphenoxy) - ethyl| -1 - deoxynojirimycin (Bay o 1248) have been used to inhibit intestinal α-glucosidases and to lower postprandial glucose levels [3, 4]. Since these drugs can be absorbed from the intestines [5] they might affect glucosidases in other organs, especially in the liver.

Most plasma proteins are synthesized in the liver. Besides albumin the majority of these proteins are glycoproteins carrying asparagine-linked oligosaccharide chains of the complex type [29]. The survival of these proteins in the circulation has been shown to depend largely on their carbohydrate moieties [30–38]. Since microsomal glucosidases are involved in the synthesis of *N*-linked oligosaccharides of the complex type [6, 7], nojirimycin derivatives might inhibit their formation and thus lead to the formation of proteins altered in their biological properties. Several studies of the effects of nojirimycin, 1-deoxynojirimycin and *N*-methyl-1-deoxynojirimycin on the biosynthesis of glycoproteins have been published [13, 14, 39–43].

Nojirimycin has been described to inhibit the *de*

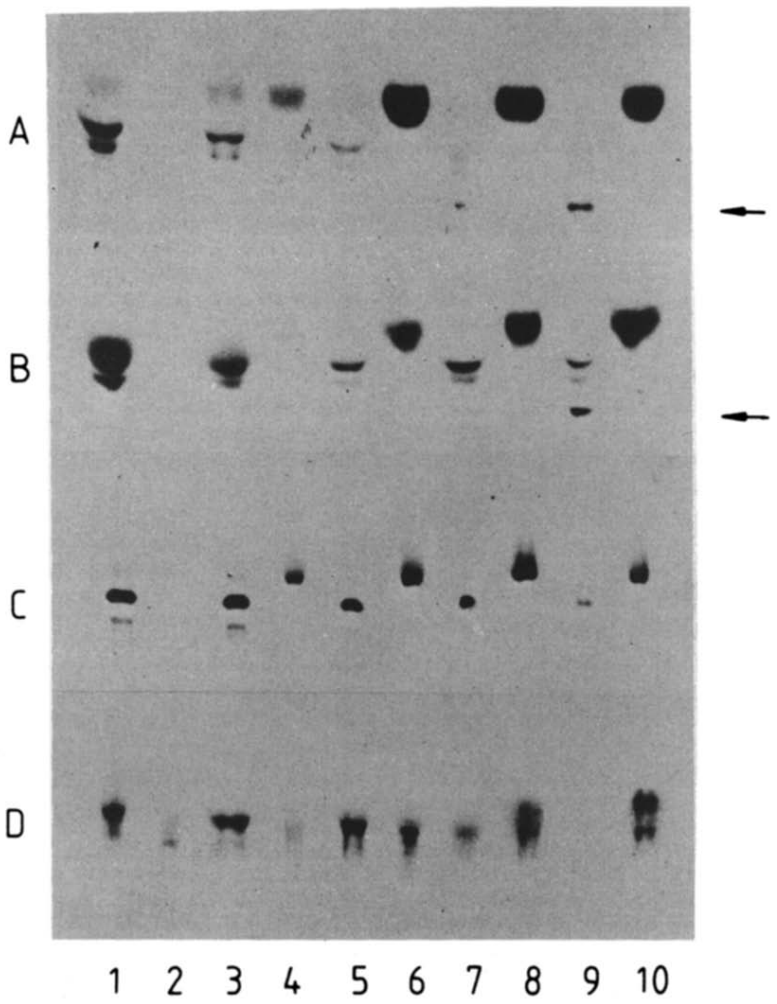

Fig. 4. Pulse-chase kinetics of α_1-antitrypsin and α_1-acid glycoprotein from control and Bay m 1099 treated hepatocytes. Rat hepatocytes primary cultures were incubated with [^{35}S]methionine (925 kBq/dish) for 10 min followed by a chase with unlabeled methionine at a final concentration of 3 mM (A and C). Half the cultures were treated with 4 mM Bay m 1099 1 hr prior to the pulse. Bay m 1099 treatment was continued during the pulse and chase periods (B and D). α_1-Antitrypsin (A, B) and α_1-acid glycoprotein (C, D) were immunoprecipitated from the cells (lanes 1, 3, 5, 7, 9) and from the media (lanes 2, 4, 6, 8, 10), 10 min (lanes 1, 2), 30 min (lanes 3, 4), 60 min (lanes 5, 6), 90 min (lanes 7, 8), and 120 min (lanes 9, 10) after the chase.

novo synthesis of *N*-linked oligosaccharides leading to the formation of drastically shortened oligosaccharide side chains which cannot be further trimmed [39].

In contrast to nojirimycin 1-deoxynojirimycin inhibits only partly *de novo* glycosylation of proteins. It has been shown that in the presence of 5 mM 1-deoxynojirimycin α_1-antitrypsin carrying two and three instead of three and α_1-acid glycoprotein with two to five instead of six oligosaccharide chains were formed [13]. Lemansky *et al.* [40] found increased amounts of a β-chain precursor of β-hexosaminidase lacking one of its carbohydrate side chains in the presence of 1-deoxynojirimycin. 1-Deoxynojirimycin inhibits the processing glucosidases and the formation of complex type oligosaccharides. This inhibitory effect, however, is not complete. Thus in the presence of 1-deoxynojirimycin there are always complex type carbohydrates remaining [13, 14, 40–42]. An inhibition of the secretion of altered glycoproteins synthesized in the presence of 1-deoxynojirimycin has been described [13, 43].

Unlike 1-deoxynojirimycin, *N*-methyl-1-deoxynojirimycin did not have any effect on *de novo* glycosylation of proteins [14]. *N*-methyl-1-deoxynojirimycin inhibited partly the formation of complex type oligosaccharides by inhibition of the processing glucosidases, but in contrast to 1-deoxynojirimycin *N*-methyl-1-deoxynojirimycin did not impair the secretion of α_1-antitrypsin or α_1-acid glycoprotein [14].

In the present work the effect of the deoxynojirimycin derivative *N*-hydroxyethyl-1-deoxynojirimycin (Bay m 1099) on the glycosylation and secretion of α_1-antitrypsin and α_1-acid glycoprotein

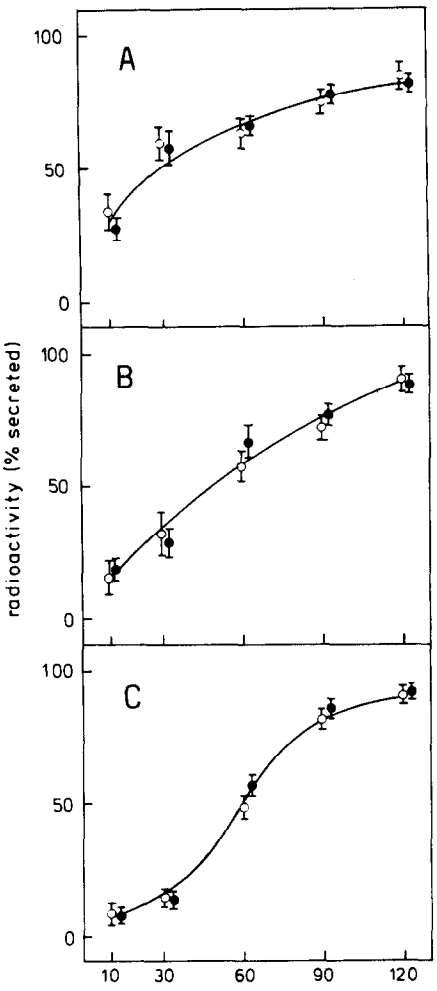

Fig. 5. Effect of Bay m 1099 on the secretion of α_1-antitrypsin, α_1-acid glycoprotein and rat serum albumin by hepatocytes. Rat hepatocyte primary cultures were incubated without (control, open circles) or with 4 mM Bay m 1099 (filled circles) for 2 hr. Then the cells were incubated with [^{35}S]methionine (925 kBq/dish) for 10 min, followed by a chase with unlabeled methionine at a final concentration of 3 mM. In Bay m 1099 treated cultures Bay m 1099 was present in the culture medium during the labeling and the chase period in a concentration of 4 mM. At the times indicated α_1-antitrypsin (A), α_1-acid glycoprotein (B) and albumin (C) were immunoprecipitated from the cells and media as described in Materials and Methods and their radioactivities were determined in a liquid scintillation spectrophotometer. For each protein the ratio of extracellular to intra- plus extracellular radioactivity is given for the various times after chase. Data are given as means ± SE (N = 5).

was studied in rat hepatocyte primary cultures. Bay m 1099 did not inhibit *de novo* N-glycosylation of newly synthesized proteins. Oligosaccharide processing was only partly inhibited by Bay m 1099 leading to the formation of α_1-antitrypsin and α_1-acid glycoprotein carrying a mixture of both high-mannose and complex type oligosaccharide side chains. This effect of Bay m 1099 could only be observed at relatively high concentrations (≥0.5 mM). After removal of the drug the inhibitory effect of Bay m 1099 on oligosaccharide trimming was reversible.

In conclusion Bay m 1099 affects the biosynthesis of complex type glycoproteins in a similar way as *N*-methyl-1-deoxynojirimycin. The effect of *N*-substituted derivatives of 1-deoxynojirimycin on the biosynthesis of *N*-linked oligosaccharides is thus different from that of 1-deoxynojirimycin which in turn differs from that of nojirimycin.

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