Interference with Glycosylation of Glycoproteins

INHIBITION OF FORMATION OF LIPID-LINKED OLIGOSACCHARIDES *IN VIVO*

By Roelf DATEMA and Ralph T. SCHWARZ

Institut für Virologie, Justus-Liebig-Universität Giessen, 6300 Lahn-Giessen, Germany

(Received 12 February 1979)

Influenza-virus-infected cells were labelled with radioactive sugars and extracted to give fractions containing lipid-linked oligosaccharides and glycoproteins. The oligosaccharides linked to lipid were of the 'high-mannose' type and contained glucose. In the glycoprotein fraction, radioactivity was associated with virus proteins and found to occur predominantly in the 'high-mannose' type of glycopeptides. In the presence of the inhibitors 2-deoxy-D-glucose, 2-deoxy-2-amino-D-glucose (glucosamine), 2-deoxy-2-fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose incorporation of radiolabelled sugars into lipid- and protein-linked oligosaccharides was decreased. Kinetic analysis showed that the inhibitors affected first the assembly of lipid-linked oligosaccharides and then protein glycosylation after a lag period. During inhibition by deoxyglucose and the fluoro sugars lipid-linked oligosaccharides were formed that contained oligosaccharides of decreased molecular weight. No such aberrant forms were found during inhibition by glucosamine. In the case of inhibition by deoxyglucose it was shown that the aberrant oligosaccharides were not transferred to protein. Inhibition of formation of lipid-linked oligosaccharides by deoxyglucose and fluoro sugars was antagonized by mannose, in which case oligosaccharides of normal molecular weight were formed. The inhibition by glucosamine was reversed by its removal from the medium. The reversible effects of these inhibitors exemplify their usefulness as tools in the study of glycosylation processes.

Oligosaccharides bound *N*-glycosidically to protein are of two principal types: 'high-mannose' and 'complex' types (Kornfeld & Kornfeld, 1976). The oligosaccharides share the common core sequence:

but differ in the externally located sugar residues (Montreuil, 1975). The two types are probably derived from one precursor: the high-mannose-type oligosaccharide linked to dolichol pyrophosphate (Robbins *et al.*, 1977). This lipid-linked oligosaccharide is transferred to protein (Hemming, 1977; Waechter & Lennarz, 1976) and modified further (Kornfeld *et al.*, 1978). The assembly of the oligosaccharide on the lipid carrier possibly proceeds by stepwise additions of glycosyl residues, starting with the GlcNAc residues (Waechter & Lennarz, 1976) and the β -linked mannosyl residue derived from nucleotide derivatives. The other sugars of the lipid-linked oligosaccharide (mannosyl and glucosyl residues) are derived from dolichol phosphate-activated sugars (Hemming, 1974; Parodi & Leloir, 1976; Hemming, 1977).

Abbreviation used: Dol-P, dolichol monophosphate.

It has been suggested that glycosylation of proteins can have important roles, for example inhibition of glycosylation of the viral glycoproteins prevents maturation of enveloped viruses (Scholtissek, 1975). Deoxyglucose, glucosamine, tunicamycin and fluoro sugars have been tested as inhibitors of glycosylation in virus-infected cells (Schmidt *et al.*, 1976*a,b*). However, which steps in the complex series of reactions are blocked by these inhibitors is not yet clear, except in the case of tunicamycin (Lehle & Tanner, 1976; Tkacz & Lampen, 1975; Takatsuki *et al.*, 1975). Our previous studies (Schmidt *et al.*, 1974, 1978) showed that GDP-deoxyglucose prevents formation of the lipid-linked oligosaccharides (Schwarz *et al.*, 1978*b*; Datema & Schwarz, 1978). However, evidence that the prime target of the inhibition of glycosylation by deoxyglucose in the living cell is the synthesis of lipid-linked oligosaccharides is still lacking.

The situation with the inhibitor glucosamine is less clear. Glucosamine itself, rather than a derivative of it, inhibits glycosylation (Koch *et al.*, 1979) and it was found that whole oligosaccharide chains were lacking (Schwarz *et al.*, 1977). Therefore the amino sugar possibly influences the synthesis of lipid-linked oligosaccharides. The fluoro sugars 2-deoxy-2-

fluoro-D-glucose and 2-deoxy-2-fluoro-D-mannose are both converted to the UDP- and GDP- derivatives (Schmidt *et al.*, 1978). As mannose serves as antagonist to these inhibitors (Schmidt *et al.*, 1976b), it is possible that the GDP- derivatives are the inhibiting agents, being competitors for GDP-mannose in the synthesis of the lipid-linked oligosaccharides.

To us it appeared necessary to study the effects of the above-mentioned inhibitors *in vivo* to be able to interpret data on the use of these inhibitors on whole-cell systems. For these studies we used influenza-virus-infected chick-embryo cells, which have been shown to be a useful model system (Schwarz *et al.*, 1979).

Experimental

Materials

UDP-*N*-acetyl-[¹⁴C]glucosamine (231 Ci/mol), GDP-[¹⁴C]mannose (220 Ci/mol) and UDP-[¹⁴C]-glucose (300 Ci/mol) were obtained from New England Nuclear (Dreieich, Germany). Unlabelled UDP-*N*-acetylglucosamine and GDP-mannose were purchased from Boehringer (Mannheim, Germany). [6-³H]Glucosamine hydrochloride, [1-³H]glucosamine hydrochloride, [2-³H]mannose and [1-³H]-galactose were from Amersham Buchler (Braunschweig, Germany) (specific radioactivities were 2–5 Ci/mmol). Glucosamine hydrochloride and 2-deoxyglucose came from Serva (Heidelberg, Germany). The enzymes α -mannosidase (from jack beans, grade III), alkaline phosphatase (from calf intestine) and Pronase (from *Streptomyces griseus*) were from Sigma (München, Germany), Boehringer and Serva respectively. Endoglucosaminidase H was a gift from P. W. Robbins (M.I.T., Cambridge, U.S.A.). Sephadex G-10 and G-15 were from Pharmacia (Freiburg, Germany); Bio-Gel P-6 was from Bio-Rad (München, Germany); DEAE-cellulose (DE-52) was from Serva and thin-layer plates coated with silica-gel G-60 or with cellulose were products from Merck (Darmstadt, Germany). Tunicamycin was a gift from G. Tamura (University of Tokyo); the fluoro sugars were gifts from J. H. Westwood and A. B. Foster (Chester Beatty Research Institute, London).

Tissue culture, virus infection and labelling procedure

For labelling procedures *in vivo* monolayer cultures of primary chick-embryo cells grown in Petri dishes (diameter 14 cm) incubated at 37°C were used. The cells were infected with fowl plague virus (strain Rostock), which is an influenza A virus, at an input multiplicity of 20–50 plaque-forming units per cell. After adsorption (30 min) the cells were washed twice with warm phosphate-buffered saline (1.0 mM-KH₂PO₄, 11.5 mM-NaH₂PO₄·2H₂O, 3 mM-KCl,

170 mM-NaCl, 0.5 mM-MgCl₂·6H₂O, 0.8 mM-CaCl₂·H₂O), and the cells were flooded with 20 ml of Earle's medium (Schmidt *et al.*, 1976a). The medium contained either glucose (5 mM) or pyruvate (10 mM) as carbon source ('glucose medium' or 'pyruvate medium' respectively). Other additions to the medium (deoxyglucose, mannose, fluoro sugars, amine and tunicamycin) were done from concentrated stock solution. The glucosamine hydrochloride solution (0.5 M) was freshly prepared each time and brought to pH 7.5 with NaOH. Radioactive isotopes were added 3–4 h after infection, and cells were harvested after the indicated times (see the Results section). Incubations with [³H]galactose (50 μ Ci/ml) were done in media containing 0.5 mM-glucose. When medium changes were necessary, cells were washed three times with warm phosphate-buffered saline before addition of the new medium.

Extraction of cells

At the end of the labelling periods, plates were put on ice and the medium was removed. The cells were washed four times with ice-cold phosphate-buffered saline and scraped off in 2 ml of this buffer per plate. Cells were collected by centrifugation (5 min, 9000g) and extracted or frozen in liquid N₂, and stored at –80°C. The extraction of the cell pellet was as follows (amounts are given for cells collected from one plate). The cell pellet was extracted twice with 2 ml of chloroform/methanol (2:1, v/v). The extract was washed to remove water-soluble compounds as previously described (Schwarz *et al.*, 1978b). This organic extract is called fraction I. The cellular residue was dried under N₂, washed four times with 5 ml of water with the use of sonication to suspend the pellet, and again dried after addition of 0.2 ml of methanol. The dried material was extracted three times with 1.0 ml of chloroform/methanol/water (10:10:3, by vol.). This extract is called fraction II. The dried pellets (fraction III) were dissolved in 1.0 ml of 0.2 M-NaOH (for liquid-scintillation counting), in 'sample buffer' for polyacrylamide-gel electrophoresis (Laemmli, 1970; Schwarz *et al.*, 1978b) or digested with Pronase (see below) for glycopeptide analysis. The organic phases were counted for radioactivity after drying; water-containing samples were counted for radioactivity in a Triton X-100-containing scintillation fluid (Roth, Karlsruhe, Germany). The quantitative data presented are averages of at least three separate experiments.

Measurement of uptake of sugars

To measure the uptake of radiolabelled sugars into cells in the presence or absence of glycosylation inhibitors, two methods were used. The first procedure was to measure the radioactivity present in the aqueous phases obtained during the extraction of the

cells described above. The second procedure was measurement of the radioactivity in HClO_4 extracts of cells, as described by Schwarz & Klenk (1974).

Hydrolytic procedures

The mild acid treatment of glycolipids used to hydrolyse the (pyro)phosphoryl linkages was performed as described by Lehle & Tanner (1975). Alkaline cleavage (alkaline hydrolysis and alkaline phosphatase treatment) of glycolipids (Behrens & Táborá, 1978; Datema & Schwarz, 1978) was used whenever deoxyglucose residues might occur in the oligosaccharide moiety. Strong acid hydrolysis to obtain monosaccharides was performed in 0.75 ml of 1M- H_2SO_4 (6h, 100°C). The hydrolysate was neutralized with barium acetate. Digestions with Pronase (Datema & Schwarz, 1978) and endoglucosaminidase H plus α -mannosidase (Robbins *et al.*, 1977) were as described. The substrates for endoglucosaminidase and α -mannosidase were purified by either chromatography on Bio-Gel P-6 or centrifugation through a 5cm column of Sephadex G-10. For the proteolytic digestion of proteins in polyacrylamide gels the following procedure was used: the gel was cut into small pieces and extracted with 0.15M-Tris/HCl buffer (pH 7.8) containing 1.5mM- CaCl_2 , for 2 days at 4°C. Then Pronase (predigested for 1h) was added: 2mg and after 24h another 2mg, and after another 12h 1mg. After incubation at 37°C the residual material was spun down; the supernatant was evaporated to dryness, taken up in the Tris/HCl buffer, and incubated with 1mg of Pronase overnight at 37°C.

Separation techniques

The chromatographic systems used were the following: system 1, paper chromatograms developed with butan-1-ol/ethanol/water (10:1:2, by vol.) on Whatman number 1 paper; system 2, thin-layer plates of silica gel developed with chloroform/methanol/water (20:13:2, by vol.); system 3, thin-layer plates of cellulose developed with pyridine/ethyl acetate/acetic acid/water (9:9:2:5, by vol.); system 4, thin-layer plates of silica gel developed with chloroform/methanol/water (10:10:3, by vol.).

Gel filtration on calibrated columns (1cm $\times$ 110cm) of Sephadex G-15 and Bio-Gel P-6 was performed as previously described (Datema & Schwarz, 1978).

Ion-exchange chromatography (Spiro *et al.*, 1976) used DEAE-cellulose (DE-52) converted to the acetate form, poured into a column (1.5cm $\times$ 7cm) and equilibrated with chloroform/methanol/water (10:10:3, by vol.). Samples in this solvent were applied and eluted either batchwise with 50ml of the equilibrating solvent followed with 50mM-ammonium acetate in this solvent, or with a linear

gradient of ammonium acetate [0.0–0.5M in chloroform/methanol/water (10:10:3, by vol.)].

Polyacrylamide-slab-gel electrophoresis (Laemmli, 1970; Schwarz *et al.*, 1978b) and paper electrophoresis in borate buffer (Foster & Stacey, 1953; Montreuil & Spik, 1968) were also used.

Glycosylation in vitro

Glycosylation *in vitro* to form dolichol pyrophosphate-linked di-*N*-acetylchitobiose [$\text{Dol-P-P-(GlcNAc)}_2$] and dolichol pyrophosphate-linked oligosaccharides [$\text{Dol-P-P-(GlcNAc)}_2\text{-Man}_x$] was performed as previously described (Datema & Schwarz, 1978). For the formation of lipid-linked oligosaccharides containing glucose residues [$\text{Dol-P-P-(GlcNAc)}_2\text{-(Man)}_x\text{-(Glc)}_y$], UDP-glucose or UDP-[^{14}C]glucose was included in the assay at a concentration of 5 μM . The enzymes were present in a crude membrane fraction from chick-embryo cells, prepared as described by Krag & Robbins (1977). The extraction procedure of the lipid-linked saccharides was the one described previously (Datema & Schwarz, 1978). For the addition of glucosamine to the incubations we used freshly prepared concentrated solutions of glucosamine hydrochloride (0.35M and 1.05M) carefully neutralized to the required pH. Glycosylation *in vitro* was studied at pH 7.4, 8.0, 8.5 and 9.0, because the pH optima for the various reactions differ somewhat (R. Datema & R. T. Schwarz, unpublished results).

Other procedures

Determination of haemagglutination activity has been described by Davenport *et al.* (1960) and *N*-acetylation of amino sugars by Sweely *et al.* (1963).

Results

Analysis of cellular extracts

Influenza-virus-infected chick-embryo cells were labelled with [^3H]glucosamine or [^3H]mannose for 1–4h and extracted to give three fractions: fraction I, a chloroform/methanol (2:1, v/v) extract; fraction II, a chloroform/methanol/water (10:10:3, by vol.) extract; fraction III, the residue. Significant randomization of the labelled sugars is not observed (Schwarz *et al.*, 1977; Schwarz & Klenk, 1974), as confirmed for the three fractions obtained from cells labelled in glucose or pyruvate medium. This could be shown after strong-acid hydrolysis followed by paper chromatography (system 1) for mannose, and by *N*-acetylation and borate electrophoresis for *N*-acetylglucosamine.

Tunicamycin blocks the first step in the synthesis of lipid-linked oligosaccharides, the formation of lipid pyrophosphate-linked *N*-acetylglucosamine,

and thus prevents glycosylation of proteins (Lehle & Tanner, 1976; Takatsuki *et al.*, 1975; Tkacz & Lampen, 1975). We studied the effect of tunicamycin on the incorporation of [^3H]glucosamine into fractions I, II and III. The labelled sugar was added together with tunicamycin (1 $\mu\text{g}/\text{ml}$) to cells 3 h after infection. Control cells did not receive tunicamycin. The incorporation of radioactivity into fractions II and III after further incubation for 3 h was inhibited by 80% by the drug, whereas the incorporation into fraction I was not significantly affected. This means that fraction I contains little if any of the glycolipid precursors of protein glycosylation.

To analyse further the nature of the three fractions, influenza-virus-infected cells were labelled with [^3H]glucosamine 3 h after infection, and for 3 h in the absence of inhibitors and then extracted.

Fraction I. Over 90% of the radioactive material extracted with chloroform/methanol (2:1, v/v) ran with the front on silica-gel plates (chromatography system 2). This means that little material with the properties of the dolichol (pyro)phosphate-linked saccharides is present (Datema & Schwarz, 1978). This was confirmed by DEAE-cellulose chromatography of the extract from cells labelled with [^3H]glucosamine in pyruvate medium. Of the radioactivity applied to the column, 88–90% did not bind, and therefore is present in neutral components. The remainder was eluted from the column with 25–50 mM-ammonium acetate, suggesting the occurrence of a pyrophosphoryl group. This acidic material, after Folch washing to remove the salt, was subjected to alkaline cleavage. Water-soluble compounds were chromatographed on a column of Sephadex G-15. Most of the radioactivity applied to the column (>95%) was eluted in the monosaccharide region, and identified as *N*-acetylglucosamine with chromatography system 3; 3–5% of the radioactivity present in fraction I was recovered in this way. This analysis suggests that fraction I contains small amounts of lipid pyrophosphate-linked *N*-acetylglucosamine.

Fraction II. The chloroform/methanol/water (10:10:3, by vol.) extract contained labelled glycolipids with R_F values of 0.6–0.8 in chromatography system 4, suggesting the occurrence of dolichol pyrophosphate-linked oligosaccharides (Datema & Schwarz, 1978). This was confirmed by adsorption of the labelled material to DEAE-cellulose; it required 100–120 mM-ammonium acetate in chloroform/methanol/water (10:10:3, by vol.) for elution.

After mild-acid hydrolysis of the glycolipids in fraction II, 90–100% of the radioactivity became water-soluble. Most of this material was eluted in the 2000–1800-mol.wt. range from a Bio-Gel P-6 column (1 cm $\times$ 110 cm) (Fig. 1a).

Endo- β -*N*-acetylglucosaminidase H splits the bond between the two *N*-acetylglucosamine units in the core region of oligosaccharides of the high-

Fig. 1. Bio-Gel P-6 chromatography of oligosaccharides (a) Co-chromatography of [^3H]glucosamine-labelled oligosaccharides, obtained from fraction II by mild-acid hydrolysis, and [^{14}C]glucose-labelled oligosaccharides, obtained from incubations of membrane fraction with UDP-*N*-acetyl glucosamine, GDP-mannose and UDP- ^{14}C glucose *in vitro*. These oligosaccharides were also released by mild-acid treatment. (b) The oligosaccharide obtained by α -mannosidase treatment of material eluted in the first peak of the [^3H]glucosamine-labelled oligosaccharide of (a). (c) [^3H]Galactose-labelled oligosaccharides obtained from fraction II by mild-acid hydrolysis. (d) Oligosaccharide obtained by α -mannosidase treatment of material eluted in the first peak of the [^3H]galactose-labelled oligosaccharides of (c). Fractions containing Blue Dextran are placed under each other and are marked with a solid bar. $\circ$, ^3H ; $\bullet$, ^{14}C .

mannose type (Tarentino & Maley, 1974). The oligosaccharides obtained from fraction II by mild-

acid hydrolysis were treated with this enzyme, and then applied to the same column of Bio-Gel P-6. Half of the radioactivity applied to the column was eluted in the monosaccharide region, and was identified as *N*-acetylglucosamine (chromatography system 3). The other half was still present in oligosaccharides. So the oligosaccharide bound to lipid pyrophosphate in fraction II is of the high-mannose type, and radioactivity is present in the di-*N*-acetylchitobiosyl unit of this oligosaccharide.

Liu *et al.* (1979) showed the presence of glucose residues in the oligosaccharide bound to lipid. Although the glucose-free oligosaccharides are degraded by α -mannosidase to the Man β -GlcNAc β -GlcNAc trisaccharide at the reducing end of the chain, the glucose-containing oligosaccharides resist this extensive degradation (Hubbard & Robbins, 1979). Thus the [3 H]glucosamine-labelled oligosaccharide after treatment with α -mannosidase shifted to lower molecular weight, corresponding to a loss of only four to five saccharide units (Fig. 1*b*). To confirm the presence of glucose in the oligosaccharide isolated from fraction II, cells were labelled with [3 H]galactose (Hubbard & Robbins, 1979). The oligosaccharides obtained after mild-acid hydrolysis were separated on Bio-Gel P-6 (Fig. 1*c*). Material eluted in the main high-molecular-weight peak was hydrolysed to give the monosaccharides, and it was shown that radioactivity occurred in glucose only (chromatography systems 1 and 3). Treatment of the galactose-labelled oligosaccharide with α -mannosidase caused a similar small shift to lower molecular weights, as observed with the glucosamine-labelled oligosaccharide (Fig. 1*d*). From these results we infer that the oligosaccharide contains glucosyl units. This analysis of fraction II shows that it contains lipid-linked glucose-containing oligosaccharides.

Fraction III. Components in the residual fraction obtained after lipid extractions and washings with water were dissolved in sample buffer and separated by polyacrylamide-gel electrophoresis. A major and a minor band were seen. The compound giving rise to the major band ran like influenza-virus haemagglutinin, HA-1. From a preparative run this band was cut out of the gel, and material was digested with Pronase. The glycopeptides thus obtained were separated on the column of Bio-Gel P-6 (Fig. 2*a*). The two glycopeptides I and II had the same K_a

Fig. 2. Bio-Gel P-6 chromatography of glycopeptides obtained by Pronase digestion of material present in fraction III

(*a*) Glycopeptides derived from Pronase digests of [3 H]glucosamine-labelled virus haemagglutinin, HA-1. (*b*) Glycopeptides derived from Pronase digests of fraction III labelled with [3 H]glucosamine in the presence of deoxyglucose and mannose (see Table 2).

Table 1. Effects of inhibitors on incorporation of [3 H]mannose and [3 H]glucosamine into fractions II and III

Inhibitors were added 4h after infection, and radioactive isotopes were added after another 4h (deoxyglucose, glucosamine) or 1.5h (fluoro sugars). Labelling times were 1h ([3 H]mannose), 2h ([3 H]glucosamine in pyruvate medium) or 3h ([3 H]glucosamine in glucose medium). Tissue culture and extraction procedures are described in the Experimental section. Maximum inhibiting doses were used.

Radioactive isotope (50 μ Ci)	Carbon source in medium	Additions to the medium				Radioactivity in fraction (d.p.m.)	
		[Deoxyglucose] (mM)	[Glucosamine] (mM)	[Fluoromannose] (mM)	[Fluoroglucose] (mM)	II	I
[2- 3 H]Man	Pyruvate	0	0			1880	29700
		0	1			850	17400
		1	0			380	11000
[6- 3 H]GlcN	Glucose	0	0			1100	68400
		0	10			70	2300
		10	0			260	6600
[6- 3 H]GlcN	Pyruvate			0	0	12660	1278000
				0.5	0	1850	288000
				0	0.5	2720	267000

Fig. 3. Incorporation of radioactivity from [^3H]glucosamine into fraction II (a) and fraction III (b) in the presence or absence of deoxyglucose, as a function of the incubation time

Influenza-virus-infected cells were labelled in pyruvate medium containing either 2mM-deoxyglucose (○) or no deoxyglucose (●). [^3H]Glucosamine (200 $\mu\text{Ci}/\text{plate}$) was added 4h after infection and 1h before the addition of deoxyglucose. Extraction of cells is described in the Experimental section.

values as the complex-type and high-mannose-type glycopeptides respectively isolated in this laboratory from purified virus haemagglutinin (Schwarz *et al.*, 1977).

Fraction III was exhaustively washed with water with intermittent sonication and then digested with Pronase. Over 95% of the radioactive material

became water-soluble. Chromatography of the water-soluble compounds on Bio-Gel P-6 gave the two glycopeptide peaks described above in the same ratio, but a substance was also eluted in the void volume of the column. This latter material comprised 12% of the water-soluble compounds and might be identical with the mucopolysaccharide associated with the fowl-plague-virus particle (Klenk *et al.*, 1977; Schwarz *et al.*, 1978a).

So radioactivity in fraction III of [^3H]glucosamine-labelled-virus-infected cells is for the greater part associated with viral glycoproteins and occurs mainly in high-mannose glycopeptides. Having established the nature of the fractions, the effects of glycosylation inhibitors on the synthesis of lipid-linked oligosaccharides were studied.

Effects of deoxyglucose in vivo

Table 1 shows that treatment of influenza-virus-infected cells with deoxyglucose reduced the incorporation of [^3H]glucosamine and [^3H]mannose into lipid-linked oligosaccharides (fraction II) and into glycoproteins (fraction III). It has been shown previously that the uptake of the radiolabelled sugars is not significantly affected by deoxyglucose (Schwarz & Klenk, 1974).

To find out whether the synthesis of lipid-linked oligosaccharides is the prime target affected by deoxyglucose, we studied the inhibition as a function of the time the cells were in contact with deoxyglucose. The labelled sugar, [^3H]glucosamine, was added 4h after infection in pyruvate medium and 1h before the addition of deoxyglucose (2mM). The results of this experiment (Fig. 3) show that the incorporation into lipid-linked oligosaccharides decreased, and the incorporation of radioactivity into glycoproteins first increased but then levelled off. Apparently deoxyglucose first affects the biosynthesis of lipid-linked oligosaccharides, and subsequently the glycosylation of protein is inhibited.

To study the oligosaccharides formed during inhibition by deoxyglucose, cells were labelled with [^3H]glucosamine in pyruvate medium for 45 min, and then deoxyglucose (2mM) was added for 20 min. Fraction II was isolated and the material was subjected to alkaline cleavage. The oligosaccharides were fractionated on a column of Bio-Gel P-6. The pattern of oligosaccharides (Fig. 4a) clearly differed from that of control incubations (Fig. 1a). Oligosaccharides of decreased molecular weight were formed, and the major oligosaccharide eluted in fractions 80–90 (see Fig. 1a) had decreased in amount.

The shortened lipid-linked oligosaccharides do not accumulate under these labelling conditions in these cells, because radioactivity in fraction II is decreased, and fraction I (analysed as described above) does not contain increased amounts of lipid pyrophosphate-linked saccharides.

Fig. 4. Bio-Gel P-6 chromatography of oligosaccharides labelled with [^3H]glucosamine under various conditions

(a) Oligosaccharides obtained by alkaline cleavage of material in fraction II labelled in the presence of inhibitory concentrations of deoxyglucose. (b) Oligosaccharides obtained by alkaline cleavage of material in fraction II of cells labelled in the presence of mannose and deoxyglucose (cf. Table 2). (c) Oligosaccharides obtained by mild-acid hydrolysis of material in fraction II of cells under conditions of inhibition by fluoromannose. (d) Oligosaccharides obtained by mild-acid hydrolysis of material in fraction II of cells labelled in the presence of mannose and fluoromannose. (e) as (c), except for inhibition by

Reversion of the inhibition by deoxyglucose in vivo

Kaluza *et al.* (1973) have shown that inhibition of virus formation by deoxyglucose is reversed by the addition of mannose to the culture medium. Influenza-virus-infected cells were labelled with [^3H]glucosamine in pyruvate medium containing mannose and/or deoxyglucose as shown in Table 2. In the presence of deoxyglucose, virus formation was inhibited, as measured by the haemagglutination assay; also formation of lipid-linked oligosaccharides was reduced. In the presence of deoxyglucose plus mannose, where virus formation was observed, increased incorporation into lipid-linked oligosaccharides was found relative to that in cells grown in the presence of deoxyglucose only (Table 2).

The oligosaccharides present in fraction II of cells labelled in the presence of mannose plus deoxyglucose (Table 2) were released by alkaline cleavage, and then fractionated on Bio-Gel P-6. The pattern obtained (Fig. 4b) shows the very low amounts of oligosaccharides of normal size (arrow in Fig. 4b). In contrast with the oligosaccharide pattern, the pattern of the glycopeptides isolated from fraction III does show the presence of glycopeptides of normal size; the elution profile is shown in Fig. 2(b). Apparently, oligosaccharides of physiological size once formed are transferred immediately to protein. Material eluted around fraction 140 in Fig. 2(b) was of the size of monosaccharides and possibly arose by contamination of fraction III with the original label.

Cells were treated with deoxyglucose (0.2 mM in pyruvate medium) for 3 h; then mannose (0.5 mM) and [^3H]glucosamine (500 μCi) were added. In cells treated thus glycopeptides eluted between fractions 60 and 90 (see Fig. 2) were not discovered until after 2 h of labelling. In cells not treated with deoxyglucose, these glycopeptides were found after less than 10 min. So inhibition of glycosylation by deoxyglucose is slowly reversed by mannose.

Effects of glucosamine in vivo

When influenza-virus-infected cells were treated with glucosamine, incorporation of [^3H]glucosamine or [^3H]mannose into lipid-linked oligosaccharide (fraction II) and into glycoprotein (fraction III) was decreased (Table 1). Uptake of [^3H]mannose into cells was increased in the presence of glucosamine. In the presence of 5 mM-glucosamine this stimulation was 1.6-fold which was allowed for in

fluoroglucose. (f) as (d), except cells were labelled in the presence of mannose and fluoroglucose. The arrow points to the position of the main oligosaccharide formed under non-inhibiting conditions (see Fig. 1a).

Table 2. *Effects of deoxyglucose and mannose on the incorporation of [^3H]glucosamine into fractions II and III*
Cells were labelled in pyruvate medium with 200 μCi of 6- ^3H]glucosamine per plate. Labelling time was 3 h. The labelled sugar was added 4 h after infection. For extraction see the Experimental section.

Haemagglutinating activity	Additions to the medium		$10^{-3} \times$ Radioactivity in fraction (d.p.m.)	
	[Deoxyglucose] (mM)	[Mannose] (mM)	II	III
32	0	0	89	11100
64	0	0.4	81	7900
0	0.2	0	11	980
16	0.2	0.4	23	2300

subsequent calculations of the inhibition by glucosamine.

Inhibition of incorporation of labelled sugars into lipid-linked oligosaccharides and into protein attained its maximum value (80%) when equimolar amounts of glucosamine and glucose (carbon source) were present (results not shown). The time-dependence of inhibition was studied. At 3 h after infection, cells were labelled with [^3H]mannose in glucose medium. Then 1 h later glucosamine was added to achieve a final concentration of 10 mM, and cells were harvested at various times after the addition of the inhibitor. The pattern of incorporation of radioactivity into fractions II and III (Fig. 5) shows that the synthesis of lipid-linked oligosaccharides was first affected. The incorporation into protein levelled off only after 20 min. This suggests that inhibition of protein glycosylation by glucosamine is caused by inhibition of formation of lipid-linked oligosaccharides.

The products extracted into chloroform/methanol/water were analysed by t.l.c. (systems 2 and 4). No aberrant forms were detected among the lipid-linked oligosaccharides synthesized during inhibition. Fraction II of cells after 30 min of glucosamine inhibition was isolated and the material was treated with mild acid to release the oligosaccharides. Chromatography of the oligosaccharides on a column of Bio-Gel P-6 revealed that only those oligosaccharides were present that were also found in the absence of glucosamine (Fig. 1a), but in smaller amounts, as indicated in Fig. 5.

Products in fraction I were analysed after removal of the neutral components by DEAE-cellulose chromatography (see above). The products eluted with a gradient of ammonium acetate (0–0.5 M) were the same as those synthesized in the absence of glucosamine (see analysis of fraction I above). This result suggests that glucosamine exerts its effect early in the biosynthesis of lipid-linked oligosaccharides, because intermediates between lipid-linked *N*-acetylglucosamine and the full-size lipid-linked oligosaccharides were found not to accumulate.

After removal of the glucosamine-containing medium, and a 'chase' in a glucosamine-free medium,

Fig. 5. *Incorporation of radioactivity from [^3H]mannose into fraction II (a) and fraction III (b) in the presence or absence of glucosamine as a function of the incubation time*
Influenza-virus-infected cells were labelled in glucose medium containing either 10 mM-glucosamine (○) or no glucosamine (●). [^3H]Mannose (200 μCi /plate) was added 3 h after infection and 1 h before the addition of glucosamine. Extraction of cells is described in the Experimental section.

onset of glycosylation was observed after 10–15 min (Koch *et al.*, 1979; Wöllert, 1975). This rapid incorporation of carbohydrate into protein was paralleled by a similar rapid increase in the incorporation of [^3H]mannose into lipid-linked oligosaccharides (results not shown). Apparently, after the removal of glucosamine, no products are present that prevent reinitiation of oligosaccharide assembly and transfer of oligosaccharide to protein.

Effects of glucosamine on the synthesis of lipid-linked oligosaccharides in vitro

Koch *et al.* (1979) showed that glucosamine itself inhibits protein glycosylation. So we studied the effects of this amino sugar on the synthesis of lipid-linked oligosaccharides *in vitro*. A particulate fraction from chick-embryo cells was incubated with the following mixtures of nucleotide sugars (Datema & Schwarz, 1978): (1) UDP-[^{14}C]GlcNAc to give Dol-*P-P*-(^{14}C)GlcNAc $_2$; (2) UDP-GlcNAc and GDP-[^{14}C]mannose to give mannose-containing lipid-linked oligosaccharides labelled in mannose

and dolichol phosphate [^{14}C]mannose; (3) UDP-GlcNAc, GDP-mannose plus UDP-[^{14}C]glucose to give glucose- and mannose-containing lipid-linked oligosaccharides labelled in glucose as well as dolichol phosphate [^{14}C]glucose. The products synthesized in reactions (1) and (2) have previously been identified (Datema & Schwarz, 1978). The elution pattern of the glucose-containing oligosaccharides, isolated after mild-acid hydrolysis, is shown in Fig. 1(a). They are co-eluted with the oligosaccharide isolated from whole cells. Dolichol phosphate glucose was identified by previously established procedures (Schwarz *et al.*, 1978b).

Addition of glucosamine (in concentrations ranging from 5 to 100 mM) did not interfere with either of these reactions, nor did it prevent the incorporation of radioactivity into protein. Apparently, for the expression of the inhibitory effect of glucosamine, an intact cell system is required.

Effects of fluoro sugars in vivo

Treatment of virus-infected cells with fluoro sugars (2-deoxy-2-fluoro-D-mannose and 2-deoxy-2-fluoro-D-glucose) prevented the incorporation of radioactivity from [^3H]glucosamine into lipid-linked oligosaccharides (fraction II) and into protein (fraction III), as shown in Table 1. A study of the time-dependence of the inhibition showed that both 2-fluoroglucose and 2-fluoromannose exerted their effects more slowly than did deoxyglucose (Figs. 6a and 6b). The reason for this might be that fluoro sugars are more slowly converted to the inhibiting agents than is deoxyglucose. When incorporation was corrected for the uptake of the radiolabelled sugar, it was found that the fluoro sugars first affected the incorporation into lipid-linked oligosaccharides and then the incorporation into protein (Figs. 6c and 6d).

Oligosaccharides linked to lipid and isolated from fraction II of cells during inhibition by fluoroglucose or fluoromannose were analysed by gel filtration (Bio-Gel P-6). Thus the cells were labelled with [^3H]glucosamine for 45 min; inhibitors were then added (0.5 mM) and 15 min afterwards cells were harvested. The inhibition obtained in these cases was about 40% for each inhibitor. The elution profiles (Figs. 4c and 4e) show that, in addition to the oligosaccharides found under non-inhibitory conditions (arrow), oligosaccharides of lower molecular weight were present.

Schmidt *et al.* (1976b) have reported that inhibition of the biological properties of enveloped viruses by fluoro sugars is antagonized by mannose. So cells were labelled with [^3H]glucosamine (45 min), and then fluoro sugars (0.5 mM) plus or minus mannose (1 mM) were added for 15 min. It was found that inhibition of the incorporation of [^3H]glucosamine into lipid-linked oligosaccharides was overcome if

Fig. 6. Incorporation of radioactivity from [^3H]glucosamine into fraction II (a), fraction III (b), into fraction II corrected for uptake (c) and into fraction III corrected for uptake (d) in the presence or absence of fluoro sugars, as a function of the incubation time

Influenza-virus-infected cells were labelled in pyruvate medium containing 0.5 mM-fluoroglucose (○), 0.5 mM-fluoromannose (■) or no fluoro sugars (●). [^3H]Glucosamine (100 $\mu\text{Ci}/\text{plate}$) was added 3 h after infection and 45 min before the addition of the fluoro sugars. Extraction of cells and measurement of uptake are described in the Experimental section. Correction for uptake was done by dividing the amount of radioactivity in each fraction by the amount of radioactivity in the aqueous extracts of the cells.

mannose was added to the culture medium containing either fluoroglucose or fluoromannose. From analysis of the oligosaccharides from fraction II of cells labelled in the presence of fluoro sugars plus mannose it follows that under these conditions the normal-size oligosaccharides are present in much higher amounts (Figs. 4*d* and 4*f*). So there exists a correlation between inhibition of glycosylation and formation of oligosaccharides (linked to lipid) with decreased molecular weight.

Discussion

When deoxyglucose is added to tissue culture cells, it is converted to the GDP- derivative, which is the inhibitor of glycosylation (Schwarz *et al.*, 1979). It is shown here that this inhibition manifests itself in the first instance by a decreased formation of lipid-linked oligosaccharides. Subsequently, glycosylation of protein is decreased (Fig. 3). This means that *in vivo* GDP-deoxyglucose must inhibit the synthesis of lipid-linked oligosaccharides. Studies on the effect of GDP-deoxyglucose on the synthesis of lipid-linked oligosaccharides *in vitro* have shown that termination of the elongation of the oligosaccharide linked to dolichol pyrophosphate occurs (Datema & Schwarz, 1978). These shortened oligosaccharides are not transferred to protein *in vitro*. Similar mechanisms to those found *in vitro* might apply in intact cell systems described in the present paper.

We have noted the formation of shortened lipid-linked oligosaccharides (Fig. 4*a*); these shortened saccharides are not transferred to protein. Thus proteins are glycosylated with oligosaccharides of normal size when the amount of inhibitor (GDP-deoxyglucose) is decreased relative to that of the physiological sugar donors (Fig. 2*b*). This relative decrease is obtained by adding mannose to the culture medium (cf. Schmidt *et al.*, 1976*a*). On the other hand, under these conditions oligosaccharides linked to lipid are of various sizes (Fig. 4*b*). Apparently, only oligosaccharides of proper size are selected. How strict this selection is, and what relationship exists between the various oligosaccharides, needs to be investigated.

In cells under conditions of deoxyglucose inhibition synthesis of lipid-linked saccharides is decreased. A possible explanation for this is the conversion of GDP-dGlc to Dol-*P*-dGlc. Dolichol phosphate needed for the synthesis of lipid-linked oligosaccharides is then used up. Indeed it is known from studies *in vitro* that the formation of Dol-*P*-dGlc competes with the formation of Dol-*P*-Man (Schwarz *et al.*, 1978), Dol-*P*-*P*-(GlcNAc)₂ and Dol-*P*-Glc (R. Datema & R. T. Schwarz, unpublished work).

Like deoxyglucose the fluoro sugars primarily affect the synthesis of lipid-linked oligosaccharides

(Fig. 6). Also for the fluoro sugars, we were able to correlate the formation of shortened lipid-linked oligosaccharides and the inhibition of glycosylation of protein. During inhibition by fluoro sugars oligosaccharides of decreased molecular weight are formed, and, if the inhibition is reversed by adding mannose, the oligosaccharides formed are shifted to higher molecular weight. We do not yet know whether the inhibiting fluoro sugar derivatives act as inhibitors of the enzymes involved in the assembly of the oligosaccharide, or whether they cause precocious chain termination of the oligosaccharide by incorporation of fluoro sugar residues.

Inhibition by glucosamine occurs not via inhibition of cellular metabolism (Scholtissek, 1975), but, as shown here, by inhibition of the formation of lipid-linked oligosaccharides (Fig. 5). In line with the presumed precursor-product relationship between oligosaccharides linked to lipid and those linked to protein, glycosylation of protein is affected after a certain delay.

Aberrant forms of lipid-linked oligosaccharides are not detected during inhibition by glucosamine. This means that the inhibition occurs early in the assembly of the lipid-linked oligosaccharide or that this synthesis is shut off immediately by some regulatory process. Our studies on the effects of glucosamine on the biosynthesis of lipid-linked oligosaccharides *in vitro* could not discriminate between these possibilities. The synthesis of lipid-linked di-*N*-acetylchitobiose [(GlcNAc)₂], lipid-linked mannose oligosaccharides [(GlcNAc)₂-(Man)_x] and lipid-linked glucose oligosaccharides [(GlcNAc)₂-(Man)_x-(Glc)] were not influenced by glucosamine up to a concentration of 100 mM. [The intracellular concentration of glucosamine at complete inhibition of glycosylation is the same as the extracellular concentration, usually 10 mM; see Koch *et al.* (1979).] So glucosamine functions as an inhibitor only in intact cell systems. The free diffusion of glucosamine across the cell membrane facilitates its use as a reversible inhibitor (Koch *et al.*, 1979). This reversion of the synthesis of lipid-linked oligosaccharides was shown to be rapid and quantitative.

We acknowledge the competent technical assistance of Juliana Winkler and Angelika Treetz. We appreciate the interest and encouragement from Dr. R. Rott and Dr. C. Scholtissek. Thanks are also due to Dr. G. Tamura for a sample of tunicamycin, to Dr. A. B. Foster and Dr. J. H. Westwood for the fluoro sugars, and to Dr. P. W. Robbins for endoglucosaminidase H. The research was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47).

References

- Behrens, N. H. & Tábor, E. (1978) *Methods Enzymol.* **50**, 402-435

- Datema, R. & Schwarz, R. T. (1978) *Eur. J. Biochem.* **90**, 505–516
- Davenport, R. H., Rott, R. & Schäfer, W. (1960) *J. Exp. Med.* **112**, 765–782
- Foster, A. B. & Stacey, M. (1953) *J. Appl. Chem.* **3**, 19–21
- Hemming, F. W. (1974) in *Biochemistry of Lipids* (Goodwin, T. W., ed.), pp. 39–97, Butterworths, London
- Hemming, F. W. (1977) *Biochem. Soc. Trans.* **5**, 1223–1231
- Hubbard, S. C. & Robbins, P. W. (1979) *J. Biol. Chem.* **259**, 4568–4576
- Kaluza, G., Schmidt, M. F. G. & Scholtissek, C. (1973) *Virology* **54**, 179–189
- Klenk, H.-D., Rott, R. & Orlich, M. (1977) *J. Gen. Virol.* **36**, 151–161
- Koch, U., Schwarz, R. T. & Scholtissek, C. (1979) *Eur. J. Biochem.* **94**, 515–522
- Kornfeld, R. & Kornfeld, S. (1976) *Annu. Rev. Biochem.* **45**, 217–237
- Kornfeld, S., Li, E. & Tabas, I. (1978) *J. Biol. Chem.* **253**, 7771–7778
- Krag, S. S. & Robbins, P. W. (1977) *J. Biol. Chem.* **252**, 2621–2629
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lehle, L. & Tanner, W. (1975) *Biochim. Biophys. Acta* **399**, 364–374
- Lehle, L. & Tanner, W. (1976) *FEBS Lett.* **71**, 167–170
- Liu, T., Stetson, B., Turco, S., Hubbard, S. C. & Robbins, P. W. (1979) *J. Biol. Chem.* **254**, 4554–4559
- Montreuil, J. (1975) *Pure Appl. Chem.* **42**, 431–477
- Montreuil, J. & Spik, G. (1968) *Monogr. Lab. Chim. Biol. Fac. Sci. Lille no. 2: Microdosage des Glucides*, 224
- Parodi, A. J. & Leloir, L. F. (1976) *Trends Biochem. Sci.* **1**, 50–59
- Robbins, P. W., Hubbard, S. C., Turco, S. J. & Wirth, D. F. (1977) *Cell* **12**, 893–900
- Schmidt, M. F. G., Schwarz, R. T. & Scholtissek, C. (1974) *Eur. J. Biochem.* **49**, 237–247
- Schmidt, M. F. G., Schwarz, R. T. & Scholtissek, C. (1976a) *Eur. J. Biochem.* **70**, 55–62
- Schmidt, M. F. G., Schwarz, R. T. & Ludwig, H. (1976b) *J. Virol.* **18**, 819–823
- Schmidt, M. F. G., Biely, P., Krátký, Z. & Schwarz, R. T. (1978) *Eur. J. Biochem.* **87**, 55–68
- Scholtissek, C. (1975) *Curr. Top. Microbiol. Immunol.* **70**, 101–119
- Scholtissek, C., Rott, R. & Klenk, H.-D. (1975) *Virology* **63**, 191–200
- Schwarz, R. T. & Klenk, H.-D. (1974) *J. Virol.* **14**, 1023–1034
- Schwarz, R. T., Schmidt, M. F. G., Anwer, U. & Klenk, H.-D. (1977) *J. Virol.* **23**, 217–226
- Schwarz, R. T., Fournet, B., Montreuil, J., Rott, R. & Klenk, H.-D. (1978a) *Arch. Virol.* **56**, 251–255
- Schwarz, R. T., Schmidt, M. F. G. & Lehle, L. (1978b) *Eur. J. Biochem.* **85**, 163–172
- Schwarz, R. T., Schmidt, M. F. G. & Datema, R. (1979) *Biochem. Soc. Trans.* **7**, 322–326
- Spiro, M. J., Spiro, R. & Bhoyroo, V. D. (1976) *J. Biol. Chem.* **251**, 6400–6408
- Sweely, C. C., Bently, R., Nakita, M. & Wells, W. W. (1963) *J. Am. Chem. Soc.* **85**, 2497–2507
- Takatsuki, A., Kohno, K. & Tamura, G. (1975) *Agric. Biol. Chem.* **39**, 2089–2091
- Tarentino, A. L. & Maley, F. (1974) *J. Biol. Chem.* **249**, 811–816
- Tkacz, J. S. & Lampen, J. O. (1975) *Biochem. Biophys. Res. Commun.* **65**, 246–257
- Waechter, C. J. & Lennarz, W. J. (1976) *Annu. Rev. Biochem.* **45**, 95–112
- Wöllert, W. (1975) Ph.D. Thesis, University of Giessen