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Properties of baby-hamster kidney (BHK) cells treated with Swainsonine, an inhibitor of glycoprotein processing

Comparison with ricin-resistant BHK-cell mutants

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Baby-hamster kidney (BHK) cells were grown continuously in long-term monolayer culture in the presence of Swainsonine, an inhibitor of α-mannosidase II, a processing enzyme involved in glycoprotein biosynthesis. The asparagine-linked oligosaccharides (*N*-glycans) were isolated from Pronase-digested cells by gel filtration, ion-exchange chromatography and affinity chromatography on concanavalin A–Sepharose and lentil lectin–Sepharose. The major *N*-glycans, analysed by 500 MHz ^{1}H-n.m.r. spectroscopy, were identified as hybrid structures containing five mannose residues and neutral high-mannose *N*-glycans. The major hybrid species contained a core-substituted fucose α(1→6) residue and a NeuNAcα(2→3)Galβ(1→4)GlcNAc terminal sequence; smaller amounts of non-sialylated and non-fucosylated hybrid structures were also detected. Swainsonine-treated cells also produced neutral oligosaccharides containing a single reducing *N*-acetylglucosamine residue substituted with polymannose sequences. The glycopeptide composition of Swainsonine-treated BHK cells resembles closely that of the ricin-resistant BHK cell mutant, Ric^{R}21 [P. A. Gleeson, J. Feeney and R. C. Hughes (1985) Biochemistry **24**, 493–503], except the hybrid structures of Ric^{R}21 cells contain three, not five, mannose residues. Like Ric^{R}21 cells, Swainsonine-treated BHK cells showed a greatly increased resistance to ricin cytotoxicity, but not to modeccin, another galactose-binding lectin. These effects were readily reversed on removal of Swainsonine and growth in normal medium.

INTRODUCTION

Swainsonine, a trihydroxylated indolizidine alkaloid, is a potent inhibitor of α-D-mannosidase activities (Dorling *et al.*, 1980; Tulsiani *et al.*, 1985). When added to cells in culture, Swainsonine accumulates in lysosomes to produce a deficiency similar to genetic α-mannosidosis (Cenci di Bello *et al.*, 1983). Additionally, Swainsonine is known to induce alterations in the assembly of asparagine-linked oligosaccharides (*N*-glycans) of glycoproteins. The normal conversion of high-mannose *N*-glycans to complex *N*-glycans is prevented and, instead, hybrid structures are formed (Elbein *et al.*, 1981, 1982; Tulsiani *et al.*, 1982, 1985; Arumugham & Tanzer, 1983; Gross *et al.*, 1983). Formation of hybrid structures is consistent with the inhibition by Swainsonine of α-mannosidase II, a Golgi glycosidase involved in processing of *N*-glycans during assembly of fully glycosylated glycoproteins (Tulsiani *et al.*, 1982).

Recent work from our laboratory has shown that hybrid *N*-glycans are present in minor amounts in the glycoproteins of normal BHK cells (Hughes & Mills, 1985), although the predominant processing pathways in these cells lead to conventional complex *N*-glycans. Several ricin-resistant mutants of BHK cells produce exclusively hybrid *N*-glycans after processing (Hughes *et al.*, 1983; Gleeson *et al.*, 1985), the structures of which account satisfactorily (Baenziger & Fiete, 1979) for the inability of ricin to bind to the mutant cell surface and hence the resistance of the cells to the toxin. The relationship between the assembly of hybrid structures, the rounded morphology and the reduced adhesiveness of ricin-resistant BHK cells (Edwards *et al.*, 1976; Pena & Hughes, 1978) is less clear.

In order to relate the altered glycosylation patterns of ricin-resistant BHK cells to these biological properties, we have examined the effects of Swainsonine on normal BHK cells.

EXPERIMENTAL

Materials

Chemicals and biochemicals. Bio-Gel P2 (100–200 mesh) and Chelex 100 (100–200 mesh, Na^+ form) were from Bio-Rad Laboratories. Con A–Sepharose and lentil lectin–Sepharose were from Pharmacia. [^{35}S]Methionine (1.34Ci/mmol), D-[2-^{3}H]mannose (13.4 Ci/mmol), L-[5,6-^{3}H]fucose (64 Ci/mmol) and 2-amino-2-deoxy-D-[1-^{14}C]-glucose hydrochloride (56.8 Ci/mmol) were from Amersham International. Ricin was from Miles Laboratories and modeccin from Pierce. Jack-bean (*Canavalia ensiformis*) glycosidases, prepared as described by Li & Li (1972), were generously provided by Mr. Gary Mills of this Institute. *Vibrio cholerae* neuraminidase (1 i.u./ml) and Pronase CB were from Calbiochem–Behring Corp. and endo-β-*N*-acetylglucosaminidases D and H were from Miles Laboratories. Bovine pancreatic DNAase 1 (1800 Kunitz units/mg) was from Sigma. The $(Man)_5(GlcNAc)_2Asn$ glycopeptide was prepared from ovalbumin and labelled with [1-^{14}C]acetic anhydride as described previously (Gleeson *et al.*, 1985).

Abbreviations used: BHK, baby-hamster kidney; DNAase, deoxyribonuclease; Con A, concanavalin A;
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Swainsonine was generously provided by Dr. P. R. Dorling, Murdoch University, Perth, Western Australia, Australia, and Dr. B. Winchester, Queen Elizabeth College, London, U.K. The alkaloid was dissolved in phosphate-buffered saline (see below) at 100 μg/ml, passed through a 0.22 μm-pore-size Millipore filter and stored at 2 °C. Preliminary experiments showed that jack-bean α-mannosidase-catalysed hydrolysis of *p*-nitrophenyl α-mannoside (Sigma) was inhibited completely by 1 μM-Swainsonine (0.173 μg/ml). Phosphate-buffered saline contained NaCl (8.0 g), KCl (0.29 g), Na_2HPO_4 (1.159 g), KH_2PO_4 (0.29 g), $CaCl_2$ (0.19 g) and $MgCl_2$ (0.19 g) diluted to 1 litre with water (pH 7.04). Buffer A contained 10 mM-Tris/HCl, pH 7.5, 0.1 M-NaCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 1 mM-$MnCl_2$, and 0.02% NaN_3. Buffer B contained 50 mM-Tris/HCl, pH 7.5, 10 mM-$CaCl_2$ and 0.02% NaN_3.

Cells. BHK fibroblasts (BHK21 C13) were grown at 37 °C in monolayer cultures either on standard plasticware or in glass roller bottles (800 cm^2 surface area). In the latter cultures the medium was supplemented with 5 mM-Hepes to maintain a correct pH without CO_2/air gassing. When indicated, the growth medium was supplemented with Swainonine (2–5 μg/ml). No difference in growth rate or plating efficiency was noted when Swainsonine-supplemented medium, rather than normal medium, was used, even over 1–2 months of continuous culture. For subculturing, cell monolayers were trypsin-treated as described by Meager *et al.* (1976), pelleted by centrifugation at 800 *g* for 5 min, resuspended in complete serum-supplemented medium and added at approx. 1:4 dilution in complete medium for re-growth at 37 °C. Cells used for preparation of glycopeptides were harvested by first rinsing the monolayer cultures with phosphate-buffered saline, and then scraping off the cells with a rubber policeman. The cells were pelleted at 800 *g* for 10 min and washed several times with phosphate-buffered saline. Metabolic labelling of cells was carried out by growth of the cells at 37 °C in media supplemented as indicated with [^{3}H]fucose (4 μCi/ml), [^{3}H]mannose (20 μCi/ml) or [^{14}C]glucosamine (5 μCi/ml).

Methods

Pronase digestion. In small-scale experiments, cell pellets were suspended in Buffer B (0.5 ml), heated at 100 °C for 5 min and incubated under toluene at 37 °C with Pronase [50 μl; 0.4% (w/v) in Buffer B]. Fresh aliquots (50 μl each) of Pronase were added after 24 h and 48 h incubation at 37 °C. The mixtures were again heated as described above after 72 h, centrifuged at 10000 *g* for 10 min, and portions of the supernatants were analysed. In the large-scale experiments, the cell pellet (approx. 10^9 cells) was extracted once with 100 ml of chloroform/methanol (2:1, v/v) at room temperature for 2 h to remove glycolipids. The residue, recovered by centrifugation at 8000 *g* for 30 min, was treated with chloroform/methanol a second time and the residue was suspended in Buffer B (60 ml). After heating at 100 °C for 5 min, the solution was incubated at 37 °C with Pronase (30 mg). Additional portions (30 mg) of Pronase were added each day for a total incubation time of 4 days. The mixture was then heated at 100 °C for 5 min, cooled and incubated at 37 °C for 1 h with bovine pancreatic DNAase (0.002 mg/ml) to reduce the viscosity. The mixture was again heated at 100 °C for 5 min and centrifuged at 8000 *g* for 30 min. The residue was washed twice with Buffer B and the supernatants were removed and pooled for preparation of glycopeptides.

Purification of glycopeptides. Portions (40 ml) of the supernatant (80 ml) from Pronase digestion were applied to a column (4 cm × 112 cm) of Sephadex G-25, which was washed with water at room temperature. Fractions (6 ml) were collected and aliquots (0.2 ml) were counted for ^{3}H/^{14}C radioactivity. Fractions S1 and S2 (Fig. 1*a* below) were pooled, freeze-dried and dissolved in water (3 ml). Approx. 85–90% of the total radioactivity was recovered. Fractions S1 and S2 were applied separately to a column (1.4 cm × 36 cm) of Con A–Sepharose equilibrated with Buffer A. Fractions (2 ml)were collected by elution, first with Buffer A, then with 10 mM-α-methyl glucoside, and finally with 200 mM-α-methyl mannoside. The major fractions obtained from Con A–Sepharose chromatography of S1 (Fig. 1*b*) and S2 (Fig.. 1*c*) represented 65–73% of the radioactivity applied. The pooled fractions were freeze-dried, dissolved in water (2 ml) and desalted by passage through a column (2 cm × 27 cm) of Bio-Gel P2. Peak radioactive fractions 15–23 (each 2 ml) eluted from Bio-Gel P2 with water were again freeze-dried, dissolved in water (2 ml) and separated further by ion-exchange chromatography. A column (1 cm × 20 cm) of DEAE-Sephacel was equilibrated with 50 mM-acetic acid at 2 °C. Glycopeptide samples (2 ml) were applied to the column, which was washed first with water (22 ml), followed by a salt gradient made up by running 0.5 M-NaCl in 50 mM-acetic acid (45 ml) into 50 mM-acetic acid (45 ml). Fractions (1.4 ml) were collected and samples (10–25 μl) assayed for ^{3}H/^{14}C radioactivity. Peak fractions were pooled (Figs 1*d* and 1*e*), freeze-dried and dissolved in water (2 ml). The recovery of radioactivity was more than 95%. In the final step of purification, fractions obtained from DEAE-Sephacel were applied separately to a column (0.9 cm × 12 cm) of lentil lectin–Sepharose equilibrated at room temperature in Buffer A. Column fractions (1 ml) were collected by elution with Buffer A followed by 200 mM α-methyl glucoside. Aliquots (10–25 μl) were assayed for radioactivity, and peak fractions (Figs. 1*f*–1*h*) were pooled, freeze-dried and dissolved in water. Each fraction was desalted on Bic-Gel P2 as described above. The recovery of radioactivity from lentil lectin–Sepharose was greater than 88%.

Analytical chromatography. Glycopeptide fractions (usually 0.05–0.10 ml; 1000–15000 c.p.m. of ^{3}H; 200–1000 c.p.m. of ^{14}C)were applied to columns (approx. 2.5 ml bed volume) of Con A–Sepharose. The columns were washed with Buffer A at room temperature and five 1 ml fractions were collected. The columns were then washed with 10 mM-α-methyl glucoside (9 ml) followed by 500 mM-α-methyl mannoside (9 ml). Column fractions (1 ml) were assayed for radioactivity. Analytical chromatography of glycopeptides on lentil lectin–Sepharose columns (approx. 2.5 ml) was performed similarly, except that the columns were washed first with Buffer A (5 ml) followed by 200 mM-α-methyl glucoside (9 ml).

Lectin cytotoxicity. BHK cells grown at 37 °C for at least 3 days in 75 cm^2 plastic flasks in Glasgow-modified minimal essential medium or medium supplemented with Swainsonine (5 μg/ml) were harvested by trypsin

treatment and dispensed into 9.6 cm^2 dishes containing 1 ml of medium with or without Swainsonine. The cells were grown to near confluence over 1–2 days at 37 °C, the growth medium was removed and replaced with either ricin or modeccin (0–20 μg/ml) in Glasgow-modified minimal essential medium (1 ml) with or without Swainsonine (5 μg/ml). After incubation at 37 °C for 2–15 h, the medium was replaced with 1 ml of [^{35}S]methionine (5 μCi/ml) in methionine-depleted Glasgow-modified minimal essential medium. Labelling was carried out at 37 °C for 1 h, after which the monolayers were rinsed four times at 2 °C (2 ml each time) with phosphate-buffered saline, followed by 10% (v/v) $HClO_4$/2% (w/v) phosphotungstic acid and finally ethanol. The residues were dissolved in hot (80 °C) 1M-NaOH and samples (0.05–0.2 ml) were assayed for protein (Bradford, 1976) and radioactivity.

Glycosidase digestion. Treatments with endo-β-*N*-acetylglucosaminidase H or D (10 munits) were carried out in 100 μl of 0.1 M-sodium citrate buffer, pH 5.5, or 0.15 M-sodium phosphate buffer, pH 6.5, respectively, at 37 °C for up to 48 h. The reactions were stopped by boiling for 5 min and the products were fractionated on analytical columns of Con A–Sepharose as described above.

T.l.c. of oligosaccharides. Samples Onof cell oligosaccharides (1620 c.p.m. of [^{14}C]glucosamine) and authentic neutral oligosaccharides (Abraham *et al.*, 1983) isolated from the urine of Swainsonine-treated rats or guinea pigs (1–10 μl; 1 mg/ml) of known structure (Sadeh *et al.*, 1983; Tulsiani & Touster, 1983) were applied to silica-gel plates (F 1500/LS 254, Schleicher and Scheull G.m.b.H., Einbeck, Germany) and chromatographed in propan-1-ol/water (8:3, v/v). Standards were located with orcinol (0.2%, w/v)/H_2SO_4 (5% w/v) in methanol. The radioactive oligosaccharides were located by autoradiography on Fuji X-ray film.

N.m.r. spectroscopy. Glycopeptide samples (30–55 nmol) were prepared for spectroscopy and analysed as described previously (Gleeson *et al.*, 1985), except the samples were freeze-dried between each cycle of exchange with ^2H_2O.

Hexosamine analysis. The hexosamine content of glycopeptides was analysed (Hughes, 1970) after hydrolysis of samples in 4M-HCl at 100 °C for 3 h. The hydrolysates were evaporated to dryness before chromatography on a column (1 cm × 20 cm) of Dowex 50 (X8; 200–400 mesh), equilibrated with 0.1 M-pyridine/acetate buffer, pH 2.8. Fractions (2 ml) were collected during elution first with the same buffer (60 ml), followed by 0.133 M-pyridine/acetate buffer, pH 3.85. Total hexosamines were determined by a modified Elson–Morgan reaction (Hughes, 1970). Reduction of oligosaccharides was carried out with 1% (w/v) $NaBH_4$ in 50 mM-sodium borate buffer, pH 8.9 (75 μl) at 37 °C for 2 h. Excess reagent was destroyed with 1 M-HCl (100 μl).

Other procedures. Liquid-scintillation counting of sample (up to 0.5 ml) radioactivity was carried out with EP (Beckman) scintillant (10 ml).

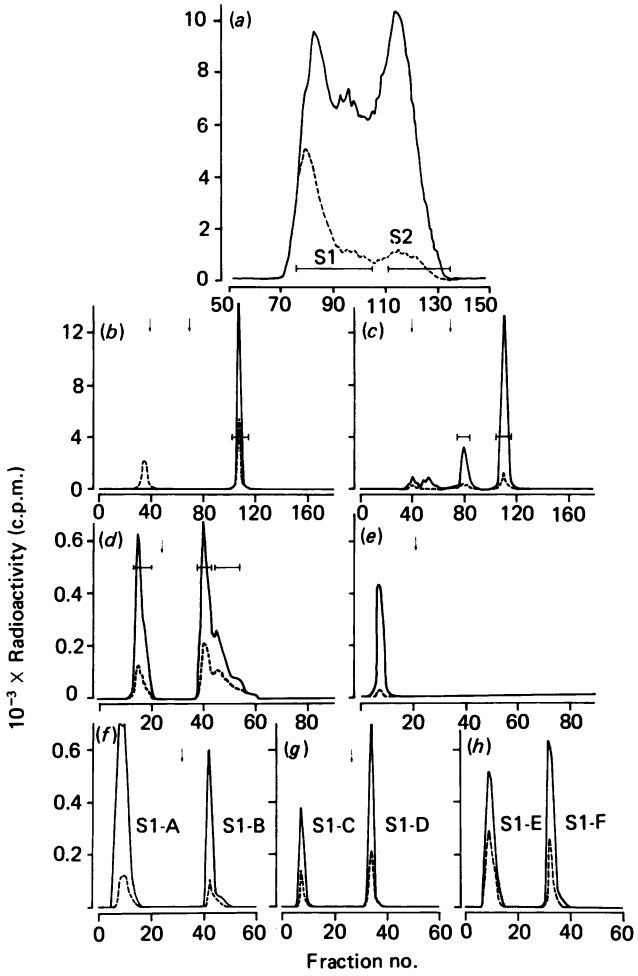


Fig. 1. Fractionation of glycopeptides from Swainsonine-treated BHK cells

(*a*) Glycopeptide samples containing 1.15×10^6 c.p.m. of [^{3}H]mannose and 0.28×10^6 c.p.m. of [^{14}C]glucosamine were applied to a Sephadex G-25 column and eluted with water. (*b*, *c*) Fractions S1 (*b*) or S2 (*c*) were applied to a Con A–Sepharose column and eluted sequentially with buffer followed (arrows) by 10 mM-α-methyl mannoside and 200 mM-α-methyl mannoside. (*d*, *e*) The major pooled fractions from Con A–Sepharose (*b* and *c* respectively) chromatography were separated by ion-exchange chromatography on DEAE-Sephacel. (*f*–*h*) The pooled fractions from (*d*) were separately applied to a lentil lectin–Sepharose column and eluted with buffer followed (arrows) by 200 mM-α-methyl glucoside: (*f*) neutral fraction; (*g*) major acidic fraction; (*h*) minor acidic fraction. ——, ^{3}H; – – – –, ^{14}C.

RESULTS

Glycopeptide fractionation

The [^{3}H]mannose/[^{14}C]glucosamine-labelled glycopeptide mixture obtained from Swainsonine-treated BHK cells was separated into two major fractions by gel filtration (Fig. 1*a*). Fraction S1 was enriched in ^{14}C radioactivity relative to fraction S2. Affinity chromatography of fraction S1 on Con A–Sepharose produced (Fig. 1*b*) an unretarded peak (column fractions 32–38)

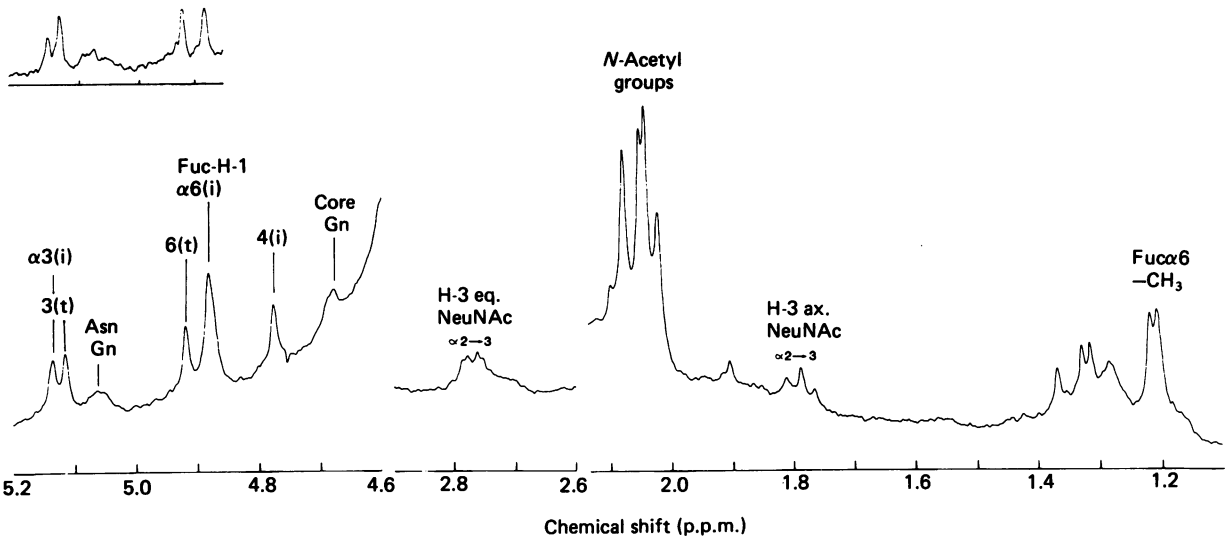


Fig. 2. 500 MHz ^{1}H-n.m.r. spectrum of glycopeptide S1-D and (inset) S1-C at 70 °C

Abbreviations: (i), internal residue; (t), terminal residue; Gn, *N*-acetylglucosamine; eq., equitorial; ax., axial.

Table 1. Chemical shifts of protons derived from 500 MHz n.m.r. spectroscopy at 70 °C: hybrid structures

Abbreviations used: (t), terminal residue; (i), internal residue. Data for Ric^{R}21 glycopeptides are from Gleeson *et al.*, (1985).

Species...	Chemical shift (p.p.m.)			
	Fucosylated		Non-fucosylated	
Protons	Glycopeptide S1-D	Ric^{R}21, hybrid A	Glycopeptide S1-C	Ric^{R}21, hybrid Cl
H-1 of:				
Manα(1→3) (t)	5.113	–	5.118	–
Manα(1→3) (i)	5.134	5.138	5.135	5.135
Manα(1→6) (t)	4.916	4.921	4.918	4.919
Manα(1→6) (i)	4.880	–	4.880	–
Manβ(1→4) (i)	4.775	4.771	4.776	4.772
H-1 of:				
Asn-GlcNAc	5.052	5.057	5.060	5.047
Core GlcNAc	4.680	4.687	4.629	4.629
ArmGlcNAc	4.589	4.599	4.589	4.595
H-1 of Fuc	4.880	4.874	–	–
CH_3 of Fuc	1.202	1.206	1.199*	–
H-3a of NeuNAc	1.784	1.783	1.787	1.785
H-3e of NeuNAc	2.764	2.769	2.772	2.761
N-Acetyl of:†				
Asn-GlcNAc	2.017	2.018	2.018	2.016
Core GlcNAc	2.074	2.085	2.064	2.075
Arm GlcNAc	2.049	2.048	2.048	2.048
NeuNAc	2.038	2.040	2.039	2.040

* Very small signal (< 0.1 integral).

† The 'Asn-GlcNAc' refers to the GlcNAc residue linked to asparagine; 'core GlcNAc' is the residue substituted by a β(1→4)mannose unit; 'arm GlcNAc' is the residue linked β(1→2) to an α(1→3)-linked mannose residue.

containing only ^{14}C radioactivity, presumably representing *O*-glycans, and a peak (column fractions 105–111) eluted with 200 mM-α-methyl mannoside. The fraction of S1 that was absorbed to Con A–Sepharose contained neutral glycopeptides (column fractions 12–20), and a mixture of acidic glycopeptides that was resolved into a major peak (column fractions 28–43) and a more acidic trailing peak (column fractions 44–54) by chromatography on DEAE-Sephacel (Fig. 1*d*). Each peak from ion-exchange chromatography was separated by lentil lectin–Sepharose chromatography (Figs. 1*f*–1*h*) into glycopeptides S1-A, C and E (without affinity for lentil lectin) and glycopeptides S1-B, D and F (eluted with 200 mM-α-methyl glucoside). The recoveries of [^{3}H]-

mannose in the purified glycopeptide fractions were (in ratio): S1-A, 1.00; S1-B, 0.054; S1-C, 0.092; S1-D, 0.229; S1-E, 0.061; S1-F, 0.037.

Fraction S2 from Sephadex G-25 (Fig. 1*a*) gave several peaks when subjected to chromatography on Con A–Sepharose (Fig. 1*c*). The major peak, S2-D, representing 72% of total radioactivity, was eluted with 200 mM-methyl α-mannoside and minor peaks (S2-A, S2-B and S2-C) with weak affinity for Con A were eluted with buffer or 10 mM-α-methyl glucoside. Fractions S2-C and S2-D contained only neutral glycopeptides, as shown by DEAE-Sephacel chromatography (Fig. 1*e*), and were eluted unretarded from lentil lectin–Sepharose (results not shown).

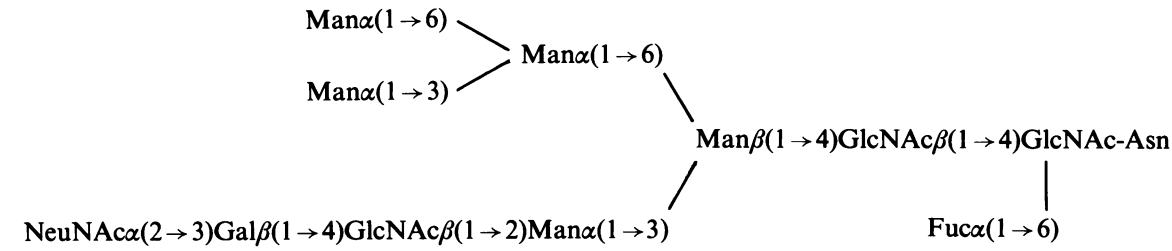
Structures of S1 glycopeptides

The n.m.r. spectra of the major acidic glycopeptides S1-C and D (Fig. 2) are explicable in terms of a closely similar structure for the two compounds: glycopeptide S1-D differs from glycopeptide S1-C in containing a fucose α(1→6) unit substituted to the asparagine-linked *N*-acetylglucosamine residue of the core sequence, in agreement (Kornfeld *et al.*, 1981) with the separation of these glycopeptides on lentil lectin–Sepharose (Fig. 1*g*). Assignments of the resonances throughout this analysis are based on the published data of Carver *et al.* (1981), Grey *et al.* (1982), Allen *et al.* (1984) and Gleeson *et al.* (1985).

The major anomeric hydrogen signals and their assignments are shown in Table 1. Two major differences between the spectra of glycopeptides S1-C and D were observed: the ratio of intensities of the signals at 4.880 p.p.m. relative to those at 4.916 p.p.m. were 1:1 for S1-C and > 2:1 for S1-D (Fig. 2). The additional signal at 4.880 p.p.m. in glycopeptide S1-D may be assigned to the anomeric proton of Fucα(1→6). This assignment is supported further by the CH_3 fucose signal at 1.202 p.p.m. in S1-D, present only in trace amount in S1-C. Secondly, the resonances assigned to the core GlcNAc in glycopeptides S1-D (4.680 p.p.m.) and S1-C (4.629 p.p.m.) are typical for fucosylated and non-fucosylated species respectively. The other anomeric resonances consist of: a GlcNAcβ(1→2)-substituted Manα(1→3) residue (5.134–5.135 p.p.m.), the substitution being identified by a downfield shift (+0.014) compared with an unsubstituted residue, a terminal Manα(1→3) residue (5.113–5.118 p.p.m.), a broad peak due to asparagine-linked GlcNAc residue at 5.052–5.060 p.p.m., a terminal Manα(1→6) residue at 4.916–4.918 p.p.m., an internal Manα(1→6) residue at 4.880 p.p.m. and an internal Manβ(1→4) residue at 4.775–4.776 p.p.m. In glycopeptide S1-D the ratios of these intensities were integral, indicating the presence of a single major component containing five mannose residues. An H-1 signal at 4.589 p.p.m. supports the presence of a GlcNAc in β(1→2) linkage to the Manα(1→3) residue of the core sequence. The chemical shifts of the NeuNAc H-3 signals (H-3a at 1.784–1.787 and H-3e at 2.764–2.772 p.p.m.) are characteristic of a NeuNAcα(2→3)Gal sequence. No signals were present at 2.669 and 1.718 p.p.m., indicating the absence of a NeuNAcα(2→6)Gal sequence.

The chemical shifts of the *N*-acetyl groups show signals for Asn-linked GlcNAc and core GlcNAc at 2.017–2.018 and 2.074 (S1-D) or 2.064 (S1-C) p.p.m. respectively. The upfield shift (+0.011 p.p.m.) for the core GlcNAc *N*-acetyl group signal of glycopeptide S1-D compared with the Man_3 hybrid A of Ric^R21 cells (Table 1) is consistent with a high-mannose structure in the former glycopeptide. The additional upfield shift (+0.010 p.p.m.) for the core GlcNAc *N*-acetyl group of glycopeptide S1-C is expected for a non-fucosylated species. The remaining signals in this region are S1-D and S1-C *N*-acetyl groups of an antennary (arm) GlcNAc at 2.049 p.p.m. and NeuNAc at 2.038 p.p.m.

Therefore the structure of glycopeptide S1-D is a sialylated fucosylated Man_5 hybrid:



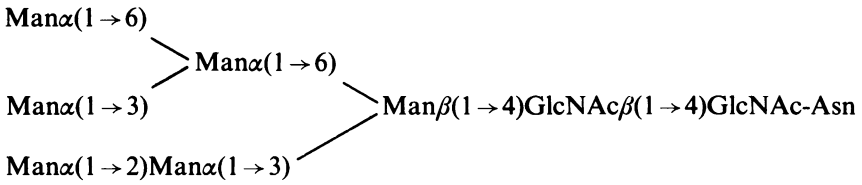
and the major component of S1-C is the non-fucosylated derivative.

The n.m.r. spectrum shows peaks from 1.282 to 1.366 p.p.m., which may be assigned to aliphatic signals of amino acids in the peptide moiety.

Insufficient amounts of glycopeptides S1-B, E and F were obtained for n.m.r. analysis. Glycopeptide S1-B is a neutral component that shows affinity for lentil lectin. The $^3H/^{14}C$ ratio (4.67:1) for this glycopeptide was intermediate between high-mannose glycopeptide S1-A (5.80:1) and the sialylated hybrid glycopeptides S1-C (2.72:1) and S1-D (2.98:1). Assuming approximately equal labelling of *N*-acetylneuraminic acid and *N*-acetylglucosamine by growth of cells in [^{14}C]glucosamine, these results are consistent with S1-B being a non-sialylated core fucosylated hybrid structure containing five mannose residues. Glycopeptides S1-E and S1-F are more acidic than the monosialylated hybrid glycopeptides S1-C and S1-D, as shown by DEAE-Sephacel chromatography (Fig. 1*d*) and also contain more glucosamine label ($^{14}C/^3H$ ratios: S1-E, 1.61:1; S1-F, 2.23:1). Since glycopeptide S1-E was converted into a neutral species by neuraminidase treatment (results not shown), the increased acidity may be explained by additional sialylation. As one possibility, glycopeptide S1-E may represent a branched hybrid structure containing two NeuNAc-Gal-GlcNAc sequences substituted β(1→2) and β(1→4) to the α(1→3)-linked mannose unit of the core sequence. Similar branched hybrid structures have been detected in the glycoproteins of ricin-resistant Ric^R21 cells (Gleeson *et al.*, 1985). Unlike the latter glycopeptides, however, the proposed branched hybrid glycopeptide retains affinity for Con A and hence contains at least three interacting mannose residues. This structural feature is

consistent with a branched hybrid composition containing a total of five mannose units.

Glycopeptide S1-A is the fraction of Con A-binding glycopeptides that did not bind to either DEAE-Sephacel or lentil lectin (Fig. 1*f*). The absence of fucose and sialic acid was confirmed by n.m.r. analysis, which showed no resonance peaks at 1.202 p.p.m. (fucose) or 2.764 p.p.m. and 1.784 p.p.m. (sialic acid). The fraction would be expected to contain high-mannose glycopeptides and any non-sialylated–non-fucosylated hybrids. Small amounts of the latter were detected (less than about 10%) by minor signals at 5.133 p.p.m., indicative of a Manα(1→3) residue substituted by GlcNAc in β(1→2)-linkage and at 2.049 p.p.m., indicative of an arm GlcNAc residue. The major signals were identical with the analogous neutral glycopeptide fraction isolated from Ric^{R}21 cells (Gleeson *et al.*, 1985), identified as a Man_6 structure:



Structure of S2 components

The major components S2-C and S2-D of this fraction consist of neutral compounds that bind to Con A (Fig. 1*c*), but not to lentil lectin, and contain less [^{14}C]-glucosamine label than the glycopeptides of Sephadex G-25 fraction S1 (^{3}H/^{14}C ratios: S2-C, 7.40:1; S2-D, 6.86:1; S1-A, 5.80:1). The glucosamine is present exclusively at the reducing end of the oligosaccharides (Fig. 3). After reduction of S2-D with $NaBH_4$ followed by acid hydrolysis, all of the ^{14}C radioactivity was eluted from a Dowex-50 column in the position (Hughes, 1970) of glucosaminitol. Analysis of fractions S2-C and S2-D by t.l.c. showed (Fig. 4) that the predominant oligosaccharide migrated with a $Man_5GlcNAc$ standard with minor amounts migrating with $Man_4GlcNAc$ and in S2-D, $Man_3GlcNAc$. The 500 MHz ^{1}H-n.m.r. spectra at 70 °C showed a complex pattern in the anomeric region in agreement with the presence of several oligomannosidic components as identified by t.l.c. However, the *N*-acetyl region showed one abundant singlet at 2.056 p.p.m. confirming (Mutsaers *et al.*, 1985) the presence of a single reducing GlcNAc residue in these components.

Labelling of cells with [^{3}H]fucose

The alteration in protein *N*-glycosylation in Swainsonine-treated BHK cells was confirmed by analysis of the glycopeptides obtained from cells labelled by growth in the presence of [^{3}H]fucose. BHK cells were incubated in medium containing Swainsonine (5 μg/ml) for 5 h, then [^{3}H]fucose was added for 24 h at 37 °C. The glycopeptides obtained by Pronase digestion of disrupted cells were analysed by chromatography on Con A–Sepharose (Fig. 5). The predominant glycopeptide species labelled in the control cells were present in fractions eluted unretarded from the column (65% of total) or eluted with 10 mM-α-methyl glucoside (29% of total). Previous work (Hughes & Mills, 1983) has established these structures as respectively, tri- and tetra-antennary complex *N*-glycans and bi-antennary complex *N*-glycans. In contrast, about two-thirds of the radioactive material obtained from Swainsonine-treated cells was bound tightly to Con A–Sepharose and was eluted with 500 mM-α-methyl mannoside. Approx. 90% of this material was bound to a lentil lectin–Sepharose

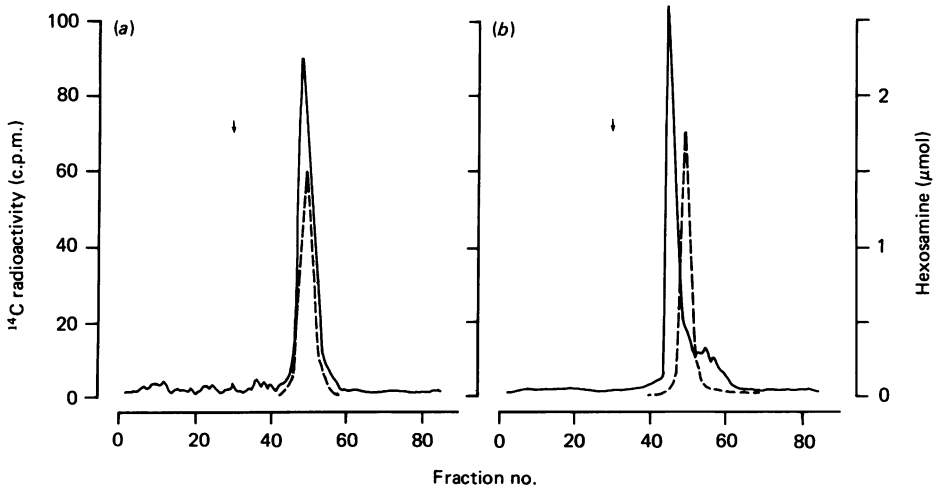


Fig. 3. Reduction of oligosaccharide fractions S2-D with $NaBH_4$

Samples containing 1620 c.p.m. of [^{14}C]glucosamine and 11126 c.p.m. of [^{3}H]mannose were hydrolysed before (*a*) and after (*b*) treatment with $NaBH_4$. The hydrosylates were mixed with unlabelled glucosamine (4 μmol) and chromatographed on Dowex-50 resin with 0.1 M-pyridine acetate, pH 2.8, followed (arrows) by 0.133 M-pyridine/acetate, pH 3.3. Column fractions were analysed for ^{14}C radioactivity —— and hexosamine (----). ^{3}H radioactivity was eluted between fractions 5 and 10.

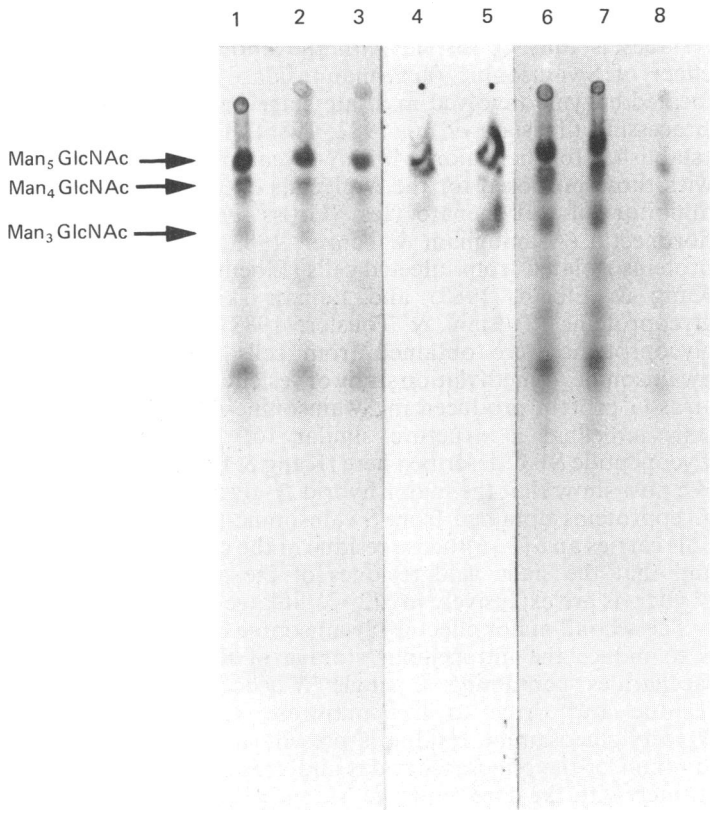


Fig. 4. T.l.c. of oligosaccharides S2-C and S2-D

Tracks 1–3, urine oligosaccharides from Swainsonine-treated guinea pigs (5, 3 and 1 μl respectively); track 4, fraction S2-C; track 5, fraction S2-D; tracks 6–8, urine oligosaccharides from Swainsonine-treated rats (5, 3 and 1 μl respectively). Tracks 1–3 and 6–8 were developed with orcinol reagent; tracks 4 and 5 were exposed for radioactivity. The migration of known standard oligosaccharides ($Man_nGlcNAc$) is indicated by the arrows.

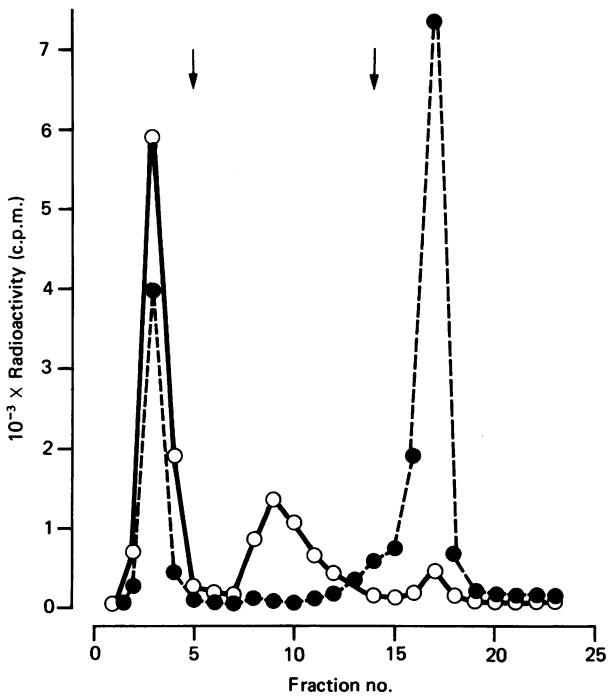


Fig. 5. Con A–Sepharose chromatography of [^{3}H]fucose-labelled glycopeptides from untreated (○, 14000 c.p.m.) or Swainsonine-treated (●, 17200 c.p.m.) BHK cells

Samples (50 μl) were applied and eluted with buffer (5 ml), followed (arrows) by 10 mM-α-methyl glucoside (9 ml) and 500 mM-α-methyl mannoside (9 ml). The fraction size was 1 ml.

column (2 ml) and eluted with 200 mM-α-methyl glucoside in agreement with the structures deduced by n.m.r. spectroscopy for the major hybrid glycopeptides.

Endo-*N*-acetylglucosaminidase H cleaved the hybrid glycopeptides of Swainsonine-treated BHK cells to produce a [^{3}H]fucose-labelled fragment, presumably Fucα(1→6)GlcNAc-Asn, without affinity for Con A–Sepharose (Fig. 6). The rate of hydrolysis of this hybrid glycopeptide fraction by endo-*N*-acetylglucosaminidase H was similar to that of ovalbumin glycopeptide $Man_5(GlcNAc)_2$-Asn and considerably faster than the hydrolysis of the [^{3}H]fucose-labelled hybrid glycopeptide obtained from Ric^R21 cells (Fig. 6). The latter glycopeptide contains only three mannose residues (Gleeson *et al.*, 1985) in contrast with the five mannose residues present in the hybrid structures from Swainsonine-treated BHK cells. Other work has shown that, compared with $Man_5(GlcNAc)_2Asn$, the rate of hydrolysis of $Man_3(GlcNAc)_2$-Asn by endo-*N*-acetylglucosaminidase H is slower by four orders of magnitude (Tarentino & Maley, 1975; Tai *et al.*, 1977). Our results show that core fucosylation of a high-mannose hybrid glycopeptide apparently does not significantly inhibit hydrolysis by endo-*N*-acetylglucosaminidase H, since the ovalbumin glycopeptide lacks fucose.

Endo-β-*N*-acetylglucosaminidase D failed to hydrolyse the [^{3}H]fucose-labelled glycopeptide fraction (results not shown), in agreement with the substitution of the Manα(1→3) residue of the core sequence (Gleeson *et al.*, 1985).

Effects of toxic lectins on Swainsonine-treated cells

BHK cells grown continuously in the presence of Swainsonine (5 μg/ml) were tested for sensitivity to the toxic effects of ricin and modeccin, two galactose-binding lectins, on cellular protein synthesis. As Fig. 7 shows, the treated cells exhibited a striking increase in ricin resistance, whereas Swainsonine treatment had no protective effect against the cytotoxicity of modeccin (Fig. 7). The protective effect of Swainsonine against ricin cytotoxicity was readily reversed by removal of the drug from the cells for 1–2 days (Fig. 8). By contrast, the protective effect required prolonged incubation of the cells with Swainsonine; little or no protection was afforded by exposure of the cells to the drug over 24–30 h (Fig. 8). Presumably these results indicate that complete substitution of a normal complement of cell-surface carbohydrate receptors for ricin by non-binding carbohydrate chains is relatively slow and that a small amount of appropriate ricin receptors at the cell surface is sufficient to mediate the cytotoxic response.

DISCUSSION

The findings described here show that Swainsonine has two separate effects on glycoprotein metabolism in BHK cells. The complete inhibition of formation of normal, branched complex *N*-glycans and the induction of

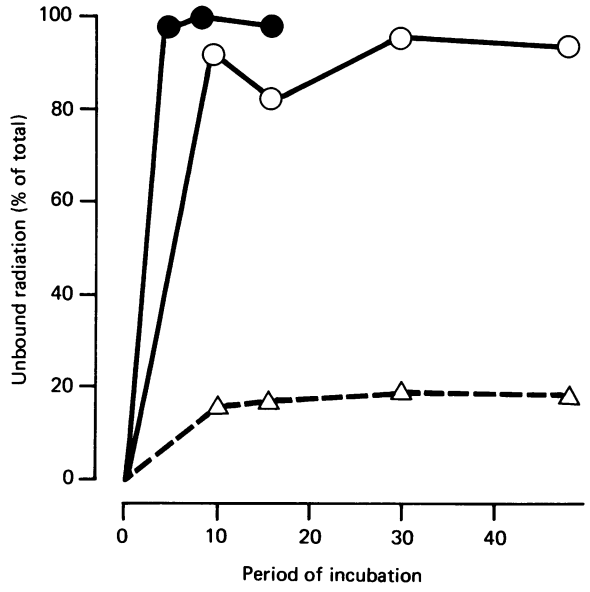


Fig. 6. Hydrolysis by endo-β-acetylglucosaminidase H of [^{3}H]-fucose-labelled hybrid glycopeptides of Swainsonine-treated BHK cells (○) and Ric^{R}21 cells (△)

The hybrid glycopeptides were isolated by Con A–Sepharose chromatography as shown in Fig. 5. The hydrolysis of ovalbumin glycopeptide $Man_5(GlcNAc)_2Asn$ is also shown (●). Samples (0.1 ml, 2000 c.p.m.) were treated with endo-β-acetylglucosaminidase H in 0.1 M-sodium citrate buffer, pH 5.5 at 37 °C for the times indicated. The digestion products were separated by analytical chromatography on Con A–Sepharose and the extent of hydrolysis was measured by the proportion of radioactivity eluted with buffer.

synthesis of hybrid *N*-glycans containing five mannose residues is fully consistent with the known inhibitory effect of Swainsonine on α-mannosidase II, a Golgi-located enzyme involved in a late stage in glycoprotein processing (Tulsiani *et al.*, 1982, 1985). The structures established for the major hybrid *N*-glycans are consistent with those proposed for the *N*-glycans of α_1-antitrypsin inhibitor of rat hepatocytes (Gross *et al.*, 1983), fibronectin (Arumugham & Tanzer, 1983), viral glycoproteins isolated from infected cells (Elbein *et al.*, 1982; Kang & Elbein, 1983) and human skin fibroblast glycoproteins (Tulsiani & Touster, 1983) when these glycoproteins were obtained from cells treated with Swainsonine. A methylation study of vesicular-stomatitis-virus-G protein produced in Swainsonine-treated BHK cells indicated a structure similar to the sialylated glycopeptide S1-C described here (Kang & Elbein, 1983). We now show that the major hybrid *N*-glycan species of glycoproteins obtained from Swainsonine-treated BHK cells carries an α(1→6)fucose residue in the core sequence and that the sialic acid residues of the acidic hybrid *N*-glycans are exclusively in α(2→3)-linkage to galactose.

The second major effect of Swainsonine on BHK cells is to induce the intracellular storage of neutral oligosaccharides containing a single *N*-acetylglucosamine residue and three to five mannose residues. The *N*-acetylglucosamine residue is present at the reducing terminus of the oligosaccharides and presumably is substituted with the core sequence Manα(→3)[Manα(1→6)]-Manβ(1→4), since the glycopeptides retain affinity for Con A. Accumulation of these oligosaccharides can be attributed to the effect of Swainsonine, since they were not detected during analysis (Gleeson *et al.*, 1985) of the *N*-glycans of mutant (Ric^{R}21) BHK cells.

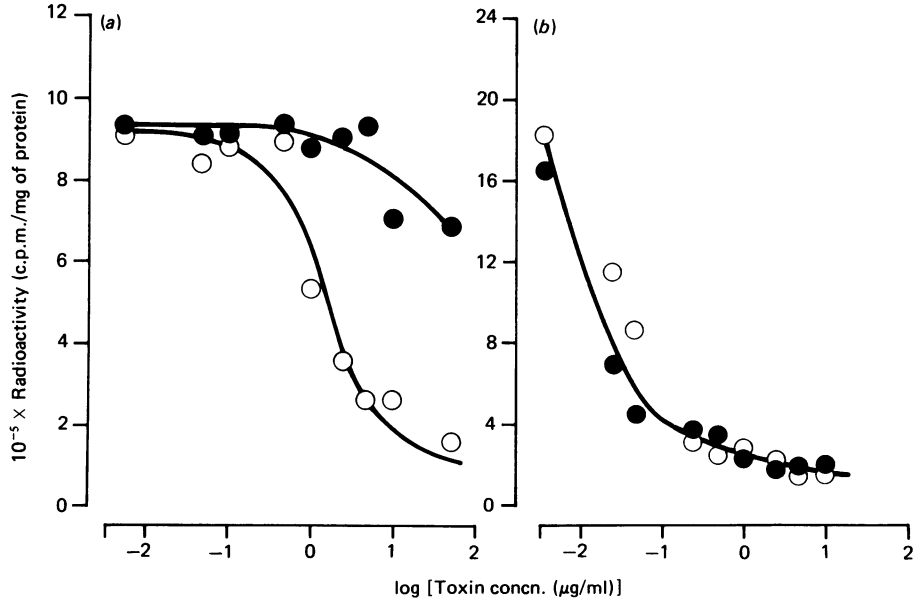


Fig. 7. Effect of ricin (*a*) or modeccin (*b*) on protein synthesis on BHK cells (○) or Swainsonine-treated BHK cells (●)

Monolayer cultures were treated at 37 °C for 1 h with toxins at various concentrations and the incorporation of [^{35}S]methionine into acid precipitable protein was measured.

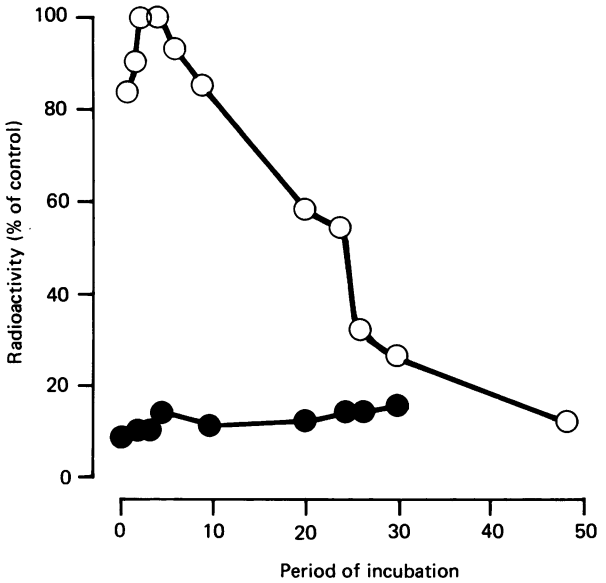


Fig. 8. Time course and reversal of Swainsonine-induced effects

○, BHK cells grown in the presence of Swainsonine for 3 days were rinsed with medium lacking Swainsonine and incubated at 37 °C for the times shown before addition of ricin (10 μg/ml). ●, BHK cells were incubated with Swainsonine (5 μg/ml) at 37 °C for the times shown before the addition of ricin. After further incubation at 37° for 1 h, the extent of incorporation of [^{35}S]methionine into acid-precipitable protein was measured.

Previous work (Cenci di Bello *et al.*, 1983; Sadeh *et al.*, 1983; Warren *et al.*, 1983) has indicated the presence of neutral oligosaccharides in Swainsonine-treated human fibroblasts that may be structurally similar to those found in our study. The origin of the neutral oligosaccharides found in Swainsonine-treated BHK cells is not known, but could be due to the known inhibition of lysosomal α-mannosidase by Swainsonine and hence incomplete catabolism of cellular glycoproteins. An endo-β-*N*-acetylglucosaminidase has been detected in cell extracts that presumably accounts for the cleavage of *N*-glycans from protein moieties and the exposure of *N*-acetylglucosamine reducing groups in the neutral oligosaccharides (Overdijk *et al.*, 1981). Since we find small amounts of neutral oligosaccharides containing three and four mannose residues in Swainsonine-treated cells, some residual α-mannosidase activity must be present in these cells, although concentrations of Swainsonine similar to those used in our study inhibit completely *in vitro* both α-mannosidase II and lysosomal α-mannosidase (Dorling *et al.*, 1980; Tulsiani *et al.*, 1985).

The hybrid *N*-glycans of Ric^{R}21 cells (Gleeson *et al.*, 1985) differ from the major hybrid species identified in Swainsonine-treated BHK cells only in the absence of two terminal mannose residues substituted on to the Manα(1→6) branch of the core sequence. The increased resistance of Swainsonine-treated BHK cells to ricin supports other reports (Elbein *et al.*, 1983) and indicates, in agreement with previous work (Gleeson *et al.*, 1985), that a 3-*O*-substituted galactose residue binds ricin poorly. Previously, we have shown that ricin- or modeccin-resistant mutants of BHK cells retain sensitivity to the non-selective lectin (Sargiacomo & Hughes, 1982; Gleeson & Hughes, 1985) in agreement with the present data. Evidently, ricin and modeccin bind to distinctly different carbohydrate sequences and only the ricin-binding receptors are altered in the mutants or during Swainsonine treatment.

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