Biological Activities of Isolated Tunicamycin and Streptovirudin Fractions†

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ABSTRACT: The nucleoside antibiotics tunicamycin and streptovirudin were separated by high-performance liquid chromatography into a series of 256-nm-absorbing peaks. Most of the streptovirudin peaks eluted from a Biosil ODS column earlier than those of tunicamycin, indicating that they were less hydrophobic. With the exception of the first peak, 17 other tunicamycin peaks were potent inhibitors of the formation of dolichylpyrophosphoryl-*N*-acetylglucosamine with 50% inhibition of the solubilized GlcNAc-1-P transferase requiring about 10 ng of antibiotic per mL. These fractions also inhibited the synthesis of dolichylphosphorylglucose, but in these cases about 500 ng/mL was necessary to achieve 50% inhibition. In MDCK cells in culture, the four major tunicamycin peaks inhibited the incorporation of [2-^{3}H]mannose into protein by 50% at about 0.2–0.5 μg/mL, but [^{3}H]leucine incorporation into protein was unaffected, except at high levels of antibiotic (5–10 μg/mL). Essentially the same results were observed with the streptovirudin fractions except that they were somewhat less active and some inhibition of protein synthesis was observed with several of these peaks.

Tunicamycin has been widely used in cell physiology, cell biology, and biochemistry as a tool to probe the role of carbohydrates in glycoprotein functions. Tunicamycin (Takatuski et al., 1971) and streptovirudin (Eckardt et al., 1975) are both streptomycete antibiotics that have been shown to be potent inhibitors of glycosylation in those proteins that have a GlcNAc-asparagine-linked oligosaccharide. Thus, both antibiotics block the formation of dolichylpyrophosphoryl-GlcNac by specifically inhibiting the first enzyme in the dolichol pathway, the UDP-GlcNAc-dolichyl-P:GlcNAc-1-P transferase (Tkacz & Lampen, 1975; Struck & Lennarz, 1977; Waechter & Harford, 1977; Ericson et al., 1977; Lehle & Tanner, 1976; Heifetz et al., 1979). These antibiotics also inhibit the transfer of glucose from UDP-glucose to dolichylphosphate, but this inhibition requires about 100-fold higher concentrations of antibiotic than does inhibition of GlcNAc-1-P transfer (Elbein et al., 1979).

The structure of tunicamycin has recently been elucidated (Takatsuki et al., 1979). It was shown to be a nucleoside antibiotic containing the base uracil to which an 11-carbon sugar named tunicamine is attached in an N-glycosidic linkage. Attached to the tunicamine is a fatty acid, which may vary in chain length and arrangement, and an *N*-acetylglucosamine. Tunicamycin is produced as a family of closely related compounds that differ in the nature of the fatty acid. These compounds have been separated by high-performance liquid chromatography (HPLC) (Ito et al., 1980). The streptovirudins are composed ot two series of compounds; series I streptovirudins contain glucosamine but lack uracil, while series II components have both glucosamine and uracil (Eckardt et al., 1980). These workers reported that the streptovirudin series II components were separated by HPLC into four components, of which three were identical with the tunicamycin components. Although the exact relationship of series II streptovirudins to tunicamycin is not clear, the fact that they both have the same mechanism of action and are equally susceptible to periodate oxidation suggests that they are structurally related (Elbein et al., 1979).

The separation of tunicamycin into discrete components by high-performance liquid chromatography was recently reported (Mahoney & Duskin, 1979; Ito et al., 1980). Mahoney and Duskin found that tunicamycin separated into two major and eight minor peaks, all of which inhibited protein glycosylation. However, one of the major components still inhibited protein synthesis while the other had a negligible effect. In this paper, we describe the separation of tunicamycin into 15 or more peaks and of streptovirudin into four major fractions. Almost all of these individual fractions were potent inhibitors, in vitro, of the formation of dolichylpyrophosphoryl-GlcNAc, and they also inhibited the formation of dolichylphosphorylglucose. However, in contrast to the studies of Mahoney and Duskin, five of the major tunicamycin fractions showed little or no inhibitory activity on [^{3}H]leucine incorporation into protein in MDCK cells at levels where [^{3}H]mannose incorporation was inhibited more than 50%. However, at high levels of these fractions, some inhibition of leucine incorporation may occur. Also in the case of streptovirudin, several of the fractions did show some inhibition of protein synthesis, but this required much higher levels of antibiotic than necessary to inhibit protein glycosylation. Other workers have postulated a regulatory link between the glycosylation of the protein moiety and its biosynthesis (Hasilik & Tanner, 1978; Schwaiger & Tanner, 1979).

Materials and Methods

Materials. UDP-[^{3}H]GlcNAc (6.6 Ci/mmol), UDP-[^{3}H]glucose (5 Ci/mmol), and [^{3}H]leucine (110 Ci/mmol) were obtained from New England Nuclear. 2-[^{3}H]Mannose (20 Ci/mmol) was purchased from Amersham. Dolichylphosphate, unlabeled sugar nucleotides, DEAE-cellulose, and other biochemicals were from Sigma. Tunicamycin was from Eli Lilly, and streptovirudin was from Dr. K. Eckardt, Zentralinstitut fur Mikrobiologie and Experimentalle Therapie, Jena, East Germany.

Separation of Tunicamycin and Streptovirudin by HPLC. A Waters Model 6000A instrument equipped with a 256-nm detector was employed for the HPLC separations. A Biosil ODS-10 column, 4 × 250 mm, was eluted at a flow rate of 1 mL/min, using either solvent A (methanol–25 mM sodium

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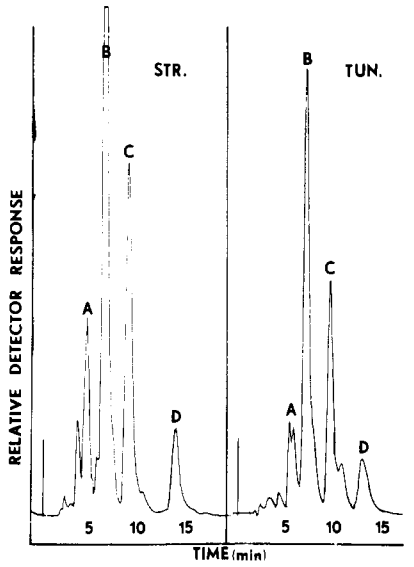

FIGURE 1: HPLC separation of tunicamycin and streptovirudin. Conditions were as described in the text using solvent B for tunicamycin and a solvent A for streptovirudin. Letters indicate the peaks collected.

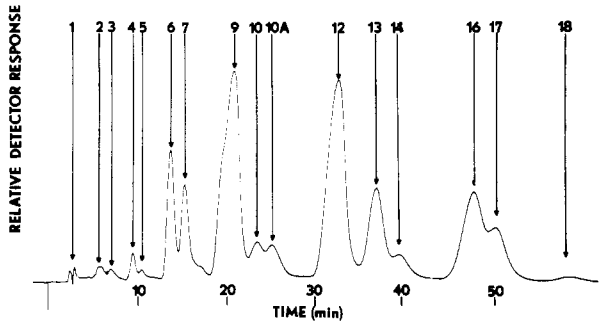

FIGURE 2: HPLC separation of tunicamycin. Conditions were as in Figure 1 except solvent A was used. Numbers and letters refer to the fractions collected.

acetate buffer, pH 5.0, 7:3 v/v) for streptovirudin and tunicamycin, solvent B (methanol–25 mM sodium acetate buffer, pH 5.0, 4:1 v/v) for tunicamycin, or solvent C (methanol–water, 3:1 v/v) for streptovirudin and tunicamycin. Fractions were collected at the peaks shown in Figures 1 and 2, concentrated to dryness, resuspended in 0.01 N NaOH, and diluted to the appropriate concentrations in 0.001 N NaOH. The quantities of materials in each peak were determined from their adsorption at 260 nm by using an extinction coefficient of 9650 and assuming a molecular weight of 870 (Takatsuki et al., 1971).

Mass Spectrometry and Nuclear Magnetic Resonance Determinations. Field desorption spectra were determined on a Varian-MAT731 mass spectrometer using carbon dendrite emitters grown on 10 μM tungsten wires. The tunicamycins and streptovirudins field desorbed at emitter currents of approximately 18 mA at an ion source temperature of 120 °C. The ^1H NMR spectra were obtained on a Varian HA100 instrument with the samples in pyridine-d_6 at 7–10 mg/mL.

Preparation and Assay of Enzyme Fractions. The intimal layer from fresh pig aorta was homogenized and a particulate enzyme preparation was isolated as previously described (Chambers & Elbein, 1975). The particulate enzyme was solubilized by mixing it with Nonidet P-40 (NP-40), to a final concentration of 0.5%, and allowing the mixture to stir in an ice bath for several minutes (Heifetz & Elbein, 1977). The mixture was centrifuged at 100000*g* for 45 min and the supernatant liquid was used as the enzyme source.

Assay mixtures with the particulate enzyme contained the following components in a final volume of 0.5 mL: Tris buffer, pH 7.5, 10 μmol, $MnCl_2$, 2.5 μmol, various concentrations of tunicamycin or streptovirudin as indicated in the figures and tables, 100000 cpm of either UDP-[^3H]GlcNAc or UDP-[^3H]glucose, and 100 μL of particulate enzyme (1–2 mg of protein). In some experiments, enzyme and antibiotic were allowed to preincubate for 1–2 min before the addition of the radioactive sugar nucleotides, but since this did not change the results to any extent, preincubation was usually omitted. For assay with the solubilized enzyme, it was necessary to add dolichylphosphate as a glycosyl acceptor. In these experiments, 5 μg of dolichylphosphate, usually dissolved in 20 μL of $CHCl_3$, was added first to the assay tubes and the solvent was removed under a stream of air. One hundred microliters of 1% NP-40 was added to each tube to suspend the lipid, and the tubes were mixed vigorously. The other reaction components were then added (Tris buffer, $MnCl_2$, antibiotic, and sugar nucleotide) in the same order with thorough mixing between each addition. The reactions were initiated by the addition of 100 μL of soluble enzyme (0.5–1 mg of protein).

The incubations were done at 37 °C for varying periods of time, but usually 10 min was used. Reactions were stopped by the addition of 2 mL of $CHCl_3$–CH_3OH and 0.5 mL of water. After thorough mixing, the phases were separated by centrifugation and dolichylpyrophosphoryl-GlcNAc and dolichylphosphorylglucose were isolated by the extraction method described previously (Chambers & Elbein, 1975; Heifetz & Elbein, 1977). The radioactive content of these lipids was determined by scintillation counting as a measure of their synthesis. Various controls were done in which tunicamycin or streptovirudin were added at the end of the incubation to be certain that they did not affect the extraction of the various lipids. Other controls were also done in which various amounts of 0.001 N NaOH were added to be certain that alkali did not affect the enzymatic activity. These controls were all similar to incubations done in the absence of antibiotic.

Growth and Assay of MDCK Cells. A stable canine kidney cell line (MDCK) was used to study the in vivo effect of these antibiotics. The cells were maintained on Eagle's basal medium supplemented with 10% fetal calf serum, 0.03% glutamine, and 20 μg/mL neomycin. Forty-eight hours before the start of an experiment, the cells were plated into six-well Limbro dishes in the above medium and incubated at 34 °C in air containing 5% CO_2. When the cells had reached confluency (usually 48 h), each well contained about 5×10^6 cells. Cell viability was determined by the Trypan Blue dye exclusion method. The confluent cells were infected with the NWS strain of influenza virus so that the effect of antibiotic on the synthesis of the viral coat glycoproteins could be measured (Pan et al., 1979).

After allowing 1 h for virus penetration to occur, various concentrations of the antibiotics were added and the cells were allowed to incubate at 34 °C for 1 h. Then 5 μCi of [^3H]-mannose or 5 μCi of [^3H]leucine was added to each culture and the cells were allowed to incubate with the radioactive precursors for 3 h. At the end of this time, the media was removed by aspiration and the cell monolayers were washed 3–4 times with phosphate-buffered saline solution. The cell monolayers were removed from the plates by incubation with a trypsin–EDTA solution, and the dislodged monolayers were placed in tubes. Two milliliters of $CHCl_3$–CH_3OH (1:1) was added to each tube along with 0.1 mL of aorta particulate enzyme to aid in the extraction procedure. After thorough mixing, lipid-linked monosaccharides and lipid-linked oligo-

Table I: Identity of Tunicamycin Fractions

fraction	factor designation	M_r[a] (FDMS)	fatty acid type[b]
TM6	A_1	816	I
TM7	A_2	816	N
TM9	B_1	830	I
TM12	C_1	844	I
TM13	C_2	844	N
TM16	D_1	858	N

[a] The molecule is cationized during the FD process and is observed at (M + Na), e.g., for A_1 at m/z 839. [b] I = iso fatty acid [δ 0.87 (doublet) indicated isopropyl group]; N = normal fatty acid.

saccharides were extracted as described previously (Chambers & Elbein, 1975; Pan et al., 1979). After extraction of the lipids, the cell debris was washed with 5% trichloroacetic acid, suspended in 1 mL of Protosol, and counted as a measure of protein synthesis or protein glycosylation.

Results

Separation of Tunicamycin and Streptovirudin by HPLC. In the early experiments, we used solvent B to elute tunicamycin from the Biosil columns and were able to resolve the antibiotic into four major 256-nm-absorbing components, as shown in Figure 1. These fractions, labeled A, B, C, and D, were each tested to determine their biological activity both in vitro and in vivo (see below). However, in later experiments we found that solvent A gave better resolution and could separate tunicamycin into at least 15 fractions (Figure 2). In order to compare the peaks in Figure 1 to those of Figure 2, peaks A, B, C, and D were rechromatographed on the Biosil column and eluted with solvent A. Peak A (Figure 1) eluted in the positions of peaks 6 and 7 (Figure 2); peak B eluted in the positions of peaks 6 and 7 (Figure 2); peak B eluted like peak 9; peak C like peaks 12 and 13; and peak D like peaks 16 and 17. Each of the peaks shown in Figure 2 and designated 6 (A_1), 7 (A_2), 9 (B_1), 12 (C_1), 13 (C_2), 16 (D_1), and 17 (D_2) (see Table I for identity) was tested to determine whether it inhibited the solubilized GlcNAc-1-P transferase, and the major fractions were also tested for their ability to inhibit protein glycosylation in MDCK cells (see below).

Streptovirudin was also chromatographed on the Biosil ODS column in solvent A as shown in Figure 1. Two of the streptovirudin components eluted prior to the tunicamycin components, indicating that these streptovirudin fractions were less hydrophobic. Figure 1 shows that streptovirudin also separated into four major fractions, labeled A, B, C, and D. These peaks were tested for biological activity in various systems as discussed below.

The resolved tunicamycin peaks were shown to be homologues by field-desorption mass spectrometry (FDMS) (Table I). The fatty acid type of each factor (Table I) was determined by NMR to be *iso* acids [δ 0.87 (doublet)] and *normal* acids. The presence of these fatty acids was indicated previously by Takatsuki et al. (1979). The streptovirudin peaks were also shown to be homologues by FDMS (Table II). Peak A (M_r 774) had not been reported previously (Eckardt et al., 1971), peak C (M_r 802) was identical with minor tunicamycin components, and peak D (M_r 816) was identical with tunicamycin A_1. The identity of the components was confirmed by HPLC of streptovirudin and tunicamycin using solvent C and agreed with the results of Eckardt et al. (1980).

Effect of Tunicamycin and Streptovirudin Fractions on the Soluble UDP-GlcNAc-dolichyl-P:GlcNAc-1-P Transferase. Figure 3 shows the effect of increasing amounts of the tunicamycin fractions on the incorporation of GlcNAc from UDP-[^{3}H]GlcNAc into dolichylpyrophosphoryl-GlcNAc. In these experiments, the soluble GlcNAc-1-P transferase was used as the enzyme source. With the exception of fraction 1, all of the tunicamycin peaks shown in Figure 2 were active in inhibiting GlcNAc-1-P transfer, although some differences were noted between the different fractions. It can be seen in Figure 3 that the peaks emerging the earliest from the Biosil column (fractions 1–3) were somewhat less active than those which eluted in the middle (fractions 7–14). Although not shown in the figure, peaks 4–6 were also less active as inhibitors. These earlier peaks are somewhat less hydrophobic and probably have shorter chain fatty acids. Thus, the size and configuration of the fatty acids may influence the inhibition. Nevertheless, all of the fractions showed good inhibition at 2.5–5 ng/incubation (5–10 ng/mL or about 1×10^{-8} M). In these experiments, the amount of antibiotic required for 50% inhibition is much lower than that seen in previous studies, but it should be noted that these studies were done with the solubilized GlcNAc-1-P transferase and that the tunicamycin fractions were more highly purified.

Table II: Molecular Weight of Streptovirudin Fractions

fraction	M_r[a] (FDMS)
A	774
B	788
C	802
D	816

[a] The molecule is cationized during the FD process and is observed at (M + Na), e.g., for A at m/z 797.

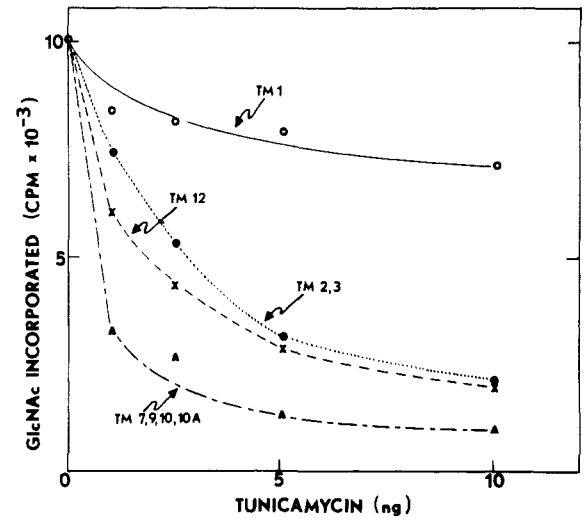

FIGURE 3: Effect of tunicamycin fractions on the formation of dolichylpyrophosphoryl-GlcNAc by the aorta solubilized enzyme. Assays were as described in the text and contained the indicated amounts of the tunicamycin fractions seen in Figure 2. Soluble enzyme (1 mg of protein) was used with UDP-[^{3}H]GlcNAc and dolichyl-P. Lipids were isolated as described.

The four streptovirudin fractions shown in Figure 1 were also tested as inhibitors of dolichylpyrophosphoryl-GlcNAc synthesis, as shown in Figure 4. Peaks B, C, and D all appeared to have about the same activity, giving 50% inhibition at about 10 ng/incubation (20 ng/mL or about 2×10^{-8} M). Peak A was of lower activity and required 30% more material for the same degree of activity. This peak emerged earliest from the HPLC and is probably the least hydrophobic of the series.

In these experiments, it was not possible to block GlcNAc incorporation any greater than 80–85% even at very high

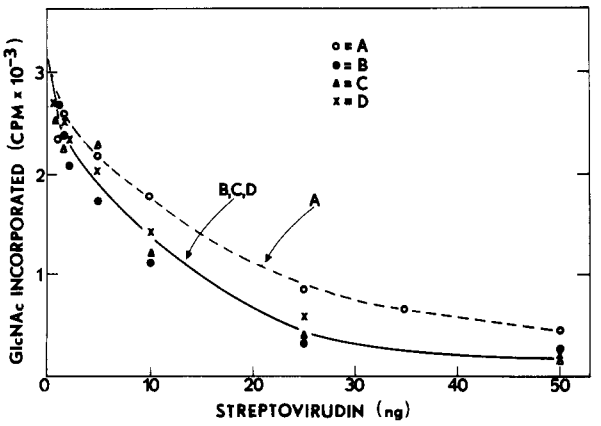

FIGURE 4: Effect of streptovirudin fractions on the formation of dolichylpyrophosphoryl-GlcNAc by the soluble enzyme. Protocol was as indicated in Figure 3 with the various streptovirudin fractions.

Table III: Effect of Tunicamycin Fractions on the Particulate Glc Transferase[a]

fraction	inhibition (%) at[b] 0.1 μg	0.5 μg	2.0 μg	10 μg
4	16	44	72	-
6	13	24	71	80
7	29	59	75	83
9	36	63	76	80
10	28	55	68	79
10A	34	53	78	-
12	11	25	65	75
13	20	46	71	78
16	20	54	73	80
17	24	37	69	79
18	22	43	63	76

[a] Tunicamycin fractions tested were those shown in Figure 2. Control incubations with particulate enzyme incorporated about 14 000 cpm of glucose from UDP-[^{3}H]glucose into lipid. [b] Amount of fraction tested.

concentrations of antibiotic. This is probably due to the fact that the solubilized enzyme contains small amounts of dolichylpyrophosphoryl-GlcNAc which can serve as an acceptor of a second GlcNAc to produce dolichylpyrophosphoryl-*N*,*N*′-diacetylchitobiose. In a previous study we showed that the remaining GlcNAc residues are in fact found in the dolichylpyrophosphoryl-*N*,*N*′-diacetylchitobiose (Elbein et al., 1979). The enzyme which adds the second GlcNAc is not sensitive to tunicamycin (Lehle & Tanner, 1976).

Effect of Tunicamycin and Streptovirudin Fractions on Glucose Transfer. In previous studies, both tunicamycin and streptovirudin were shown to inhibit the formation of dolichylphosphorylglucose (Elbein et al., 1979). However, glucose incorporation into lipid required 50–100-fold higher concentrations of antibiotic for the same degree of inhibition as observed with GlcNAc-1-P transfer. Table III presents the activities of the tunicamycin fractions on glucose transfer from UDP-glucose to lipid by the particulate enzyme from aorta. All of the fractions tested were active in inhibiting this reaction, showing 50% inhibition at about 0.5–1 μg/incubation (1–2 μg/mL or about 2×10^{-6} M). Just as in the case of the GlcNAc-1-P transferase, the earlier peaks were the least active while the later peaks were more inhibitory.

Figure 5 shows the activities of the four streptovirudin components with regard to glucose transfer to lipid. In this case, the solubilized glucosyl transferase was used. Distinct differences were observed between the fractions, with A being the least active and D the most active. Glucose incorporation into lipid could not be inhibited more than 80% even at high

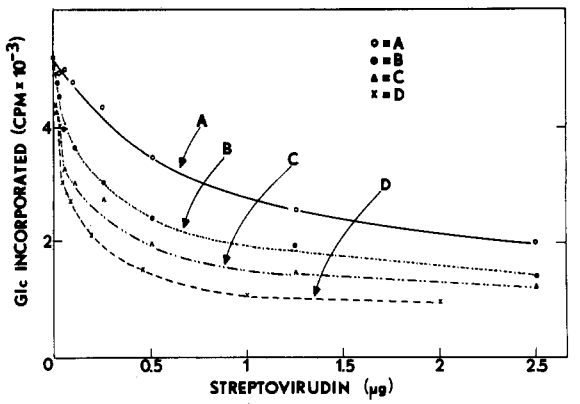

FIGURE 5: Effect of streptovirudin fractions on the formation of dolichylphosphorylglucose by the solubilized enzyme. Assays with UDP-[^{3}H]glucose were as described in the text and contained 100 μL of soluble enzyme (1 mg of protein) and 5 μg of dolichyl-P. Streptovirudin fractions were added as indicated. Lipids were isolated as described in the text.

Table IV: Effect of Tunicamycin Fractions on the Incorporation of [^{3}H]Mannose and [^{14}C]Leucine into Protein by MDCK Cells

tunicamycin	concn (μg/mL)	[^{3}H]mannose incorporated (cpm) into Dol-P-Man	LLO	protein	[^{14}C]leucine incorporated into protein (cpm)
control[a]		762	3800	34 400	25 900
TM 7	0.1	713	2390	31 400	26 200
	0.5	846	330	9 050	16 900
	2.0	756	183	6 050	15 900
	10.0	570	136	5 700	17 300
TM 9	0.1	866	827	9 830	23 700
	0.5	912	224	8 590	23 200
	2.0	820	261	5 620	16 000
	10.0	680	169	7 070	23 900
TM 10	0.1	769	1800	31 730	28 200
	0.5	870	500	16 210	26 300
	2.0	650	670	6 590	19 400
	10.0	520	136	5 400	18 500
TM 13	0.1	830	800	19 500	24 900
	0.5	864	308	12 010	27 000
	2.0	695	197	5 870	18 500
	10.0	590	173	6 100	19 000
TM 16	0.1	890	824	21 500	28 400
	0.5	792	556	10 000	23 500
	2.0	690	204	5 600	17 800
	10.0	568	208	6 000	20 400

[a] Average of triplicate samples.

antibiotic concentrations. The apparent reason for this is that glucose is also incorporated into a second lipid which appears to be a glucosylceramide (Kang et al., 1978; Elbein et al., 1979). This lipid, which accounts for 10–15% of the total radioactivity incorporated, is not sensitive to tunicamycin.

Effect of Tunicamycin Fractions on Protein Synthesis and Protein Glycosylation. The tunicamycin complex has been shown to cause some inhibition of protein synthesis in various cell cultures (Hart & Lennarz, 1978; Hickman et al., 1977), and one of the fractions obtained by HPLC was reported to inhibit protein synthesis (Mahoney & Duskin, 1979). Therefore, it was important to test the effect of our major tunicamycin fractions on protein synthesis and protein glycosylation in cell cultures. Table IV presents the results of such an experiment using a kidney cell line that had been infected with influenza virus. In this system, the major proteins synthesized are the viral glycoproteins hemagglutinin and neuraminidase (Schwarz et al., 1976). After infection, the cell cultures were incubated with [^{3}H]mannose to measure

Table V: Activity of Streptovirudin Fractions on the Incorporation of [^{3}H]Mannose or [^{3}H]Leucine into Protein in MDCK Cells[a]

amount of streptovirudin added (μg/mL)	[^{3}H]leucine incorporated (cpm × 10^{-3}) streptovirudin fraction tested			
	A	B	C	D
	65.3	57.0	55.0	53.7
0.25	73.5	62.5	57.8	54.5
1.0	61.5	52.0	54.1	45.9
5.0	59.1	47.9	48.4	41.6
15	56.3	47.5	40.2	–
25		42.8	36.2	–
	[^{3}H]mannose incorporated (cpm × 10^{-3})			
	8.2	12.6	11.0	11.4
0.05	8.2	14.0	13.0	12.0
0.25	8.6	12.1	12.2	4.9
1.0	8.6	8.6	4.1	2.6
5.0	8.4	2.0	1.3	2.4
12.0	–	1.2	1.1	1.2

[a] Conditions were described in the text.

protein glycosylation or [^{3}H]leucine to measure protein synthesis. In this experiment, we also measured the incorporation of mannose into the lipid-linked saccharides, dolichylphosphorylmannose (Dol-P-Man) and lipid-linked oligosaccharides (LLO).

The results show that tunicamycin fractions 7, 9, 10, 13, and 16 were potent inhibitors of mannose incorporation into LLO and into protein, inhibiting more than 50% at 0.5 μg/mL. Fraction 9 appeared to be the most active. However, there was little inhibition of mannose incorporation into Dol-P-Man except at fairly high concentrations of antibiotic (10 μg/mL). In terms of [^{3}H]leucine incorporation into protein, a fair degree of variability was noted even among the control incubations (i.e., controls ranged from 22 000 cpm of leucine incorporated to 27 000 cpm). This variability may in part be explained by the fact that the cell monolayers vary to some extent in their degree of confluency (i.e., the actual number of cells). However, there was not consistent decline in the leucine incorporation as the antibiotic concentration was raised. This experiment was repeated several times at varying tunicamycin concentrations with results similar to those shown in Table IV. Thus, these fractions do not appear to inhibit protein synthesis at levels where mannose incorporation is blocked. However, high concentrations (10 μg/mL or higher) of these antibiotics may inhibit protein synthesis since it has been postulated that the synthesis of the protein portion of glycoproteins is specifically inhibited when glycosylation is prevented (Schwaiger & Tanner, 1979).

Effect of Streptovirudin Fractions on Protein Synthesis and Protein Glycosylation in MDCK Cells. The four streptovirudin fractions were tested in MDCK cells to determine their effect on [^{3}H]leucine and [^{3}H]mannose incorporation into protein. The results of this experiment are shown in Table V. Fractions B, C, and D were fairly effective in inhibiting mannose incorporation into protein, inhibiting by 50% or more at 1 μg/mL. Fraction A, which was tested in a separate experiment and therefore shows a different level of incorporation, demonstrated little inhibitory activity except perhaps at high concentrations. These results are consistent with the results of in vitro inhibition. With regard to leucine incorporation into protein, these fractions did appear to have some inhibitory activity especially with fractions B and C. However, even in this case at concentrations of antibiotic that inhibited mannose incorporation by 80% or more (i.e., 5 μg/mL), leucine incorporation was only inhibited 10–15%.

Discussion

Tunicamycin, streptovirudin, and related antibiotics have proven to be valuable tools for studying the role of protein glycosylation in a variety of cellular events. These antibiotics block the lipid-linked saccharide pathway by inhibiting the formation of the first lipid intermediate, dolichylpyrophosphoryl-GlcNAc (Tkacz & Lampen, 1975; Struck & Lennarz, 1977; Waechter & Harford, 1977; Ericson, et al., 1977; Lehle & Tanner, 1976; Elbein et al., 1979). Therefore they prevent glycosylation of those proteins having an oligosaccharide attached to protein in a GlcNAc–asparagine bond (Waechter & Lennarz, 1976; Elbein, 1979). Since these proteins are not glycosylated in the presence of these antibiotics, one can study the "nonglycosylated" protein to determine how it functions in various cell systems.

Tunicamycin and streptovirudin are both produced by streptomycetes as a series of closely related antibiotics that differ from each other in the fatty acid moiety (Takatsuki et al., 1977). Although these various compounds have been separated by high-performance liquid chromatography (Ito et al., 1980; Mahoney & Duskin, 1979), only a few of the different fractions have been tested for biological activity. In this report, we show that all of the major tunicamycin and streptovirudin fractions are potent inhibitors of the UDP-GlcNAc-dolichyl-P:GlcNAc-1-P transferase, inhibiting this solubilized enzyme by 50% at concentrations of 5–10 ng/mL. This level of inhibition is much greater than that seen in other experiments, probably because the purified antibiotic fractions are more active and because the solubilized enzyme is more sensitive. The various antibiotic fractions also inhibited the formation of dolichylphosphorylglucose in vitro and the incorporation of mannose into protein in cells grown in culture. However, in these experiments some differences were observed in the inhibitory activity of the different fractions. These differences are probably related to differences in the fatty acids and may reflect how these antibiotics interact with the transferases or how well they are taken up by whole cells. It has been suggested that the mechanism of action of tunicamycin is due to the fact that it mimics the substrate, dolichylphosphate (Keller et al., 1979). Thus the length and configuration of the fatty acid could play a key role in inhibition.

While the tunicamycin complex has been shown to cause some inhibition of protein synthesis in various cell cultures (Hart & Lennarz, 1978; Hickman et al., 1977), we found little inhibition of leucine incorporation in MDCK cells at levels of antibiotic where mannose incorporation was almost completely inhibited. Thus although there was some scattering in the data, probably due to variations in the number of cells in the different monolayers, increasing concentrations of antibiotic did not lead to increasing inhibition of leucine incorporation. These data are in contrast to those of Mahoney & Duskin (1979) who found that one of their major tunicamycin fractions still inhibited protein synthesis. It may be that the tunicamycin complex also contains a specific inhibitor of protein synthesis which was separated from the tunicamycin components in our HPLC systems, or it may be that different cell types show different susceptibility to these antibiotics. From our observations with several different cell lines, there does seem to be a difference in the amount of tunicamycin required to inhibit mannose incorporation in different cells (unpublished observations). These differences may be due to different rates of uptake by cells. However, at fairly high concentrations of these antibiotics (5–10 μg/mL or above), there may be some inhibition of protein synthesis. This may be due to the fact that protein synthesis is somehow linked to

glycosylation as originally proposed by Hasilik & Tanner (1977) and Schwaiger & Tanner (1978). Schwaiger and Tanner found that tunicamycin inhibited the glycosylation and secretion of amylase in barley aleurone layers, but no amylase was found to accumulate intracellularly. They discussed the possibility that the synthesis of the protein is inhibited when glycosylation is blocked. In another system, Hasilik and Tanner found that tunicamycin inhibited the glycosylation of carboxypeptidase Y and also specifically reduced the amount of carbohydrate-free carboxypeptidase Y found in the cells. Since this product was found to be metabolically stable, these authors considered that there might be a regulatory link between the glycosylation of the protein moiety and its biosynthesis.

Streptovirudin has been reported to be a tunicamycin-like antibiotic (Eckardt et al., 1975), but its structure has not been reported. Since two of its components elute from HPLC prior to the tunicamycin fractions, they are apparently less hydrophobic and contain shorter chain fatty acids. On the basis of mass spectrometry, they are related to tunicamycin but have shorter chain fatty acids. The streptovirudin fractions from HPLC show greater variation in activity, both in vivo and in vitro, than did the tunicamycin fractions. Thus, the most hydrophobic streptovirudins (fractions C and D) were more active than the least hydrophobic component. In the case of these compounds, the inhibition of leucine incorporation was more noticable, but again at levels of antibiotic where mannose incorporation was markedly inhibited, leucine inhibition was only slight.

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