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Stimulation as well as inhibition by antibiotics of the formation of GlcNAc-lipids of the dolichol pathway

Edward L. Kean* and Zenglu Wei

Departments of Ophthalmology and Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, USA

The antiobiotics, diumycin, amphomycin, bacitracin, and showdomycin have been shown previously to block the synthesis of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol. In view of inconsistencies in the literature concerning the sites of inhibition, we have reinvestigated the influence of these drugs on the formation of these early intermediates of the dolichol pathway. Unexpectedly, when the individual products of the reactions were examined, instead of inhibition, showdomycin and bacitracin were found to stimulate the formation of GlcNAc-P-P-dolichol, and diumycin stimulated at low concentrations. Three derivatives of showdomycin were examined with similar results, showing stimulations of GlcNAc-P-P-dolichol formation of up to two-fold over controls. Amphomycin specifically inhibited GlcNAc-P-P-dolichol formation, an effect that was reversed by a high concentration of dolichyl phosphate. In contrast, with the exception of amphomycin, each compound directly inhibited the formation of GlcNAc-GlcNAc-P-P-dolichol. Using chemically synthesized GlcNAc-P-P-dolichol as substrate, the kinetics of inhibition of GlcNAc-GlcNAc-P-P-dolichol formation by showdomycin, bacitracin and diumycin was examined. The apparent K_i values calculated from these studies indicated that showdomycin was the most active inhibitor. These findings provide a new understanding of the action of these compounds on the GlcNAc-transferases of the dolichol pathway.

***Keywords*: dolichol pathway, GlcNAc-P-P-dolichol, GlcNAc-GlcNAc-P-P-dolichol, antibiotics, inhibition, stimulation, retina, showdomycin, diumycin, amphomycin, bacitracin, microsomes, embryonic chick, kinetics, Dionex**

***Abbreviations*: GlcNAc-P-P-dolichol, *N*-acetylglucosaminylpyrophosphoryldolichol; GlcNAc-GlcNAc-P-P-dolichol, *N*-acetylglucosaminyl-*N*-acetylglucosaminylpyrophosphoryldolichol; TX-100, triton X-100; Tes, 2-{[*tris*-(hydroxy-methyl)-methyl]-amino}-ethanesulfonic acid; chitobiose, GlcNAc-GlcNAc**

Introduction

Although the dolichol pathway has been long established as the means whereby the core region of asparagine-linked glycoproteins is assembled [1], many of the properties of this complex series of events remain to be explored. The task of dissecting the intricacies of many of the reactions has been aided greatly by the availability of a variety of compounds that inhibit specific reactions [2]. Among the first discovered was the nucleoside antibiotic, tunicamycin, shown to block the initial reaction of the dolichol pathway, the formation of GlcNAc-P-P-dolichol catalysed by the enzyme UDP-GlcNAc: dolichyl phosphate *N*-acetylglucosamine-1-phosphate transferase (GPT-1) [3]. Subsequently, other antibiotics such as showdomycin, amphomycin, diumycin and bacitracin were observed to function at this site and also on the enzyme catalysing the addition of the second GlcNAc residue, UDP-GlcNAc: GlcNAc-P-P-dolichol, GlcNAc-transferase (GT-2). However, in view of a lack of agreement between different reports (see Results and Discussion) concerning the site of and nature of the effect of these drugs, and also as part of our studies investigating control mechanisms of this phase of glycoprotein biosynthesis [4], we reexamined their effects on the formation of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol. Unexpectedly, with the exception of amphomycin, we encountered stimulatory effects as well as inhibition. The influence of these antibiotics on the formation of these early intermediates of the dolichol pathway is the topic of this report.

Methods and materials

Enzymatic incubation; assay

The enzyme system used in these studies was the microsome from the retina of the embryonic chick which was prepared

*To whom correspondence should be addressed. Room 653 Wearn Bldg., Center for Vision Research, Department of Ophthalmology, Case Western Reserve University, 11100 Euclid Ave., Cleveland, Ohio 44106, USA. Tel.: (216) 844-3613; Fax: (216) 844-7899; E-mail: elk2@po.cwru.edu.

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as described previously [5]. GlcNAc-lipid synthesis was determined after incubating the microsomes (0.1–0.2 mg protein) at 37 °C for 15 min in the presence of dolichyl phosphate (15–19 μM), TX-100 (0.15%), $MgCl_2$ (27 mM), Tes buffer (0.2 M, pH 7.5), UDP-[^{3}H] GlcNAc (50 μM, 170×10^6 dpm μmol^{-1} in the presence or absence of the indicated test compounds (dissolved in DMSO or water) in a total volume of 0.15 ml (optimal conditions as described previously [5]). The reaction was terminated by the addition of chloroform: methanol (2 : 1, by vol) and the mixture extracted by the procedure of Folch *et al.* [6]. Incubations were also performed using chemically synthesized GlcNAc-P-P-dolichol as substrate under optimal conditions as described previously [7]. The radioactivity of the material in the washed lower phase, after evaporation to dryness, was determined by scintillation spectrometry as described previously [5]. The term 'GlcNAc-lipids' as used in this report refers to the mixture of products formed by the GPT-1 and GT-2 reactions present in the lower phase after solvent partitioning by the Folch procedure of the incubation mixture.

Product identification

After large-scale incubations (usually four-fold over that described above), the material in the lower phase after Folch-washing was evaporated to dryness and subjected to mild acid hydrolysis in 0.1 N HCl in tetrahydrofuran for 100 min at 50 °C [8]. After evaporation, the material was applied to a mixed bed ion exchange resin column composed of 0.5 ml each of Ag-2-x-8 (200–400 mesh) acetate and Ag-50 x-8 (200–400 mesh)H^+, and eluted with water, as described previously [7]. The water eluate was evaporated to dryness, redissolved in water and examined by high pH anion exchange chromatography (HPAEC, Dionex Corp., Sunnyvale, CA). The time of elution of internal standards added to each sample (fucose (5 nmol, as an early eluting marker), GlcNAc (10 nmol), and *N*,*N*′-diacetylchitobiose (10 nmol)), was measured by pulsed amperometric detection (PAD) using conditions described previously [9]. Radioactivity was measured in the corresponding fractions from the PAD cell. Product identification was also carried out by paper chromatography as described previously [10]. Radioactivity in the fractions was determined by scintillation spectrometry in the presence of 0.5 ml water and 5 ml EcoLume scintillation cocktail (ICN Research Products Division, Costa Mesa, CA).

Antibiotics

Showdomycin was obtained from Sigma Chemical Corp. (St Louis MO)‡ and D-(+)-showdomycin from Dr Sung Ho Kang, Department of Chemistry, Korea Advanced Institute of Science and Technology, Taejon Korea, 2′-Deoxyshowdomycin and *N*-benzyl-2′-deoxyeshowdomycin were obtained from Dr R.S. Hosmane, University of Maryland County, MD. Amphomycin was obtained from Bristol Laboratories, Syracuse, NY‡, and diumycin, from E.R. Squibb and Sons, Princeton, NJ‡. Bacitracin was purchased from Sigma.

‡ Showdomycin, amphomycin and diumycin are no longer available from these sources.

Data presentation

With the exception of bacitracin, the concentration of the other compounds which were investigated is presented in terms of weight per volume in order to be consistent with other reports in the literature dealing with these reactions. For the same reason, molar concentrations are used with bacitracin.

Other procedures

Protein concentration was determined by the Lowry procedure. Apparent K_i values were calculated from a non-linear least squares analysis of the data using an expression for general inhibition which was derived from an equation for mixed inhibition with a constant substrate concentration

$$v\ (\text{inhibited}) = \frac{V\ (\text{uninhibited})}{(1 + [1]/K_i\ \text{apparent})}$$

Thus, three common sets of experiments were performed on each of the four antibiotics examined in this study: (1) The effect of the antibiotic on total GlcNAc-lipid synthesis by measuring the incorporation of ^{3}H-GlcNAc into the lipid fraction after Folch-washing; (2) the nature of the saccharide components after mild acid hydrolysis, mixed bed ion exchange chromatography, and separation and quantitation by Dionex chromatography and paper chromatography (data for the latter not shown); (3) the kinetics of inhibition of the formation of GlcNAc-GlcNAc-P-P-dolichol using GlcNAc-P-P-dolichol as the substrate by measuring the incorporation of ^{3}H from UDP[^{3}H]GlcNAc into the lipid fraction after Folch-washing. Up to 10 separate experiments each for (1) and (2) were carried out on each antibiotic. Presented are representative examples of the effects noted. Due to limitations in supply of chemically synthesized GlcNAc-P-P-dolichol, single experiments were carried out on the kinetics of inhibition of the formation of GlcNAc-GlcNAc-P-P-dolichol by each antibiotic.

Results and discussion

Effect of the showdomycins on GlcNAc-lipid synthesis

As described previously [5], under the conditions used in these experiments, two GlcNAc-lipids, GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol, are synthesized

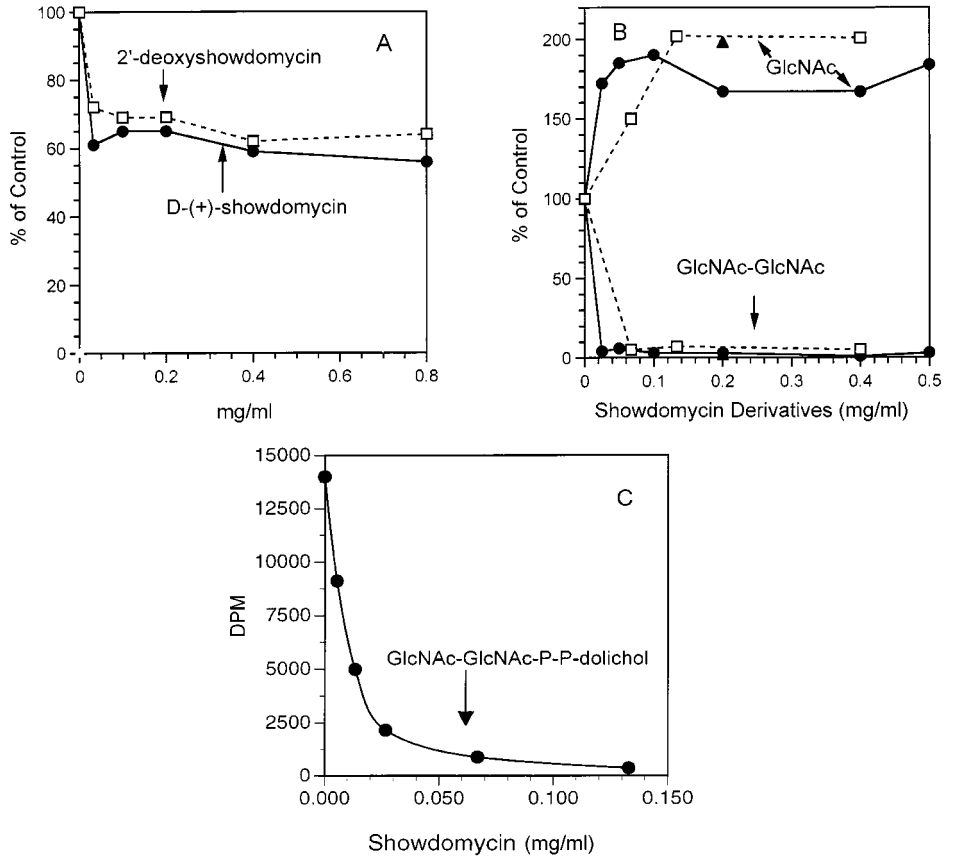

Figure 1A–C. Effect of showdomycin derivatives on total GlcNAc-lipid synthesis and on the individual products. In panels A and B, microsomes from the retina of the embryonic chick were incubated for 15 min at 37 °C in the presence of dolichol phosphate (17 μM), UDP [^{3}H] GlcNAc (50 μM, 173 dpm pmol^{-1}), Tes buffer, (0.2 M, pH 7.5), $MgCl_2$ (27 mM), TX-100 (0.15%), as described in Methods and Materials and the indicated concentrations of the showdomycin derivatives. (A) Total GlcNAc-lipid formation. 2′-deoxyshowdomycin. (□); D-(+)-showdomycin (●) In the absence of 2′-deoxyshowdomycin or D-(+)-showdomycin there were 3780 dpm and 1270 dpm incorporated, respectively. (B) Analysis of the individual products of the reactions. After extraction of the products of the reactions by the Folch procedure [6], followed by mild acid hydrolysis and mixed bed ion exchange chromatography, the constituent sugars were analysed by Dionex chromatography (HPAEC), measuring the radioactivity of fractions collected from the PAD cell, as described in Methods and Materials. 2′-deoxyshowdomycin (□); D-(+)-showdomycin (●); *N*-benzyl-2′-deoxyshowdomycin (▲). In the absence of the showdomycin derivatives there were 2630–3790 dpm in GlcNAc, and 3920–4740 dpm in GlcNAc-GlcNAc in different experiments. (C) Effect of variation in the concentration of showdomycin on the formation of GlcNAc-GlcNAc-P-P-dolichol. Incubations were performed for 15 min at 37 °C in the presence of GlcNAc-P-P-dolichol (19 μM), UDP [^{3}H]GlcNAc(140 μM, 86 dpm pmol^{-1}), Tes buffer (0.2 M, pH 7.4) TX-100 (0.15%), $MgCl_2$ (20 mM), retina microsomes and showdomycin as indicated on the abscissa in a total volume of 0.15 ml. The lipid-extractable radioactivity was determined as described in Methods and Materials.

and are present in the organic phase after extracting the incubation mixture (described above) according to the procedure of Folch *et al.* [6]. As seen in Figure 1A, the biosynthesis of the GlcNAc-lipids appeared to have been inhibited 35–40% by the nucleoside antibiotic, showdomycin. The derivatives, D-(+)-showdomycin and 2′-deoxyshowdomycin, produced similar effects as did the *N*-benzyl-2′-deoxy derivative (data for the latter not shown). The apparent inhibition, however, proved to be composed of stimulatory and inhibitory events when the nature of the individual products formed under these conditions was investigated. As seen in Figure 1B, after separation by Dionex chromatography, analysis of the individual products of the reaction revealed a previously unobserved differential effect on the formation of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol. While D-(+)-showdomycin and the 2′-deoxyshowdomycin derivative virtually abolished synthesis of GlcNAc-GlcNAc-P-P-dolichol, the formation of GlcNAc-P-P-dolichol was enhanced 1.7–2-fold. Similar results were obtained with the benzyl derivative and the preparation from Sigma. An example of the analysis by Dionex chromatography, on the basis of which these evaluations were made, is seen in Figure 2A and B. The time of elution of the radioactive peaks correspond to the PAD cell response to GlcNAc and GlcNAc-GlcNAc present as internal standards. Under basal conditions in the absence of the antibiotic, both GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol are formed, as seen in Figure 2A. In

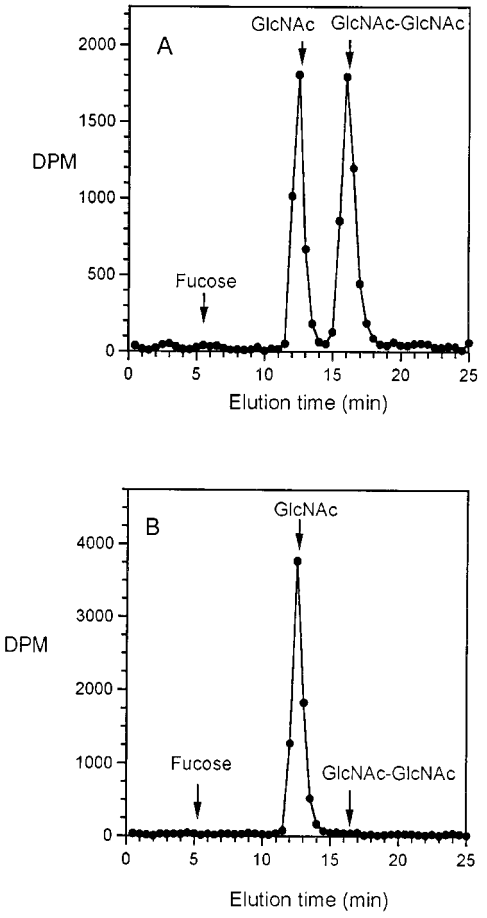

Figure 2A and B. Analysis by Dionex chromatography of the saccharide components of the GlcNAc-lipids. Incubations were carried out as described in the legend to Figure 1A, and products analyzed by Dionex chromatography as described in Figure 1B. (A) Control: analysis of the products formed in the absence of 2′-deoxyshowdomycin. (B) Analysis of the products formed in the presence of 0.133 mg ml^{-1} 2′-deoxyshowdomycin. The arrows indicate the elution time of fucose (5 nmol); GlcNAc (10 nmol); GlcNAc-GlcNAc (10 nmol), added as internal standards, while the radioactivity was that measured in the eluate from the PAD cell.

the presence of 2′-deoxyshowdomycin only GlcNAc-P-P-dolichol is formed, and in a greater amount as seen in Figure 2B. Similar results were obtained with the other showdomycin derivatives. Analysis by paper chromatography also showed a similar response (data now shown).

The enhanced formation of the first intermediate of the pathway we suggest is to a large extent a mass action consequence of a lack of inhibition of the first GlcNAc transferase coupled with the inhibition of the second GlcNAc-transferase in these sequential reactions:

dolichyl phosphate + UDP-GlcNAc →

GlcNAc-P-P-dol + UMP

GlcNAc-P-P-dolichol + UDP-GlcNAc →

GlcNAc-GlcNAc-P-P-dolichol + UDP

The kinetics of the inhibition by showdomycin of the formation of the chitobiosyl compound using chemically synthesized GlcNAc-P-P-dolichol as the substrate was examined further under incubation conditions described previously [7]. As seen in Figure 1C, saturation kinetics were observed for the inhibition by the antibiotic of the synthesis of GlcNAc-GlcNAc-P-P-dolichol. An apparent inhibition constant, seen in Table 1, was calculated from a non-linear least squares analysis of the data as described in Methods and Materials.

The inhibition by showdomycin of the formation of the chitobiosyl derivative and stimulation of the mono-GlcNAc derivative was also demonstrated by identifying the constituent sugars (by the Methods described above) in the products of incubations that contained both dolichyl phosphate and GlcNAc-P-P-dolichol as substrates. Although the embryonic chick retina microsome system readily converts GlcNAc-P-P-dolichol to GlcNAc-GlcNAc-P-P-dolichol [7], in the presence of showdomycin, the formation of the chitobiosyl derivative was completely inhibited and the formation of labeled GlcNAc-P-P-dolichol was stimulated (data not shown).

Although inhibition of GlcNAc-lipid synthesis by showdomycin has been observed previously with preparations from aorta [11], and the green alga, *Volvox carteri* f. *nagariensis* [12], a distinction had not been made between the effects on these two GlcNAc intermediates of the dolichol pathway. As shown by the present studies, the activity observed by measuring the radioactivity present in the chloroform/methanol extract of the incubation mixture (Figure 1A) reflects the net activity resulting from the stimulation of GlcNAc-P-P-dolichol synthesis and the inhibition of formation of the chitobiosyl derivative. Depending on the relative contributions from the stimulatory and inhibitory influences, misleading conclusions concerning the effect of this antibiotic could be obtained from an examination of a total lipid extract. In this regard, experiments were encountered in which there was no decrease in total lipid-extractable radioactivity as compared to controls, or even increases in activity, suggesting that inhibition had not occurred. In fact, when the products were analysed, extensive inhibition of the formation of the chitobiosyl compound was revealed accompanied by an

Table 1. Kinetics of inhibition of GlcNAc-GlcNAc-P-P-dolichol formation.

Inhibitor	*Apparent K_i* [a]	
Showdomycin	7.3 μg ml^{-1}	0.034 mM
Diumycin	105 μg ml^{-1}	(structure not known)
Bacitracin	2,050 μg ml^{-1}	1.45 mM

[a] Calculated by a non-linear least squares analysis of data from Figures 1C, 3C, and 5C, as described in Methods and Materials.

increase in the formation of the monoGlcNAc product (data not shown).

Diumycin

Incubations carried out in the presence of diumycin also showed inhibition of total GlcNAc-lipid synthesis as seen in Figure 3A, an inhibition that appeared to be greater than that obtained with showdomycin. The concentration of diumycin that brought about half maximal inhibition of total GlcNAc-lipid synthesis was about 0.087 mg ml^{-1}. A differential effect on the formation of the two GlcNAc-lipids resulting from use of this compound was also seen after the products were analysed, as seen in Figure 3B. The nature of the effect on GlcNAc-P-P-dolichol formation, however, varied with the concentration of the antibiotic; enhanced about 20% at concentraions below 0.1 mg ml^{-1} and limited inhibition (30–40%) at higher concentrations. This is consistent with the previous observation with the *Acanthamoeba castellanii* system [13] that GlcNAc-P-P-dolichol synthesis was largely uninhibited by 50 μg ml^{-1} of diumycin. In the present study, unlike the effect on the monoGlcNAc-derivative, chitobiosyl-lipid formation was inhibited at all concentrations of diumycin tested. The kinetics of the inhibition by diumycin of GlcNAc-GlcNAc-P-P-dolichol formation was examined as above using GlcNAc-P-P-dolichol as substrate, as seen in Figure 3C. From an analysis of this data as previously, the apparent inhibition constant, shown in Table 1, indicates that diumycin was 14-fold less active an inhibitor on a weight basis as compared to showdomycin for the addition of the second GlcNAc residue. The inhibition by diumycin of the synthesis of the chitobiosyl derivative was thus a combination of inhibiting the formation of its precursor, GlcNAc-P-P-dolichol, and also the direct inhibition of the second

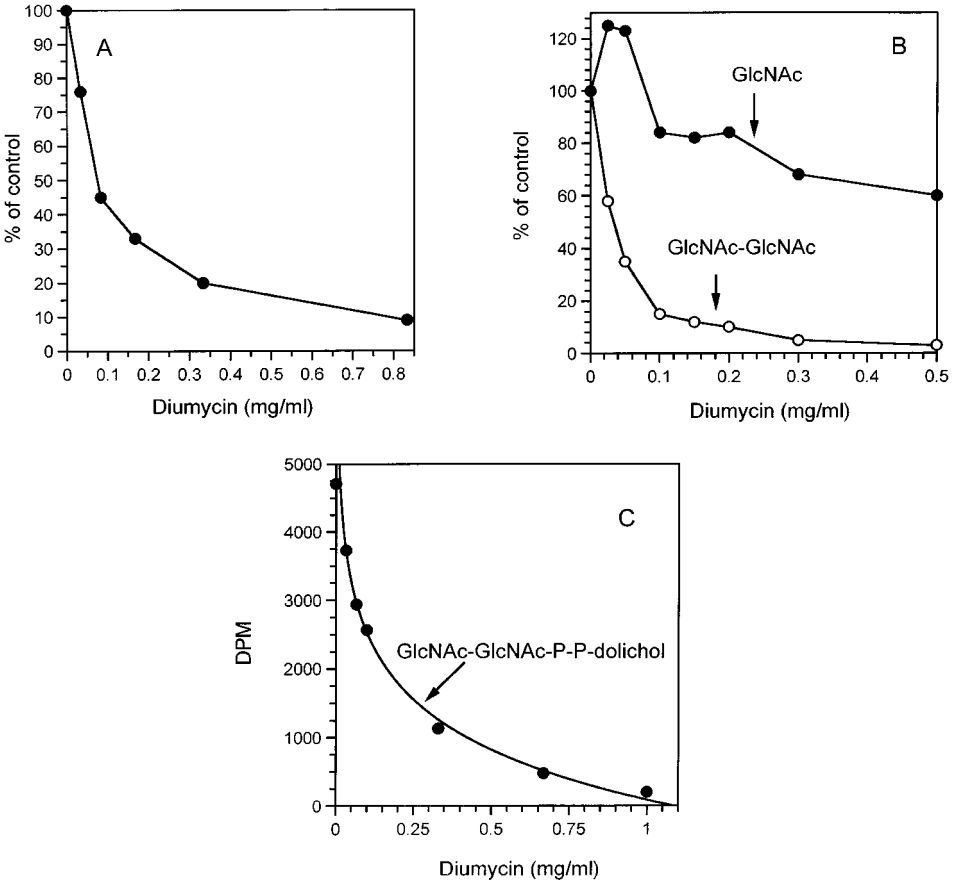

Figure 3A–C. Effect of variation in the concentration of diumycin on GlcNAc-lipid synthesis. In panels A and B, incubations were carried out using dolichol phosphate and UDP [^{3}H]GlcNAc as substrates as described in the legend to Figure 1 and in the presence of the concentrations of diumycin indicated on the abscissas. (A) Total GlcNAc-lipid formation. On the ordinate is the percent of radioactivity incorporated into GlcNAc-lipids after Folch-washing compared to that obtained the absence of the antibiotic (1800 dpm). (B) Analysis of the individual products of the reactions, as in the legend to Figure 1B. On the ordinate is the % of radioactivity in GlcNAc and GlcNAc-GlcNAc compared to the controls (absence of diumycin) taken as 100% which varied for GlcNAc from 1800 dpm to 4500 dpm, and for chitobiose, from 1900 dpm to 5000 dpm in different experiments. (C) Effect of variation in the concentration of diumycin on the formation of GlcNAc-GlcNAc-P-P-dolichol. Using GlcNAc-P-P-dolichol (1.8 μM) as substrate, incubations were performed and products analyzed as indicated in the legend to Figure 1C.

GlcNAc-transferase. These findings are consistent with and extend previous observations using an enzyme preparation from *Acanthamoeba castellanii* [13]. Even at 10-fold higher concentrations of diumycin than used in that study, greater selectivity toward inhibiting the formation of the chitobiosyl-lipid than GlcNAc-P-P-dolichol was exhibited. The basis for the shift in response from stimulation to inhibition with increasing concentration of the antibiotic is not known.

Amphomycin

Among the influences observed in plant and animal tissues on the formation of lipid-linked saccharides by the polypeptide antibiotic, amphomycin, was its inhibition of the formation of GlcNAc-containing pyrophosphoryl dolichols [14–16]. A distinction between its action on the mono-GlcNAc and chitobiosyl derivatives, however, was not made. In the present study total GlcNAc-lipid formation was strongly inhibited by amphomycin as seen in Figure 4A, with 50% inhibition obtained at about 25 $\mu g\,ml^{-1}$. This is about $\frac{1}{3}$ of that required by diumycin to achieve this effect. When the effect of amphomycin on the individual components was examined by Dionex chromatography (Figure 4B), extensive inhibition was observed for both GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol. From Figure 4B it can be estimated that 50% inhibition of the formation of GlcNAc-P-P-dolichol required about 35 $\mu g\,ml^{-1}$ of amphomycin. Further studies on the influence of amphomycin on GlcNAc-GlcNAc-P-P-dolichol formation using GlcNAc-P-P-dolichol as substrate seen in Figure 4C revealed, however, that this antibiotic had little or no direct effect on the formation of the chitobiosyl derivative. The inhibition of formation of the latter compound by amphomycin seen in Figure 4B was thus a secondary consequence of the inhibition of the synthesis of its precursor, GlcNAc-P-P-dolichol. Thus, similar to the action shown for tunicamycin [10], amphomycin did not inhibit the addition

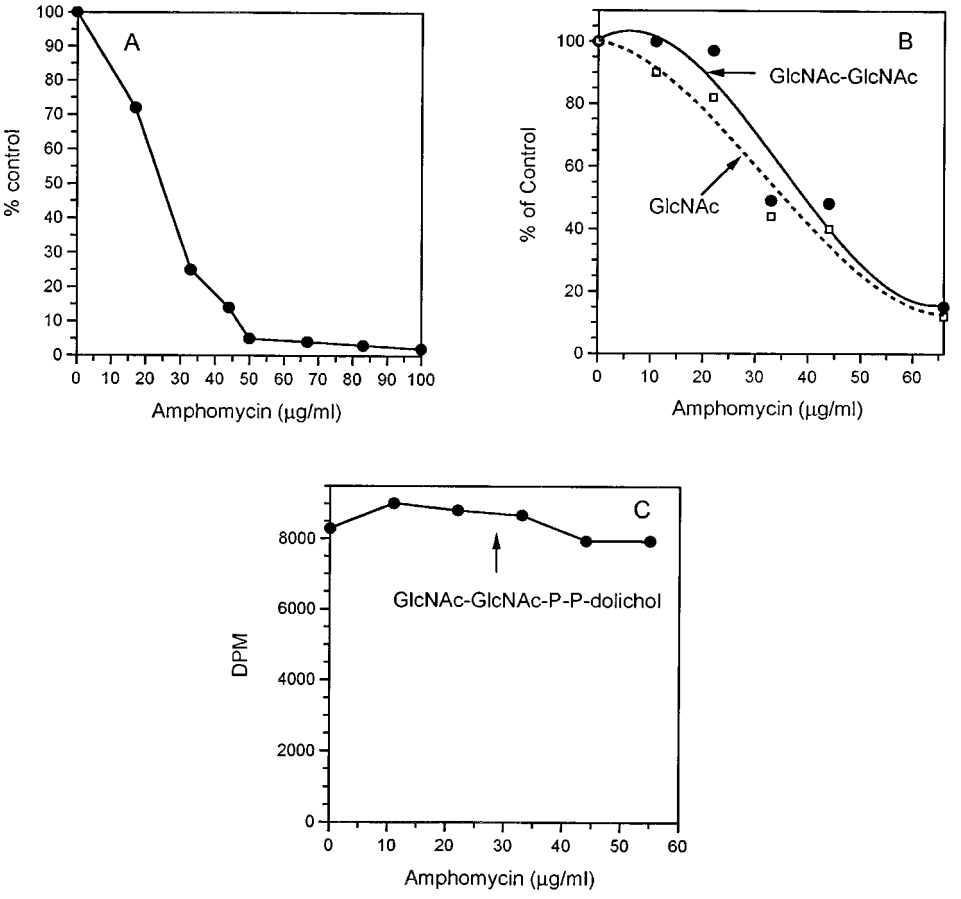

Figure 4A–C. Effect of amphomycin on total GlcNAc-lipid synthesis and on the individual products. In panels A and B, incubations were carried out as described in Figure 1A and B in the presence of the indicated concentrations of amphomycin (dissolved in DMSO). (A) Total GlcNAc-lipid synthesis. In the absence of amphomycin, 9200 dpm were present. (B) Analysis of the individual products of the reactions, as in the legend to Figure 1B. In the absence of amphomycin, there were 5860 dpm in GlcNAc and 12 300 dpm in GlcNAc-GlcNAc. (C) Effect of variation in the concentration of amphomycin on the formation of GlcNAc-GlcNAc-P-P-dolichol. Using GlcNAc-P-P-dolichol (2.3 μM) as substrate, incubations were performed and products analyzed as indicated in the legend to Figure 1C.

of the second GlcNAc residue. These findings are consistent with the observations obtained previously concerning GlcNAc-lipid synthesis by endogenous acceptors of calf brain [17].

The nature of the products formed using GlcNAc-P-P-dolichol as substrate after incubation with amphomycin was examined as previously, involving mild acid hydrolysis of the lipid extractable material, mixed bed ion exchange chromatography and analysis of the sugar component by Dionex chromatography. GlcNAc-GlcNAc accounted for 93% of the labeled products and GlcNAc 7%. This was essentially the same as that from incubations carried out in the absence of the antibiotic (data not shown). The formation of the small amount of the mono-GlcNAc derivative was probably due to small amounts of contaminating dolichyl phosphate in the preparation or to reversal of the formation of the chitobiosyl derivative.

Bacitracin

Conflicting information exists concerning the influence of bacitracin on GlcNAc-lipid synthesis. With the calf pancreas microsome system, bacitracin was observed to inhibit the formation of GlcNAc-P-P-dolichol but had little effect on the formation of GlcNAc-GlcNAc-P-P-dolichol [18]. This is in contrast to the observations with yeast membrane vesicles [19], that bacitracin inhibited the formation of the chitobiosyl derivative while having little effect on the formation of the monoGlcNAc derivative. With the porcine aorta system [20] inhibition of GlcNAc-P-P-dolichol formation by bacitracin was observed. With the hen oviduct membrane system, instead of inhibition, bacitracin brought about an increase in the formation of GlcNAc-lipid and a slight increase in $(GlcNAc)_2$-lipid formation, in addition to an increase in the mannosylated trisaccharide lipid [21]. In the present studies with microsomes from the retina of the embryonic chick, the presence of increasing concentrations of bacitracin appeared to have little effect on total GlcNAc-lipid synthesis as seen in Figure 5A. However, when the individual products of the reaction were analysed, a differential effect on the formation of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol was also observed with this antibiotic. Similar to the effect of showdomycin described above, the formation of the monoGlcNAc derivative was enhanced and that of the chitobiosyl derivative, inhibited (Figure 5B). Using exogenously added GlcNAc-P-P-dolichol as substrate, a quantitative evaluation of the latter effect was obtained as seen in Figure 5C, and summarized in Table 1. As with showdomycin, the enhanced formation of GlcNAc-P-P-dolichol to some extent may be a mass action consequence of the inhibition of the attachment of the second GlcNAc residue. Thus, depending upon the biological system under investigation, varied responses have been encountered concerning the influence of bacitracin on the GlcNAc-transferases of the dolichol pathway.

Kinetics of inhibition of GlcNAc-GlcNAc-P-P-dolichol formation

Apparent inhibition constants (K_i) were calculated from the data presented in Figures 1C, 3C and 5C as described in Materials and Methods. Values for the kinetics of inhibition by showdomycin, diumycin and bacitracin of the formation of GlcNAc-GlcNAc-P-P-dolichol using GlcNAc-P-P-dolichol as substrate are seen in Table 1. These analyses indicate that showdomycin is the most active inhibitor of this reaction among the three compounds displaying this activity, and bacitracin the least active. When calculated on a molar basis showdomycin is seen to be about 40-fold more active an inhibitor than is bacitracin. While such a calculation is not as yet possible for diumycin in view of the lack of knowledge of its structure, when compared on a weight basis, showdomycin is 14-fold more active than diumycin. The nature of the inhibitions cannot be determined from these studies. From studies on the inhibition by diumycin of the soluble transmannosylase from *Acanthamoeba castellanii* [13], mixed type kinetics were observed. It was also suggested [13] that similar type inhibition kinetics might be operating with GPT-1. Further studies in this direction are in process.

Effects of high concentrations of dolichyl phosphate on the influence of antibiotics on GlcNAc-lipid synthesis

With the calf brain microsome system [17], mention was made of the ability of high concentrations of exogenously added dolichyl phosphate to overcome the inhibition of GlcNAc-P-P-dolichol synthesis brought about by amphomycin. Such an effect would suggest a mechanism in which the initial inhibition is due to binding by the antibiotic of dolichyl phosphate, an inactivation which is overcome by competing high concentrations of dolichyl phosphate. In contrast, such a rescue was not detected in the aorta system [11]. This possibility was examined in the present studies. As seen in Figure 4A and B, concentrations of amphomycin above about $25\,\mu g\,ml^{-1}$ brought about extensive inhibition of GlcNAc-lipid synthesis. Accordingly, GlcNAc-lipid formation was examined in incubations that contained $55\,\mu g\,ml^{-1}$ amphomycin and a concentration of dolichyl phosphate ($150\,\mu M$) that was 10-fold over that usually present. As seen in Table 2, not only was the inhibition overcome, but there was a stimulation of two-fold in the synthesis of GlcNAc-P-P-dolichol and over five-fold in the formation of GlcNAc-GlcNAc-P-P-dolichol. Since amphomycin does not directly affect the formation of the chitobiosyl derivative as seen in Figure 4C, the large stimulation of its formation under these circumstances most likely was a consequence of the stimulation of the formation of its precursor, GlcNAc-P-P-dolichol, as suggested above. These findings are thus consistent with the effects observed previously with calf brain microsomes [17].

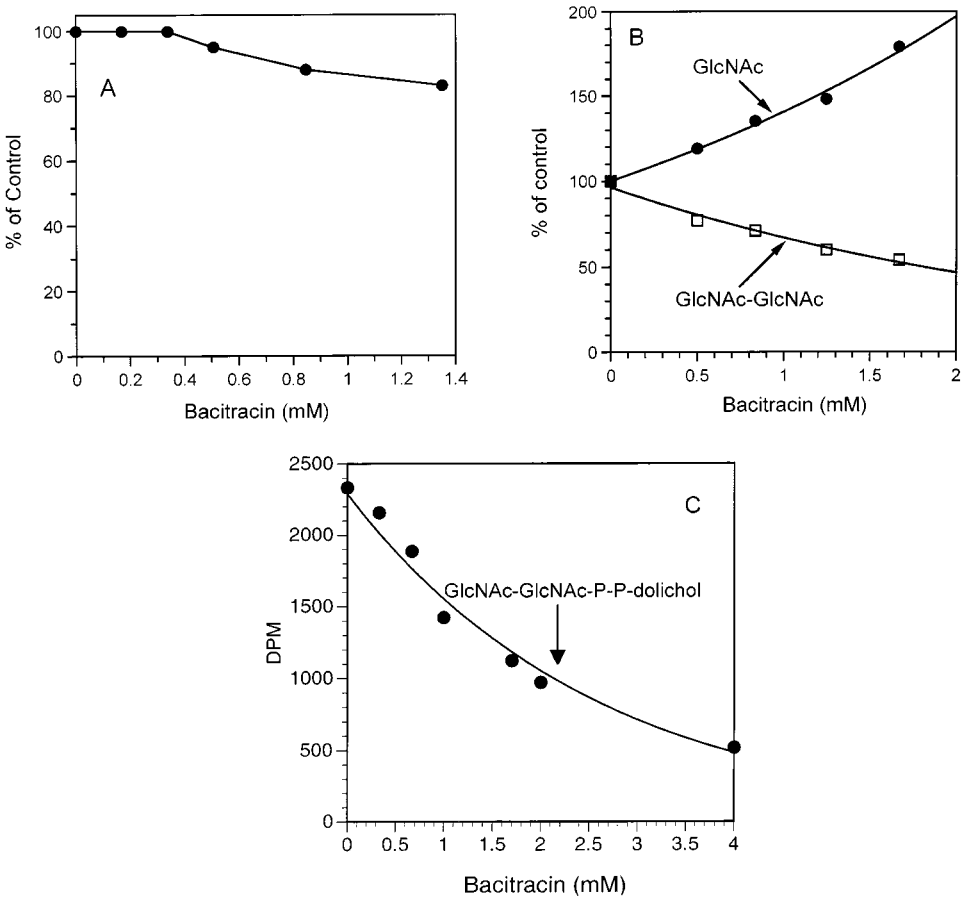

Figure 5A–C. Effect of variation in the concentration of bacitracin on GlcNAc-lipid formation. In panels A and B, incubations were carried out as described in Figure 1A and B, and the concentrations of bacitracin indicated on the abscissas. (A) Total GlcNAc-lipid synthesis. In the absence of bacitracin, 3000 dpm were present. (B) Analysis of the individual products of the reactions, as in the legend to Figure 1B. In the absence of bacitracin there were 2100 dpm present in GlcNAc and 4200 dpm in GlcNAc-GlcNAc. (C) Effect of variation in the concentration of bacitracin on the formation of GlcNAc-GlcNAc-P-P-dolichol. Using GlcNAc-P-P-dolichol (1.8 μM) as substrate, incubations were performed and products analyzed as indicated in the legend to Figure 1C.

Table 2. Effect of high concentration of dolichyl phosphate on the influence of antibiotics on GlcNAc-lipid formation. Incubations were carried out in the presence of Tes buffer, Mg^{2+}, TX-100, UDP[^{3}H]GlcNAc and microsomes (1.4 mg protein ml^{-1}), as described in Methods and Materials, 150 μM dolichyl phosphate and the indicated antibiotic. After solvent partitioning, mild acid hydrolysis and mixed bed ion exchange chromatography, analyses were performed by Dionex chromatography.

Incubation conditions	*GlcNAc-P-P-dolichol*		*$(GlcNAc)_2$-P-P-dolichol*	
	dpm	*% of control*	*dpm*	*% of control*
1. High Dol-P (150 μM)	2572	100	1154	100
+ Amphomycin (0.055 mg ml^{-1})	4970	193	6100	528
+ Diumycin (0.20 mg ml^{-1})	2290	89	177	15
+ Bacitracin (1.67 mM)	2480	97	347	30
+ Showdomycin (0.33 mg ml^{-1})	2570	100	44	4
2. High Dol-P (150 μM)	3270	100	1342	100
+ Diumycin (0.50 mg ml^{-1})	2839	87	211	16

As described previously (Figure 3B), 0.2 mg ml^{-1} and 0.5 mg ml^{-1} of diumycin inhibited the formation of GlcNAc-P-P-dolichol about 20% and 39% respectively. In the presence of the same high concentration of dolichyl phosphate as above (150 μM), there was a slight reversal of inhibition (11% and 13% inhibition at 0.2 mg ml^{-1} and 0.5 mg ml^{-1}, respectively) as seen in Table 2. There was little effect on the synthesis of GlcNAc-GlcNAc-P-P-dolichol.

Since in the present studies with the retina microsomes, the effect of showdomycin and bacitracin was to stimulate the formation of GlcNAc-P-P-dolichol, not inhibit it, an influence of this type from high concentrations of dolichyl phosphate was not to be expected with these compounds. In contrast, when examined under this condition, the stimulatory effect was not retained although the inhibition of the formation of the chitobiosyl derivative still occurred. The inhibitions by bacitracin of the formation of GlcNAc-P-P-dolichol reported for calf pancreas microsomes [18] and the porcine aorta system [20], were also not overcome by increasing concentrations of dolichol phosphate, suggesting that the action of this antibiotic was directed to the enzyme and not the substrates. Interaction with the enzyme was also suggested for the mechanism of action of diumycin in the *Acanthamoeba system* [13], and of showdomycin in studies with green algae [12]. Similar sites of action of showdomycin, diumycin, and bacitracin may be operative with retina microsomes. Further studies in this direction are in process.

The inhibition of the formation of GlcNAc-GlcNAc-P-P-dolichol and the enhanced synthesis of GlcNAc-P-P-dolichol in the same incubation by showdomycin and bacitracin argues against the notion that these effects might be due to an influence by the antibiotic on the stability of the sugar nucleotide. In the presence of a four-fold higher concentration of UDP-GlcNAc (0.2 mM) the relative stimulatory effects on the formation of GlcNAc-P-P-dolichol and the inhibition of the chitobiosyl derivative by showdomycin and bacitracin were retained (data not shown).

Concluding remarks

These studies, reexamining the influence of several antibiotics that have been used to explore the initial reactions of the dolichol pathway, have revealed differential actions as concerns their effects on the formation of GlcNAc-P-P-dolichol and GlcNAc-GlcNAc-P-P-dolichol. In addition to inhibitory effects, stimulatory influences have also been observed. A summary of these actions is seen in Table 3. The differences between results described in the present report and those from previous studies may to some extent be a reflection of the advances in oligosaccharide separation and analysis that have occurred in the intervening 20 years, as in this work in the use of Dionex chromatography. The results from the present studies have highlighted the need to examine the influence of the antibiotics on the individual products of the reactions, especially when not using individual purified enzymes (a common situation in view of their great lability as widely encountered in this area). In addition, the possibility of different responses in different tissues, as exemplified by the various studies with bacitracin, makes this requirement of even greater importance. Within these constraints, compounds of the types examined here have been and will continue to be of value in exploring the properties of individual reactions of the dolichol pathway.

Table 3. Effect of antibiotics on GlcNAc-lipid formation.

Compound	*GlcNAc-P-P-dolichol*	*$(GlcNAc)_2$-P-P-dolichol*
Tunicamycin[a]	Inhibition	No effect
Amphomycin	Inhibition	Little or no effect
Showdomycin	Stimulation	Inhibition
Diumycin	Stimulation/ Inhibition	Inhibition
Bacitracin	Stimulation	Inhibition

[a] From previous reports.

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